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SUMMARY

SUBSECTION I: AGRICULTURAL BIOTECHNOLOGY

SUBSECTION II: FOOD BIOTECHNOLOGY

SUBSECTION III: MEDICAL AND PHARMACEUTICAL BIOTECHNOLOGY

SUBSECTION IV: INDUSTRIAL AND ENVIRONMENTAL BIOTECHNOLOGY

SUBSECTION V: MISCELLANEOUS

AGRICULTURAL BIOTECHNOLOGY

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REGENERATIVE CAPACITY OF LEAVES AND STEM SEGMENTS OF SIX GENOTYPES OF *Vaccinium corymbosum* L.

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Abstract

The aim of the present study is to observe the capacity of adventitious organogenesis of leaves and stem segments of six cultivars of highbush blueberry. The effect of WPM cultural medium enriched with 3 mg/l zeatin, 3 mg/l zeatin ribozoid and 2 mg/l 2-iP on the regeneration potential, average and total regeneration rates was assessed. Direct organogenesis is different for individual genetic types of genus Vaccinium. The regenerative response in most of the variants is in favour of the stem segments as explant sources. The highest average number of regenerants was recorded in leaf segments of 'Bluejay' genotype - 5.6 numbers/explant at A_{zr} medium.

Key words*: adventitious organogenesis, in vitro, Vaccinium sp., zeatin, zeatin ribozoid, 2-iP.*

INTRODUCTION

Highbush blueberry belongs to anthocyanin-coloured small berry species that are extremely rich in valuable biologically active substances (Häkkinen et al., 1999; Häkkinen et al., 2000; Puupponen-Pimiä et al., 2002; Hung et al., 2004; Puupponen-Pimiä et al., 2005) necessary for the normal functioning of physiological processes in the human body. It is a significant commercial and biological species (Debnath, 2007).

Blueberry is a relatively new crop for Bulgaria. It finds favourable soil and climate conditions for cultivation in the mountaine and hilly regions of Bulgaria. Traditional propagation methods - through mature and green cuttings are not effective enough to provide a sufficient amount of healthy planting material. The development of modern biotechnological methods for accelerated propagation is of use in this fruit species. Many blueberry cultivars have been studied for their regenerative capacity by direct organogenesis from leaf and stem segments (Rowland and Ogden, 1992; Cao et al., 2000; Ostrolucká et al., 2002; 2009; Cao et al., 2003; Gajdošová et al., 2006).

The purpose of the present experimental work is to develop a system for *in vitro* adventitious shoot regeneration from leaf and stem segments of some highbush blueberry leaves and stem segments using different cytokinins.

MATERIALS AND METHODS

Planting material

The following genotypes were the objective of the present study: 'Bluecrop', 'Bluegold', 'Bluejay', 'Spartan', 'Patriot', and 'Toro', which have scientific and commercial interest. The selected cultivars are fertile, with a different ripening period, which makes them attractive to the market.

Cultural mediums

Stem and leaf segments were isolated from 28 daily intact *in vitro* plants cultivated on WPM cultural medium (McCown and Lloyd, 1981), with half reduced salt concentration, supplemented with 1 mg/l IAA. The leaf explants were taken from the middle layer of the clonal plants, placed horizontally, with their abaxial side to the culture medium. Both types of explants were 5-8 mm in size. The primary explants were cultured in petri dishes, each containing 10 explants and 4-5 ml cultural medium.

Cultural mediums for direct organogenesis of blueberries

In the present scientific experiment, two nutrient variants (abbreviation with A_z or A_{zr}) were used: WPM, enriched with 3 mg/l zeatin and 2 mg/l 2-iP (abbreviation with A_z), in the second case zeatin was replaced with 3 mg/l zeatin ribozoid (abbreviation with A_{zr}), containing 20 g sucrose and 6 g agar. The

acidity of the medium was adjusted to 4.2 before autoclaving.

The following indicators were evaluated to study the regeneration capacity: % regeneration, average and total number of primary explant regenerants. The regeneration response was reflected after 50 days of cultivation without subculturing the explants in fresh cultural medium.

***In vitro* cultivation**

The adventitious regenerants were grown in growth chambers at 22 ± 2°C, a photoperiod of 16/8 day/night and a light intensity of 2000-3000 lx.

Statistical methods of analysis

The data were processed by a variational-statistical method (Lidanski, 1988).

RESULTS AND DISCUSSIONS

The scientific experiment conducted shows that the studied cultivars of highbush blueberry can be successfully propagated by direct organogenesis of leaf and stem segment. Adventitious shoot regeneration depends on the cultivar features, the type of explant, and the type of cytokinin included.

Figures 1 and 2 present the regenerative potential of the tested cultivars of the presented cultural media. All cultivars included in the study successfully manifest their morphogenetic potential. 'Spartan' responded with higher rates of regeneration than other genotypes included in the study. The lowest regenerative capacity *in vitro* was observed in leaf explants with 'Bluecrop' genotype - 10% and 15%. The intensity of adventitious shoot regeneration is different for the different cultivars of *Vaccinium corymbosum* L.

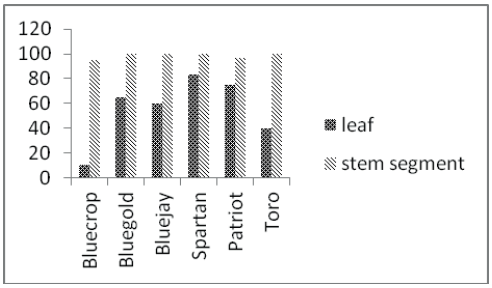

Figure 1. Percent regeneration of leaf and stem segments of different genotypes of highbush blueberries in medium A_z (%)

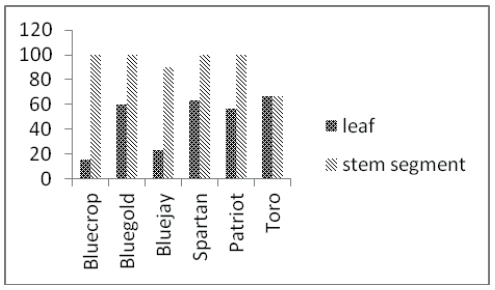

Figure 2. Percent regeneration of leaf and stem segments of different genotypes of highbush blueberries in A_{zr} medium (%)

The plant response to the regeneration rate obtained using the stem segments as explant sources was clearly better than all the variants (regardless of cytokinin used) of the study (except for 'Toro' in medium A_{zr} leaf and stem segments 66.7%) than the leaves. The observed difference was probably due to differences in the morphological structure and physiological state of both explants.

In our previous studies, we found that stem segments of 'Brigita blue' achieved their regenerative capacity *in vitro* - 92.9% in WPM enriched with 4 mg/l zeatin and 5 mg/l 2-iP (Georgieva and Kondakova, 2008).

Successful *in vitro* regeneration is dependent on the choice of the appropriate cytokinin in addition to the cultivar response. Zeatin is an effective cytokinin for the induction of adventitious organogenesis in genus *Vaccinium* and *V. vitis idaea* (Rowland and Ogden, 1992; Gonzales et al., 2000; Debnath and McRae 2002; Ostrolucká et al., 2002; Gajdošová et al., 2006; Meiner et al., 2007; Cappelletti et al., 2016). Its amount in the culture medium is fundamental to the efficiency of the regeneration process in highbush blueberry. Gajdošová et al. (2006) investigated adventitious organogenesis in 5 cultivars of highbush blueberry and reported 39.1 primary explant shoots for 'Brigitta' genotype, enriching the culture medium with 0.5 mg/l zeatin. Authors such as Ostrolucká et al. (2004) proved that the same concentration of zeatin works best for regeneration of highbush blueberry from leaf segments. Meiner et al. (2007) reported that 20 µM zeatin was more effective than TDZ and meta-topolin for induction of adventitious shoots in leaves cut from 'Ozarkblue' cultivar. Other studies have shown

that high concentrations of zeatin provoke callus formation in leaf explants (Shibli and Smith, 1996). In our study, the hormonal combination of 3 mg/l zeatin and 2 mg/l 2-iP in four genotypes ('Bluegold', 'Bluejay', 'Spartan', 'Toro') induced maximum regenerative capacity *in vitro* (100%) - in stem explants (Figure 1). The lowest regeneration capacity was observed in leaf segments of 'Bluecrop' (10%) and 'Toro' (40%) in medium A_z Similar to our experiment, other researchers such as Gonzales et al. (2000); Cappelletti et al. (2016) include 2-iP in the culture medium for blueberry morphogenesis. Zeatin is more effective than 2-iP for adventitious shoot regeneration in highbush blueberries (Rowland and Ogden, 1992; Ostrolucká et al., 2002; Gajdošová et al., 2006).

The stimulating effect of the linked cytokinin zeatin ribosoid on direct leaf organogenesis in the leaves of *Vaccinium* has been demonstrated in the scientific work of Rowland and Ogden, (1992), Cao et al. (2000; 2003). In 'Sunrise' cultivar, experimenters found that zeatin ribosoid increased shoots 2 or 5 times compared to zeatin and 2-iP (Cao et al., 2003).

In our previous studies, 2 mg/l zeatin ribozoid gave better results (96.67% regeneration in leaf explants and 100% regeneration of stem segments) compared to the same amount of zeatin in low bush blueberry (Georgieva, 2013). In the present scientific experiment, the intensity of the regeneration process is in the range of 15% ('Bluecrop') to 66.7% ('Toro') - for leaf explants in A_{zr} cultural medium (Figure 2). Analysis of the research data confirms the stimulating effect of the three types of cytokinins - zeatin and zeatin ribozoid at a concentration of 3 mg/l, combined with 2 mg/l 2-iP on the morphogenesis of *Vaccinium corymbosum*.

The results of our experimental protocol indicate that the average number of explant regenerants in most of the cultivars (regardless of the type of parent explant) is in favor of media enriched with 2-iP and zeatin ribozoid (except 'Spartan' genotype - leaf segments, 'Patriot' - stem segments and 'Toro' leaf and stem segments) (Figures 3a, 3b). The highest average number of explant regenerants was recorded for 'Bluejay' (leaf, A_{zr} medium) - 5.5 numbers/explant. The lowest values of this indicator were again registered in 'Bluecrop' - 1.5 -1.7 numbers/leaf explant. The studies of Cao et al. (2003) are in agreement with our results for the genotypic specificity of this cultivar. Similar to our results, Cao et al. (2003) received 11 shoots/leaf explant with 'Bluecrop', and 'Rowland' and 'Ogden' (1992) registered 20 shoots/leaf explant with 'Sunrise'.

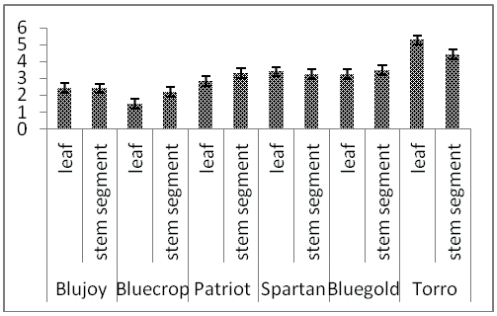

Figure 3a. Average number of regenerants in medium A_z from leaf and stem segments of different genotypes of highbush blueberries

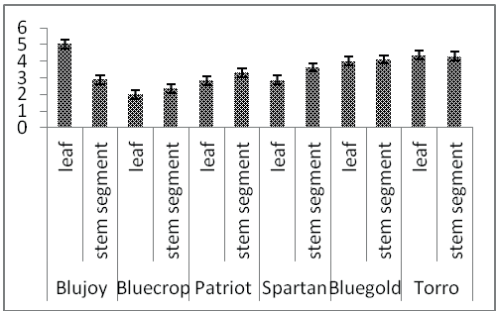

Figure 3b. Average number of regenerants of medium A_{zr} from leaf and stem segments of different genotypes of highbush blueberries

The total number of regenerants from the studied six cultivars of highbush blueberry is in favor of 'Toro' genotype - 128 numbers (stem segments, medium A_z), and 'Spartan' - 109 numbers (stem segment, medium A_{zr}). A higher total number of regenerants was found using the stem segments as explant sources (Figures 4a, 4b). In four cultivars - 'Bluecrop', 'Bluegold', 'Spartan' and 'Patriot', a positive correlation between the highest values of the average and the total number of regenerants was observed (Figures 3a, 3b, 4a, 4b). The included cytokinins had a positive effect on the adventitious organogenesis of the six varieties of *Vaccinium corymbosum* (Figures 5 and 6).

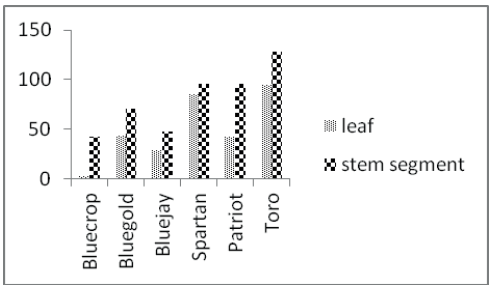

Figure 4a. Total number of regenerants of medium A_z from leaf and stem segments of different genotypes of highbush blueberries

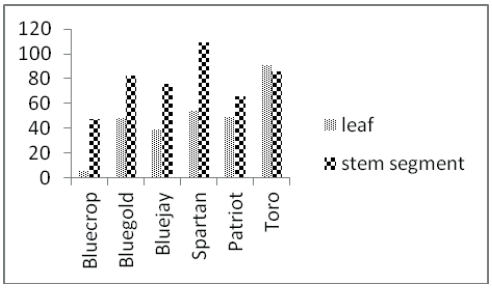

Figure 4b. Total number of regenerants of medium A_{zr} from leaf and stem segments of different genotypes of highbush blueberries

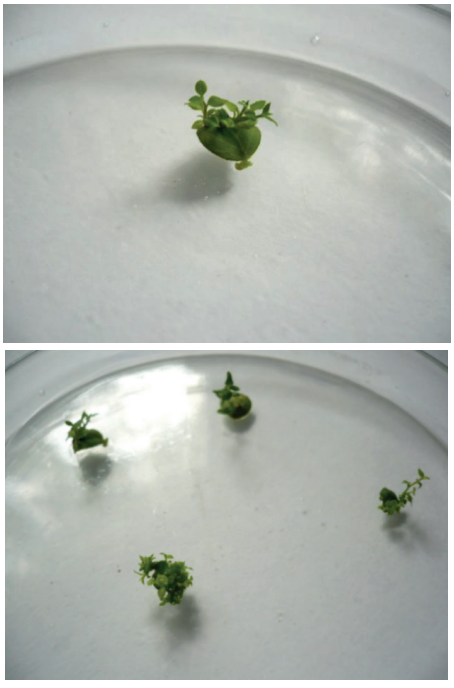

Figure 5. Adventitious shoot regeneration from leaf segment of 'Spartan' cultivar after 50 days cultivation in A_{zr} medium

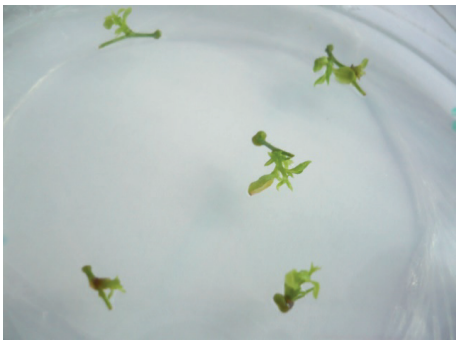

Figure 6. Adventitious shoot regeneration from stem segment in 'Bluecrop' cultivar after 50 days cultivation in medium A_z

Tables 1 to 4 present data from the mathematical processing of the results.
According to the analysis of the variant, there are no statistically proven differences among the cultivars in terms of the regenerative potential of leaf explants of both media (Table 1). With respect to stem explants, cultivar differences in regeneration potential caused by differences in environments were statistically proven (Table 2).
Differences ($P < 0.05$) in the regeneration potential between leaf and stem segments in various genotypes in zeatin media were demonstrated (Table 3). The medium enriched with zeatin ribizoid
(Table 4) is with a tendency ($P < 0.10$) to prove the differences for both types of explants of the genotypes, but without any statistical significance.

Table 1. Statistical analyzes among cultivars, media and explants

A_z	A_{zr}	A_z	A_{zr}	A_z	A_{zr}	A_z	A_{zr}	A_z	A_{zr}	A_z	A_{zr}
leaf		leaf		leaf		leaf		leaf		Leaf	
'Bluejay'		'Bluecrop'		'Patriot'		'Spartan'		'Bluegold'		'Toro'	
2.4	5.0	1.5	2.0	2.8	2.8	3.4	2.9	3.3	4.0	5.3	4.3
LSD 0.05	n.s										

Table 2. Statistical analyzes among cultivars, media and explants

A_z	A_{zr}	A_z	A_{zr}	A_z	A_{zr}	A_z	A_{zr}	A_z	A_{zr}	A_z	A_{zr}
stem segment	stem segment	stem segment	stem segment	stem segment	stem segment	stem segment	stem segment	stem segment	stem segment	stem segment	stem segment
'Bluejay'		'Bluecrop'		'Patriot'		'Spartan'		'Bluegold'		'Toro'	
2.4	2.9	2.2	2.4	3.3	3.3	3.3	3.6	3.5	4.1	4.4	4.3
LSD 0.05	2.27										

Table 3. Statistical analyzes among cultivars, media and explants

A_z	A_z	A_z	A_z	A_z	A_z	A_z	A_z	A_z	A_z	A_z	A_z
leaf	stem segment	leaf	stem segment	leaf	stem segment	leaf	stem segment	leaf	stem segment	leaf	stem segment
'Bluejay'		'Bluecrop'		'Patriot'		'Spartan'		'Bluegold'		'Toro'	
2.4	2.4	1.5	2.2	2.8	3.3	3.4	3.3	3.3	3.5	5.3	4.4
LSD 0.05	3.24										

Table 4. Statistical analyzes among cultivars, media and explants

A_{zr}	A_{zr}	A_{zr}	A_{zr}	A_{zr}	A_{zr}	A_{zr}	A_{zr}	A_{zr}	A_{zr}	A_{zr}	A_{zr}
leaf	stem segment	leaf	stem segment	leaf	stem segment	leaf	stem segment	leaf	stem segment	leaf	stem segment
'Bluejay'		'Bluecrop'		'Patriot'		'Spartan'		'Bluegold'		'Toro'	
5.0	2.9	2.0	2.4	2.8	3.3	2.9	3.6	4.0	4.1	4.3	4.3
LSD 0.05	n.s										

CONCLUSIONS

The regenerative response is genotypically dependent. The highest percentage of regeneration was distinguished for 'Spartan' cultivar, irrespective of the type of explant sources and the type of cytokinin used (100% for stem segments, 83% leaf, medium A_z and 63% leaf, medium A_{zr}).

The regeneration potential of some cultivars of highbush blueberry ('Bluecrop', 'Bluegold', 'Bluejay', 'Spartan', 'Patriot' and 'Toro') at *in vitro* level was examined using leaf and stem segments as explants. The plant response favours the use of stem segments as explant sources.

Regarding the choice of cytokinin, a higher rate of regeneration was reported in most variants using zeatin ('Bluegold' - 100%, 'Bluejay' - 100%, 'Spartan' - 100%, 'Toro' - 100% stem segment), compared to zeatin ribozoid. The highest average number of regenerants was distinguished for 'Bluejay' genotypes - 5.6 numbers/explant, leaves of A_{zr} medium and 'Toro' - 5.3 numbers/explants, leaf of medium A_z. The highest total number of regenerants (128 numbers) was found in the stem segments of 'Toro' cultivated on culture medium A_z, followed by the stem segments of the 'Spartan' genotype (109 numbers) obtained on A_{zr} medium.

REFERENCES

Cao, X., Fordham I., Douglass L., Hammerschlag, F. (2003). Sucrose level influences micropropagation and gene delivery into leaves from in vitro propagated highbush blueberry shoots. *Plant cell tissue organ culture*, 75, 255-259.

Cao, X., Hammerschlag, F. A. (2000). Improved shoot organogenesis from leaf explants of highbush blueberry. *Hortscience*, 35, 945-947.

Cappelletti, R., Sabbadini S., Mezzetti, B. (2016). The use of TDZ for the efficient in vitro regeneration and organogenesis of strawberry and blueberry cultivars, *Scientia Horticulturae*, 207, 117-124.

Debnath S. (2007). Strategies to propagate *Vaccinium* nuclear stocks for the Canadian berry industry, *Can J Plant Sci,* 87, 911-922.

Debnath, S. C. & McRae, K. B. (2002). An efficient adventitious shoot regeneration system on excised leaves of micropropagated lingonberry *(Vaccinium*

vitis-idaea L.*). J. Hort. Sci. and Biotech.*, 77, 744-752.

Gajdošová, A., Ostrolucká, M. G., Libiaková, G., Ondrušková, E., Simala. D. (2006). Microclonal propagation of *Vaccinium sp*. and *Rubus* sp. and detection of genetic variability in culture *in vitro*. *J. Fruit and Ornamental Plant Research*, 14(1), 61-76.

Georgieva, M. (2013). Micropropagation of lowbush blueberry (*Vaccinium angustifolium*), *Banat's Journal of biotechnology*, IV, (8), 42-47.

Georgieva, M., & Kondakova, V. (2008). Micropropagation of highbush blueberry /*Vaccinium corymbosum*/, International Scientific Conference- „Sustainable fruit growing: from plant to product", Jurmala-Dobele, 134-140.

Gonzalez, M. V., Lopez, M., Valdes, A. E., Ordas, R. J. (2000). Micropropagation of three berry fruit species using nodal segments from field-grown plants, Annals of Applied Biology, 137(1), 73-78.

Häkkinen, S., Heinonen, M., Kärenlampi, S., Mykkänen, H., Ruuskanen, J., Törrönen, R. (1999). Screening of selected flavonoids and phenolic acids in 19 berries. *Food Research International*, 32, 345-53.

Häkkinen, Sari H., Riitta Törrönen, A. (2000). Content of flavonols and selected phenolic acids in strawberries and Vaccinium species: influence of cultivar, cultivation site and technique. *Food Research International*, 33(6), 517-524.

Hung, H.-C., Joshipura, K. J., Jiang, R., Hu, F. B., Hunter, D., Smith-Warner, S. A., Colditz, G. A., Rosner, B., Spiegelman, D., Willett, W. C. (2004). Fruit and vegetable intake and risk of major chronic disease. *J Natl Cancer Inst.*, 96, 1577-1584.

Lidanski T. (1988). *Statistical methods in biology and in agriculture* (pp. 135-160). Sofia, Bulgaria.

McCown, B. N., Lloyd, G. (1981). Woody plant medium (WPM) - a mineral nutrient formulation for microculture of woody plant species. *HortScience,* 16, 453.

Meiners, J., Schwab, M., Szankowski, I. (2007). Efficient *in vitro* regeneration systems for *Vaccinium* species. *Plant cell, tissue and organ culture,* 89(2-3), 169-176.

Ostrolucká, M. G., Gajdošová, A., Ondrušková, E., Libiaková, G. (2009). *In vitro* propagation of several *Vaccinium corymbosum* L. and *Vaccinium vitis-idaea* L. cultivars, *Latvia-Agronomijas-Vestis* , 12, 75-80.

Ostrolucká, M.G., Gajdošová, A., Libiaková, G. (2002). Influence of zeatin on microclonal propagation of *Vaccinium corymbosum* L. *Propag. ornam. Plants,* 2, 14-18.

Ostrolucká, M. G., Libiaková, G., Ondruková, E., Gajdosová, A. (2004). *In vitro* propagation of *Vaccinium* species. *Acta Universitatis Latviensis: Biology*, 676, 207-212.

Puupponen-Pimiä, R., Aura, A. M., Oksman-Caldentey, K. M., Myllärinen, P., Saarela, M., Mattila-Sandholm, T., Poutanen, K. (2002). Development of functional ingredients for gut health. *Trends in Food Science & Technology,* 13, 3-11.

Puupponen-Pimiä, R., Nohynek, L., Hartmann-Schmidlin, S., Kähkönen, M., Heinonen, M., Määttä-Riihinen, K., Oksman-Caldentey, K.-M. (2005). Berry phenolics selectively inhibit the growth of intestinal pathogens. *J Appl. Microbiol.,* 98, 991-1000.

Rowland, L. J., Ogden, E. L. (1992). Use of a cytokinin conjugate for efficient shoot regeneration from leaf sections of highbush blueberry. *Hortscience,* 27, 1127-1129.

Shibli, R. A. & Smith, M. A. L. (1996). Direct shoot regeneration from *Vaccinium pahlae* (Othelo) and *V. myrtillus* (bilberry) leaf explants. *Hortscience*, 31, 1225-1228.

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VOLATILE COMPOUNDS IN DIFFERENT FRACTIONS OF FRUITS DISTILLATE OBTAINED BY TRADITIONAL DISTILLATION

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Abstract

To investigate the behaviour of volatile compounds during a traditional alembic distillation, large numbers of important volatile compounds were identified and quantified by GC/MS and GC/FID analysis in different fractions of fruits mash distillates made from two pears varieties, one peach variety and blackberry and cranberry fruits. The purpose of this investigation was to determinate the volatile compounds distribution of higher alcohols, esters, aldehydes and terpenes in some fruits fraction of distillates. Because of the abundance in odoriferous monoterpenes, utilization of the tail fraction as a raw material for re-distillation was considered appropriate.

Key words*: distillation fractions, volatile compounds, traditional alembic distillation, fruits distillates.*

INTRODUCTION

In Romania, delicatessen fruit alcoholic drinks are produced from autochthonous fruit berry species such as blackberry and cranberry and also from cultivated species such as pears and peaches. Together, with plum distillate which is a national beverage called *ţuică*, these fruit distillates have not been studied and there are not chemical data for many of these. An important feature of fruit distillates is the flavor of the fruit from which it originates. The most important compounds in fruit distillates that influence flavour are higher alcohols and esters along with terpenes. (Lukic et al., 2011; Stoica et al., 2015).

The final flavour of fruit distillates consists of several chemical compounds that are formed in different stages of the process. Thus, there are 4 phases: primary flavour (during fruits ripening), secondary flavour (during the fermentation process of the marc), tertiary flavour (during the distillation process of the marc) and quaternary flavour (after the maturation of the distillate) (Leaute, 1990; Spaho et al., 2013).

After the fermentation of fruits mash is finished, the distillation process follows, when alcohol and important amounts of volatile compounds are recovered. (Lukic et al., 2011; Stoica et al., 2015).

The distillation technique can significantly influence the behaviour of the volatiles compounds during distillation and therefore the chemical composition and the sensorial properties of fruit distillates (Cabaroglu and Yilmaztekin, 2011; Colonna-Ceccaldi, 2008; Tesević et al., 2005).

The traditional distillation, in comparison with the modern distillation with rectification columns, is performed with a copper pot stills called alembics (Leaute, 1990). It is a discontinuous process involving a simple separation of three important fractions which distil consecutively: the first part of the distillate (the head fraction), the middle part of the distillate (the heart) and the last part of the distillate (tail fraction).

The first (head) and the last (tail) fraction of the distillate, are usually eliminated because it contributes negatively to the quality of the distillate by certain volatile compounds found in important quantities in these fractions. (Silva et al., 2000; Prado-Ramírez et al., 2005).

The heart fraction, which contains the ethanol and the volatile compounds that add the sensory quality of the distillate, is the only one intended for consuming.

In traditional alembic distillation without computerized control, the distillation cut is determined by measuring the alcohol concentration during the process or by tasting. Therefore, the skills and the experience of the distiller have an important role (González et al., 2010; Cichi, 2015).

In the literature there are some studies which give information on the behaviour of the main volatile chemical compounds (higher alcohols, esters and terpens) during distillation (Apostolopoulou et al., 2005; Cortés-Diéguez et al., 2003; Cortés et al., 2009; Crowell and Guymon, 1973; Flouros et al., 2003; Geroyiannaki et al., 2007; Hernández-Gómez et al., 2005, 2008; Nykänen, 1986; Plutowska et al., 2010; Santos et al., 2013; Soufleros et al., 2004).

The quality of distillates depends decisively on the quality of fruit mash and fresh fruit quality used by default (Durr and Tanner, 1983).

The aim of this investigation was to determinate the chemical composition of a fermented pear mash and peach mash correlated on the fruit variety and distillation with a traditional alembic. Since, these fruit alcoholic drinks are delicatessen in Romania, this study proposes a research of distillates obtained from the peach, blackberry, cranberry and pears varieties used for this purpose.

MATERIALS AND METHODS

Fruit samples

Two pear varieties and each one variety of peaches, blackberries and cranberry were used for this study. Each variety was used for production of the fruit distillate, in Romania, especially in Oltenia region (South-West of the country).

The raw material was as follows: Williams and Favorita lui Clapp which are summer pears varieties depending on the maturation period without sclereids. The peach variety is Babygold 7, while blackberry and cranberry are wild fruits.

Fermentation

Fruits harvested from hilly areas of Oltenia (Dragasani, Valcea), were transported to the Faculty of Horticulture Craiova, Department of Horticulture and Food science laboratory. All three varieties were crushed and put into vessels with 40 L capacity each. The vessels were filled up approximate 70% of the volume. Spontaneous fermentation with indigenous yeasts has been done at 20°C ± 1°C. Monitoring of fermentation was followed every day and it lasted until the concentration of sugar decreased to 4°Brix.

Distillation

Fermented fruit mashes were distilled in a traditional copper alembic with stirrer and without dephlegmator. The alembic was hermetically sealed. This is necessary before starting distillation to prevent an escape of vapour and even an explosion.

Gradual warming of the alembic was carried out by direct fire. The distillation process was induced by strong heating, which was continued for a short time after the condensation of the distillate started.

During distillation, attained flow was maintained constant by gradually increasing the heating temperature, due to a decrease in ethanol/water ratio in the boiler and in the vapours. The flow rate obtained during the distillation process was constantly maintained by the gradual increase of the heating temperatures and the water temperature in the cooling vessel was 20-22°C throughout the distillation. A thermometer was used to measure the temperature in the pot still and in the spirits tube and the alcohol content of distillate was measured by an aerometer.

Analytical method

The first 200 ml of distillate formed the head fraction. The next fraction of distillate, collected up to an alcoholic concentration of up to 30% vol, was the heart fraction. This was divided into three 100 ml single samples, marked as heart 1, heart 2 and heart 3. The last fraction of distillate collected - the tail fraction was 200 ml at a level of alcoholic concentration of 20% vol. Thus, there were obtained the 5 main fractions: one head, one tail and three heart fractions.

All distillates were stored in dark bottles at 20°C for three months and then analyzed. All 25 samples (five for each variety) were analyzed using gas chromatography and following the method used by the Laboratory of the Department of Horticulture and Food science and the laboratory of National Institute for Cryogenics and Isotopic Technologies (I.C.S.I. Rm. Valcea).

The volatile compounds were extracted from the distillate fractions by liquid-liquid extraction following the method proposed by Lukić et al. (2010). This method consist in a 12 ml volume of a fraction sample which was diluted with 150 ml of deionised water, and 75 g of ammonium sulphate was added in order to improve the extraction efficiency. Then, a 250 µl aliquot of the internal standard solution (3-octanol, nonanoic acid, and methyl nonanoate in ethanol) was added to control the extraction. Volatile compounds were extracted with three 5 ml portions of dichloromethane. Dichloromethane extracts were combined, dried over anhydrous sodium sulphate, and concentrated to 0.5 ml. To control injection, 10 µL of a 3-heptanol ethanolic solution was added as another internal standard (Lukić et al., 2011).

Quantitative determination of volatile compounds was performed using a gas chromatography system, a VARIAN 450 gas chromatograph GC-FID detector (flame ionization detection) with a set of 275°C temperature for both the column TG-WAXMS 60 m, ID 0.32 mm, film, 0.25 mm, injector temperature 150°C, column temperature 35°C, 3 min stand, climb to 20°C/min, up to 70 to 150°C with 27°/min, stand 2 minutes, climb 200°C, stand 2 minutes, climb to 240°C with 20°C/min and stands 6 min. The carrier gas was helium (1.2 ml/min flow rate). Injection volume is 1 µl. The identification was made by comparing the retention times of standards from the calibration curve.

Chemicals and standard physicochemical analysis

Standards of volatile aroma compounds were purchased from Merck (Darmstadt, Germany) and Fluka (Buchs, Switzerland). Dichloromethane (99.8%) and sodium sulphate (99%) were supplied by Kemika (Zagreb, Croatia). Pure deionised water was obtained from an Elix 3 purification system (Millipore, Bedford, MA, USA).

RESULTS AND DISCUSSIONS

Volume fraction of methanol (%) and concentration levels (mg/L anhydrous alcohol, a.a.) of volatile compounds in different fraction of fruit distillate obtained by traditional alembic distillation are presented in Tables 1, 2, 3, 4 and 5.

Methanol

Methanol had the same pattern of distillation for all five varieties of fruits distillate (Tables 1, 2, 3, 4 and 5). In the head fraction, the methanol concentration has been increasing followed by a decrease to tail fraction which has the lowest value. Researchers Silava and Macata (2000), have shown that methanol can form azeotropic mixtures with ethanol and water, also being present in the other distillate fractions. Also, this makes it even more difficult to remove it in the case of discontinuous distillation with alambic. Other researchers (Geroyiannaki et al., 2007; Hernández-Gómez et al., 2008) found approximately equal concentrations of methanol throughout the distillation.

The content of the higher alcohols and esters in the 25 samples of the fruits distillates are presented in Tables 1, 2, 3, 4 and 5.

From a quantitative point of view, higher alcohols present the broadest group of aromatic compounds in the distillates (Lehtonen and Eriksson, 1983; Versini et al., 2012; Spaho et al., 2013). Due to lower boiling point (below 200°C), higher alcohols are distilled in the distilled head and heart fractions.

However, a non-uniform behaviour of distillation of higher alcohols has been observed. (Apostolopoulou et al., 2005; González et al., 2010).

In this study, 3-methyl-1-butanol (isoamyl alcohol) and 1-propanol were quantified as higher alcohols (Onishi et al., 1977; Cortés et al., 2009; Cabaroglu et al., 2011). These alcohols are produced by yeasts during alcoholic fermentation by converting the amino acids (leucine, isoleucine, valine) that are in this medium (Garcia-Llobodanin et al., 2007).

The highest content of methanol in the head fraction is recorded in the Favorita lui Clapp distillate and the lowest in the blackberry distillate. In all 5 distillates, Heart 1 will notice a greater amount of methanol, which might be the answer to the somewhat empirical separation of the three fractions during distillation.

Compared with methanol, higher alcohols are found in much lower amounts in all fractions, including the head fraction. From the data

presented in the tables, it can be seen from the quantitative point of view that 3-methyl 1-butanol is dominated followed by 1-propanol. It is formed by hydration of the corresponding acetals during the alcoholic fermentation of the fruit jar. Benzyl alcohol which is a high aromatic alcohol has shown somewhat non-uniform behaviour but it is found in most cases in larger quantities in the last two distillate fractions. A different behaviour of aromatic alcohols during distillation was observed by Plutowska (2010), as well as by some decreases in their concentration throughout distillation (Spaho et al., 2013).

Esters

From a qualitative point of view, the esters represent the major representative class of flavour compounds in distillates. As well as the higher alcohols, they are formed during alcoholic fermentation of fruit mash. (Lehtonen and Eriksson, 1983; Stewart, 2008). Ethyl acetate is the major ester present in alcoholic beverages. Esters, generally, are associated with a pleasant, fruity and flowery aroma (Stoica et al., 2016).

The positive influence of esters on the aroma of distillates is given by their concentration in the beverage (Onishi et al., 1977; Cortés et al., 2009). According to the data in the tables, ethyl acetate is found in significantly higher amounts comparatively with isoamyl acetate. It is worth mentioning the importance of ethyl acetate in distillates, which even in small quantities can impress a pleasant aroma of these beverages. However, in too large quantities it can print a too strong flavour that it is not always appreciated by the consumer (Silva et al., 2000; Lukic et al., 2011; Spaho et al., 2013). The concentration of ethyl acetate was significantly higher in the distillates from the pears and peach varieties than in distillates from the other two fruits varieties (blackberry and cranberries).

The second most abundant ester was isoamyl acetate. The concentration of isoamyl acetate was significantly higher in the distillates from the blackberry and cranberries varieties than in distillates from the other two fruits varieties (pears and peach). It appears that these esters could be the main predictors of the sensory profile of a brandy made from the wild fruits (blackberries and cranberries).

Monoterpenes

From the data presented in the tables (Cortés-Diéguez et al., 2003; Colonna-Ceccaldi et al., 2008; Cabaroglu et al., 2011; Cichi, 2015), the concentration of most identified terpenes decreased in fractions having a lower alcoholic strength, which is distilled at higher temperatures. The explanation would be that they are extremely volatile compounds and may be lost during distillation. The main analysed monoterpenes nerol, geraniol, limonene, are found in different amounts in pears and berries distillates. In contrast, in peaches distillates they were not identified. Aroma is one of the main characteristics that determine a brandy's organoleptic quality and style. This is the result of the contribution of hundreds of volatile compounds, including higher alcohols, esters, acids, aldehydes, ketones, terpenes, norisoprenoids and phenols. They come from volatile chemical compounds resulting from grapes and vinification and distillation process; some are derived from oak (Lukic et al., 2011). Undoubtedly the alcoholic fermentation significantly influences the flavour and final quality of the brandy (Durr and Tanner,1983; Nykänen, 1986).

Aldehydes

Concerning the concentration in acetic aldehyde, this it is found in all the fractions of the 5 analysed distillates in larger or smaller quantities. An explanation for this could be their complete solubility in both water and ethanol.

This result confirmed Geroyiannaki's conclusions (2007). Other authors (Crowell and Guymon, 1973; Lehtonen and Eriksson, 1983; Lukic et al., 2011) have found a decrease in the concentration of acetic aldehyde during the distillation process. Regarding the acetic aldehyde concentration in the fractions of the 5 distillates, it is found that in 4 of them (the pears, peach and blackberries distillates) is present in a larger quantity in the tail fraction. Exception makes the cranberry distillate where acetaldehyde is predominant in the head fraction. According to previous research (Garcia-Llobodanin et al., 2007; Colonna-Ceccaldi, 2008), the data in the tables confirm an increase in furfural content from head to tail, to all the analysed distillates.

This behavior is explained by the good solubility of this compound in water. Furfural is formed by Maillard reactions during the distillation process (Hernández-Gómez et al., 2008) and depends on the chemical composition of different fruits.
Distillation patterns of furfural were characterized by a constant increase in concentration, which was in accordance with previous results (Soufleros et al., 2004; Garcia-Llobodanin et al., 2007; Colonna-Ceccaldi, 2008). High boiling points and good solubility in water certainly supported such behaviour. Furfural is formed during distillation by Maillard reactions (Hernández-Gómez et al., 2008).

Table 1. Values of volatile compounds (mg/L a.a.) in different fraction of Williams pear distillate obtained by traditional alembic distillation

Volatile aroma compound	Distillate fraction				
	Head	Heart 1	Heart 2	Heart 3	Tail
Methanol	987.25	4261.86	1212.98	1196.60	739.69
Higher alcohols					
1-propanol	140.36	100.98	78.33	71.38	2.82
3-Methyl-1-butanol	997.06	862.09	573.06	396.61	119.18
1-hexanol	62.10	43.62	30.37	19.69	28.92
1-heptanol	3.22	4.02	3.33	1.30	0.52
1-octanol	2.82	2.77	18.77	3.13	3.54
Benzyl alcohol	-	22.02	0.95	1.99	1.22
Esters					
Ethyl acetate	429.11	305.06	844.47	144.37	60.90
Isoamyl acetate	1.23	1.36	5.12	3.02	4.12
Monoterpenes					
Nerol	-	8.22	2.14	2.60	1.00
Geraniol	3.66	-	-	-	-
Limonene	40.13	-	4.25	3.11	0.61
Aldehydes and ketones					
Acetaldehyde	769.15	513.25	619.03	654.12	1.155.02
Furfural	-	131.74	256.01	713.40	639.67

Table 2. Values of volatile compounds (mg/L a.a.) in different fraction of Favorita lui Clapp pear distillate obtained by traditional alembic distillation

Volatile aroma compound	Distillate fraction				
	Head	Heart 1	Heart 2	Heart 3	Tail
Methanol	1364.62	8945.42	1112.54	1012.45	834.24
Higher alcohols					
1-propanol	75.99	75.22	48.95	31.43	20.83
3-Methyl-1-butanol	745.93	521.71	481.93	306.15	169.17
1-hexanol	43.80	37.39	27.96	19.47	11.53
1-heptanol	1.00	5.02	0.25	2.33	4.02
1-octanol	3.87	3.51	29.80	3.31	39.32
Benzyl alcohol	0.94	1.11	-	0.98	0.15
Esters					
Ethyl acetate	1408.98	264.95	137.69	83.90	749.22
Isoamyl acetate	5.72	-	2.58	0.89	0.25
Monoterpenes					
Nerol	1.01	-	-	1.00	0.25
Geraniol	-	-	-	-	-
Limonene	0.60	2.22	-	0.65	0.11
Aldehydes and ketones					
Acetaldehyde	714.00	1087.02	912.02	1087.02	1002.25
Furfural	117.98	1066.43	512.11	512.33	315.45

Table 3. Values of volatile compounds (mg/L a.a.) in different fraction of Babygold 7peach distillate obtained by traditional alembic distillation

Volatile aroma compound	Distillate fraction				
	Head	Heart 1	Heart 2	Heart 3	Tail
Methanol	1029.56	1131.30	777.79	719.65	315.00
Higher alcohols					
1-propanol	131.98	165.66	133.33	139.87	111.82
3-Methyl-1-butanol	119.65	136.31	92.12	86.79	47.87
1-hexanol	41.97	40.08	32.98	33.17	38.48
1-heptanol	8.55	4.47	-	3.25	1.25
1-octanol	5.27	6.60	4.36	4.02	5.08
Benzyl alcohol	13.98	19.05	17.30	14.76	18.85
Esters					
Ethyl acetate	884.22	108.62	258.69	313.17	67.76
Isoamyl acetate	1.00	0.8715	0.47	6.36	2.15
Monoterpenes					
Nerol	-	-	-	-	-
Geraniol	-	-	-	-	-
Limonene	-	-	-	-	-
Aldehydes and ketones					
Acetaldehyde	959.69	487.23	1081.36	973.22	713.25
Furfural	1.90	1.90	2.15	3.88	6.41

Table 4. Values of volatile compounds (mg/L a.a.) in different fraction of blackberry distillate obtained by traditional alembic distillation

Volatile aroma compound	Distillate fraction				
	Head	Heart 1	Heart 2	Heart 3	Tail
Methanol	736.89	5229.18	987.56	733.96	346.08
Higher alcohols					
1-propanol	239.18	253.39	207.96	184.78	112.91
3-Methyl-1-butanol	1213.13	1421.01	1204.17	749.34	576.95
1-hexanol	15.79	17.43	14.28	13.42	10.33
1-heptanol	1.02	11.05	1.25	2.36	5.11
1-octanol	6.13	6.38	4.50	4.17	2.95
Benzyl alcohol	1.24	-	0.61	0.97	-
Esters					
Ethyl acetate	274.91	306.33	1456.54	125.45	1236.16
Isoamyl acetate	3.25	6.13	-	1.22	0.15
Monoterpenes					
Nerol	495.04	-	-	-	-
Geraniol	15.59	14.16	7.26	7.65	1.31
Limonene	1.01	1.54	-	1.21	-
Aldehydes and ketones					
Acetaldehyde	519.22	858.22	617.22	1235.13	881.25
Furfural	-	449.51	417.60	200.65	215.02

Table 5. Values of volatile compounds (mg/L a.a.) in different fraction of cranberry distillate obtained by traditional alembic distillation

Volatile aroma compound	Distillate fraction				
	Head	Heart 1	Heart 2	Heart 3	Tail
Methanol	1.07348	1295.32	673.73	445.42	52.34
Higher alcohols					
1-propanol	66.05	79.76	641.83	614.19	197.88
3-Methyl-1-butanol	745.80	344.58	987.25	456.12	262.78
1-hexanol	64.67	80.84	63.57	61.39	34.27
1-heptanol	5.44	3.02	4.25	4.25	12.15
1-octanol	3.11	2.01	2.74	2.33	3.89
Benzyl alcohol	1.29	1.37	1.18	1.77	2.09
Esters					
Ethyl acetate	479.97	553.41	565.22	348.37	110.42
Isoamyl acetate	4.55	12.25	7.52	1.25	-
Monoterpenes					
Nerol	1.73	1.51	1.64	1.41	1.23
Geraniol	4.44	5.29	3.74	3.00	-
Limonene	9.58	8.91	7.28	5.96	-
Aldehydes and ketones					
Acetaldehyde	1027.22	615.45	835.14	1008.44	875.00
Furfural	23.93	32.57	31.01	30.68	37.61

CONCLUSIONS

The results obtained from this study show that:
- choosing fractions separating moments during a discontinuous distillation with alembic can significantly influence the concentrations of volatile compounds in fruit distillates;
- higher alcohols and esters are volatile substances with a positive impact on the sensory quality of distillates that are influenced by fraction separation;
-traditional equipment and discontinuous distillation techniques proved to be insufficient to remove as efficiently toxic compounds as methanol and acetic aldehyde;
- in this case, it is necessary to store the fruit mash more carefully, but also to control its alcoholic fermentation.

The most important novelty of this research may be the experimental evidence for the behavior of aromatic varieties during traditional distillation. It has been shown that monoterpenes concentrations increased during distillation and reached the maximum point in the tail fraction. This makes possible the re-distillation of this fraction as a precious raw material and the enrichment with a variety of flavours.

In conclusion, it should be noted that certain deviations from the observed distillation process may be influenced by the conditions of production (type and variety of fruits, consistency and composition of the fruit mass).

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REFERENCES

Apostolopoulou A. A., Flouros A. I., Demertzis P. G., Akrida-Demertzi K., 2005. Differences in concentration of principal volatile constituent in traditional Greek distillates, *Food Control,*16, p.157-164.

Cabaroglu T., Yilmaztekin M., 2011. Methanol and Major Volatile Compounds of Turkish Raki and Effect of Distillate Source, *J.Inst. Brew*., 117, p. 98-105.

Cichi M., 2015. Pear's root system growth and location in the influence of maintenance system in Olteniasoil's, *Analele Universităţii din Craiova,* vol. XX (LVI), p. 91-96.

Colonna-Ceccaldi B., 2008. Use of terpene-producing yeasts in brandy production, Proceedings of the Worldwide Distilled Spirits Conference, Edinburgh, 2008, p. 63-69, Nottingham University Press. Nottingham.

Cortés-Diéguez S., De La Peña M. L., Gómez. E. F., 2003. Approaches to spirit aroma: Contribution of some aromatic compounds to the primary aroma in samples of Orujo spirits*, J. Agric. Food. Chem*., 51, p. 7385-7390.

Cortés S., Gil M. L., Fernández. E., 2009. Chemical affinities between the major volatile compounds

present in a grape pomace distillate, *J. Sci. Food Agric.*, 89, p.1221-1226.

Crowell. E. A., Guymon J. F., 1973. Aroma constituents of plum brandy, *Am. J. Enol. Vitic*., 24, p.159-165.

Durr. P., Tanner. H., Piggott J.R. Ed., 1983, p. 33-48, Ellis Horwood: Chichester

Flouros A. I., Apostolopoulou A. A., Demertzis P. G., Akrida-Demertzi K., 2003. Note: Influence of the Packaging Material on the Major Volatile Compounds of Tsipouro, A Traditional Greek DistillateFood, *Sci. Technol. Int.*, 9, p.371-378.

Garcia-Llobodanin L., Acherandio I., Ferrando M., Guell C., Loapez F., J., 2007. Pear Distillates from Pear Juice Concentrate: Effect of Lees in the Aromatic Composition, *Agric. Food Chem*., 55, p. 3462-3468.

Geroyiannaki M., Komaitis M. E., Stavrakas D. E., Plysiou M., Athanasopoulos P. E., Spanos M., 2007. Evaluation of acetaldehyde and methanol in Greek traditional alcoholic beverages from varietal fermented grape pomaces (*Vitis vinifera* L.), *Food Control*, 18, p. 988-995.

Hernández-Gómez L. F., Úbeda-Iranzo J., García-Romero E., Briones-Pérez A., 2005. *Food Chem.*, 90, p.115-125.

Hernández-Gómez L. F., Úbeda J., Briones. A., 2008. Characterization of wines and distilled spirits from melon (*Cucumis melo* L.), *Int. J. Food Sci. Technol.*, 43, p.644-650

Leaute R., 1990. Distillation in Alembic, *Am. J. Enol. Vitic.*,41, p.90-103.

Lehtonen. M., Eriksson. P.J., Piggott. J.R. Ed., 1983. Volatile and non-volatile compounds in the flavor of alcoholic beverages, in Flavor of distilled Beverages: Origin and Development (Pigot, J. R. Ed.), p. 64-78, Ellis Horwood: Chichester.

Lukić, I., Miličević, B., Banović, M., Tomas, S., Radeka, S., Peršurić, D., 2010. Characterization and differentiation of monovarietal grape marc distillates on the basis of varietal aroma compound composition. *J. Agric. Food Chem*., 58, p. 7351-7360

Lukic I., Srećko T., Borislav M., Radeka Sanja, Dordano P., 2011. Behavior of Volatile Compounds During Traditional Alembic Distillation of Fermented Muscat Blanc and Muskat Ruza Porecki Grape Marcs, *Journal of Institute of Brewing*, 117(3), p. 440-450.

Norio Kakiuchi, Akemi Ohmiya, 1991, Changes in the Composition and Content of Volatile Constituents in Peach Fruits in Relation to Maturity at Harvest and Artificial Ripening J. Japan. Soc. Hort. Sci., vol. 60(1), 1991, p. 209-216.

Nykänen. L., 1986. Formation and Occurrence of Flavor Compounds in Wine and Distilled Alcoholic Beverages, *Am. J. of Enol. And Vitic*., 37, p. 84-96.

Onishi M., Guymon J. F., Crowell E. A., 1977. Changes in some volatile constituents of brandy during aging, *Am. J. Enol. Vitic*., 28, p.152-158.

Plutowska B., Biernacka P., Werdencki W., 2010. Identification of volatile compounds in raw spirits of different organoleptic quality, *J. Inst. Brew*., 116, 2010, p. 433-439

Prado-Ramírez R., Gonzáles-Alvarez V., Pelayo-Ortiz C., Casillas N., Estarrón M., Gómez-Hernández H. E., 2005. The role of distillation on the quality of tequila, *Int. J. Food Sci.Technol.*, 40(7), 2005, p. 701-708.

Santos A. C.C., Duarte F.W., Carreiro S.C., Schwan F.R., 2013. Inoculated fermentation of orange juice (*Citrus sinensis* L.) for production of a citric fruit spirit, *Journal of Institute of Brewing*, 119, p. 280-287.

Silva M.L., Macedo A.C., Malcata F.X., 2000. Review: Steam distilled spirits from fermented grape pomace, *Food Science and Technology*, no.6, pp. 285-300

Spaho N., Dürr P., Grba S., Velagić-Habul E., Blesić M., 2013, Effects of distillation cut on the distribution of higher alcohols and esters in brandy produced from three plum varieties, *Journal of Institute of Brewing,* 119, p. 48-56.

Soufleros E. H., Mygdalia A. S., Natskoulis. P., 2004. Characterization and safety evaluation of the traditional Greek fruit distillated "Mouro" by flavor compounds and mineral analysis, *Food Chem.*, 86, p. 625-636.

Stewart. G.G., 2008. Proceedings of the Worldwide Distilled Spirits Conference, Edinburgh, 243-251, Nottingham University Press: Nottingham.

Stoica Felicia, Muntean Camelia, C. Băducă, L. Giurgiulescu, 2015. Researcheson Chemical Compositionand SensorialAttributesof Natural Fruit Distillates, *Analele Universităţii din Craiova*, vol. XX (LVI), p. 285-291

Stoica Felicia, Muntean Camelia, C. Băducă, 2016. Studies on chemical composition and sensory evaluation of crude and aging apples distillates, *Analele Universităţii din Craiova,* vol. XXI (LVII), p. 285-291.

Tesević V., Nikićević N., Jovanović A., Djoković D., Vujsić L.J., Vucković I., Bonić M., 2005. Volatile Components from Old Plum Brandies, Food Technol. Biotechnol., 43, p. 367-372

Versini G., Franco A.M., Moser S., Manca G., 2012. Characterization of pear distillates from wild and cultivated varieties in Sardinia, *International Journal of Food Science & Technology*, 47(12), p. 2519-2531.

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EFFECT OF STORAGE CONDITIONS ON WHEAT QUALITY PARAMETERS - A MINIREVIEW

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Abstract

Wheat is one of the most important cereals grown worldwide. After harvesting it must be stored in appropriate conditions in order to retains its nutritional and quality properties. During storage the wheat can be degraded due to improper storage conditions. The analyzes that are assessed to establish the quality of the wheat stored for a longer period are: proteins content, humidity, ash, Falling Number test and aflatoxins incidence. This paper makes an overview of the storage conditions available worldwide, the effects of the storage conditions on the quality of the wheat preserved in silos, warehouses or halls. In order to monitor the wheat storage condition it is necessary to follow the important parameters of storage, temperature and relative humidity. Optimal storage can ensure the good quality and and ventilation. Therefore, it is very important that the storage areas are properly prepared after harvesting. This preparation involves a general cleaning, followed by disinfection, derating and repair of cracks in the walls and floor, which, could become nests conducive to the development of insect larvae. Proper storage of agricultural products but also initial quality parameters of raw material represent very important factors for maintaining their quality and quantity for a long period of time.

Key words*: wheat, storage,conditions, quality parameters, silo type.*

INTRODUCTION

Wheat is one of the largest food crops consumed globally as a basic raw material. It can be stored for more than one year with a moisture content of 12-13%, however, it requires appropriate storage and environmental conditions restrict to inhibit infestation of insects, rodents and fungi. Storage is the mandatory stage in the wheat supply chain. Losses caused by insect infestation during storage account for a major part (10-30%) of post-production wheat losses (Paliwal et al., 2004), worth around $1 trillion a year (Kumar and Kalita, 2017). Quantitative loss, metabolites produced by insects, such as excreta and by-products of protein metabolism, give off an unpleasant smell. Also, during storage, wheat develops various odors, which with the passing of time is called a storage smell. During storage time, the odors generate are generally composed by aliphatic alcohols, amine compounds, ketones and other carbonyl compounds (Zhang and Wang, 2007). The main smell is produced by *Rhyzopertha dominica* and *Tribolium castaneum* (Laopongsit et al., 2014). As storage time increases, medium polarity odors also increase with a simultaneous reduction of low polarity odors (Olsson et al., 2000). As agriculture developed on an industrial scale, and farmers have learned to grow crops in greater quantities than the quantities needed for their immediate use, the need to store and transport large quantitiesof cereals was evident.

Today, cereals consumed in industrialized countries are produced by only a small proportion of the total population through highly mechanized agricultural operations. Cereals are biological materials which interact with their immediate environment. They must be stored, transported using methods which maintain the quality of the seeds, foodstuffs or raw materials.

Storage may take place either on the farm or on commercial premises outside the farm. Wheat can be stored for different periods of time, from short-term storage in which only drying takes place, to longer periods leading to its recovery and long-term storage for special stocks. Storage on farm premises is usually smaller than commercial installations.

Cereals are usually stored in cylindrical metal silos made of metal corrugated sheets which are fixed together. The size of the farm's pubs has increased significantly in recent years, and today some of the farm's large dumps are similar in size to those observed in commercial operations. Commercial grain storage facilities are often located in grain-producing areas, factories and other processing plants, at grain-handling terminals located in railway centers and in ports. Grain hoppers are usually much larger than agricultural trash used on the farm. For these facilities, concrete reinforced cylinders are commonly used, although large metal grain bins are still common. Flat-rate storage is also observed at commercial installations where cereals are stored in storage or in piles for short-term storage.The quality control of storage in silos is done indirectly through the control of moisture and air movements. Venting system reduces damage to stored grain. The cooling of cereals during storage has received increasing attention in recent years (Wilkin et al., 1990) and has been widely used in cereal stock management (Edde, 2012; Jayas, 2012; Navarro, 2012). Keeping the temperature low provides the ability to control insects with low levels of pesticide applications (Yang et al., 2017).

Moisture exchange is an important manage-ment process, as cereals adsorb or dehydrate moisture under different environmental conditions. Globally, more than two billion tons of cereals are harvested annually (D. S. Jayas, 2012). The harvested cereals must be stored safely in order to meet the food demand of the population and, in particular, to meet emergency needs such as disaster and hunger. Safe grain storage can be achieved by handling two important physical factors: Temperature and moisture content (D. S. Jayas, 2012).

Compared to temperature monitoring, grain moisture detection is more difficult for different seed distribution phases. A non-destructive and economical wheat moisture detection system has been implemented with WiFi, i.e. Wi-Wheat. The proposed system does not need any dedicated device, which means that it will cost less with easy deployment. Using WiFI and specialized apparatus, it can be experimentally proved to detect wheat moisture using amplitude and phase differences data. The detailed design of the Wi-Wheat system proposes extracting and processing amplitude data. The amplitude and difference data are extracted in front of the operating receivers of the installations. Once data is collected, the device processing module consists of pre-processing data, feature extraction, and classification of support vector technology. To pre-process data, we use external detection, data normalization, and noise elimination to obtain amplitude data that can detect the increase in stored wheat moisture (Wi-Wheat: Contact-free Wheat Moisture Detection with Commodity WiFi, 2018).

SILAGE MODELS WITH AERATION SYSTEMS

This silage model consists of a vertical cylindrical part (silo body), conical part (bunker) and roof made of zinc-coated steel. The tank and roof reinforcement rings were 50 mm wide and 10 mm thick. Silos can be considered as a rigid steel silo with smooth walls. The dimensions of the silos are 1 m high and 0,5 m in diameter, and the wall of the bunker has a tilt angle of 15 degrees with a height of 0,3 cm and a discharge opening diameter of 0,33 m. The aeration system consists of a vertically pierced pipe of the blower located centrally on the inside for the air inlet distribution along the height of the silos, the air flow control valve (Thermal and mechanical analysis of grain storage silos under forced aeration conditions using advanced modeling methods, 2019).

STRESS, TEMPERATURE AND RELATIVE HUMIDITY SENSORS

The stress measurement device consists of three vertical (Type S) and horizontal (Type L) load cells, which are calibrated at the concrete testing laboratory at the Engineering Faculty at Kafresheikh University. The temperature and humidity control method for the silos in which the seeds were stored was presented by investigating different aeration strategies and geometric shapes of the silos. The difference, between temperature measurements and grain pressure in a vertical and horizontal plane, with the response surface can effectively represent

the performance of the control system that helps to classify operating parameters. The thermal and mechanical model of silo was developed based on empirical measurement. The developed prototype may predict the heat and moisture behavior of the grain storage silos with an aeration system similar to the environment at different specific air enthalpy (Thermal and mechanical analysis of grain storage silos under forced aeration conditions using advanced modeling methods, 2019).

MEASURING WHEAT MOISTURE

Existing methods for measuring wheat moisture can be classified in: American society of Agricultural Engineers, 2001, capacity method (W. Wang et al., 2011), resistance method (Z. Liu et al., 2015), microwave method (K. Kim et al, 2006) and neutral method (Y. Yang et al., 2000).

The American society of Agricultural Engineers (2001) method is widely used. Although this method is fairly accurate, it is intended for the laboratory environment; it does not meet the requirements for online moisture detection in the field. Method for detecting capacity humidity (W. Wang et al., 2011) fairly widespread, but its performance is limited by the fact that measurement values are not only sensitive to temperature, but also to the flow rate of the grain. The method involves checking several parameters and after each use of the device its sensor must be fixed. In the case of the resistance method (Z. Liu et al., 2015), the grain moisture detector is designed based on the model of the relationship between measurement frequency and grain moisture and non-linear temperature correction method. The device consists of two computers. The lower computer mainly senses resistance values; the upper computer focuses on the conversion of humidity and frequency and non-linear temperature correction. Microwave method (K. Kim and et al., 2006) and neutral method (Y. Yang et al., 2000) these two methods show high accuracy, fast detection speed, non-destructive and non-invasive measurements and can additionally measure the inert moisture of the wheat grain, but the measurement equipment is complex at high cost.

DRYING AND STORAGE PRACTICES

Over time it was found that a superior storage is required for the impression quality of grain and not. Studies have shown the importance in improving food storage, especially in areas deficient of moisture (Nduku et al., 2013). Because of the numerous efforts to reduce postharvest losses, they were implemented related to wheat in sealed bags under optimum conditions (Nash et al., 2017).Storing quality helps in storing wheat grains, seeds quantitative prevents loss due to insects, rodents and mycotoxins (Adetunji, 2007). Most often the damage is due to quantitative contamination of grain with aflatoxins (Kang'ethe, 2011; Maina et al., 2016). It has been observed that keeping wheat in airtight bags reduced its degradation caused by insects and mycotoxins (Njoroge et al., 2014; Maina et al., 2016; Williams et al., 2014). They were subjected to a storage test of wheat two methods. The sealed plastic bags sprayed with pesticide as a first method and a second method, a plastic bag with pesticides, each method was repeated 10 timesThe storage of seeds meant drying them at 12% humidity and subjecting them to the analysis of the impurity content. Shelf lasted six months (May-October) period after harvest until the next sowing. The bags are made of high density polyethylene, reducing the transfer of gas from the outside to the inside and vice versa. This method of storage was examined six months after the making and was found: moisture content of the seeds is lower in hermetic bags treated with the pesticide. Drying the seeds prior to the time increase the quality of wheat.

STORAGE CHALLENGES

Most serious problems during storage of cereals are due to hexapod and rodents. Those who keep long-term wheat have adapted to the situation and have improved storage technologies. They also use chemicals to eliminate pests, but also using the hermetic storage increased (Nicolopoulou-Stamati et al., 2016). Due to these problems the farmers emphasizes that the research conducted under airtight storage for six months is safer and more cost-effective compared to the use of chemicals (Foy & Wafula, 2016).

AVOIDING MOLD DEVELOPMENT BY CARRYING OUT DRYING

An important factor for long-term storage under 14% humidity is low, this is achieved according to the category of grain. Adjusting the drying air temperature should be performed at each time point as the pericarp of the grain moisture content of the grain becomes impermeable to water vapor (Gaceu L., 2001; Owens, G., 2003).

THE DESIGN AND THE THERMODYNAMICS OF GRAIN STORAGE SILOS

For a period of time cereals is carried out in special silos (Figure 1). The silo consists of: 1 - air outlet; 2- grain intake; 3 - the silo roof; 4- silo walls; 5 - fan; 6 - air duct; 7 - silo foundation; 8 - circular concrete distribution system; 9 - floor support; 10 - perforated floor. During the storage, biochemical phenomena lead to enhancing the density of grain moisture and temperature, for which reason it takes a regular aeration. The process aeration of stored seeds has four general objectives - prevent mold, insects inhibited emergence, maintenance durability and reduced seed grain moisture.

Cereals without aeration retains heat long because they are a good thermal insulator. In a short period of a few days, if the air does not move will be stable temperature and humidity. During action aeration, moisture in the grain must be moved in the air. The environment must have low relative humidity to have a high percentage of moisture in the transfer process. The percentage is different depending on the variety of grains, each grain having a moisture content and stability between the relative humidity of the air. When you reach a certain point Stability Guarantee, moisture transfer does not occur.Humidity's stabilityis shown in Table 1, depending on the relative humidity of the air at 20°C (Design of silos to control mold growth, 2019).

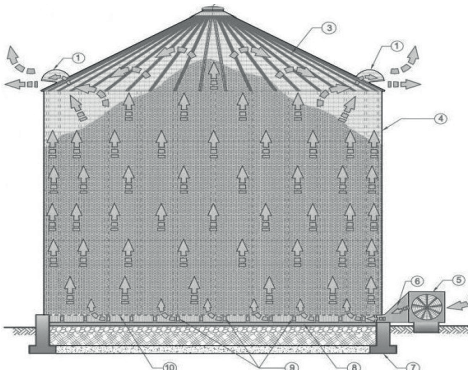

Figure 1. Typical grain silo diagram
https://www.mgtrade.ro/en/

Table 1. Equilibrium moisture [%], depending on the relative humidity of the air, at 20°C (Gaceu L., 2001)

Species	Air Relative Humidity, %				
	20	30	50	70	90
Wheat	7.8	9.2	11.8	14.3	19.9
Rye	8.3	9.5	12.2	15.2	20.8
Barley	8.3	9.5	12.0	15.2	20.9
Corn	8.2	9.4	11.9	14.9	19.2
Sunflower	-	-	5.9	7.8	11.4
Soy	-	-	6.4	8.1	12.8

THE PROCESS OF COOLING AND DRYING

The models of equipment used for aerating the cereals are designed to carry out two forms of processes: cooling with air and also drying with air. The process of cooling by aeration may be achieved by a low flow of air 2-3 liters/second/ton. As long as air drying lasts, the environment can be heated more by using a heat sink device. There are 3 modes of action aeration by moisture grain: Grains that meet market specifications have a moisture content between 12 and 14%. They may be subjected only to a cooling process, removing the drying, so much diminishes the appearance and growth of the insect, and will maintain quality during storage. Grain having a moderate humidity (16% for wheat) require drying by aeration to reduce moisture, this is done to maintain quality during storage.

When drying cannot be carried out immediately on moderately moist grains, they will cool for a period of time, thus reducing mold and insect growth until a piece of equipment is free. When drying has been carried out, cereals in the category with a moisture content of between 12 and 14 % will be cooled to retain their qualities (Grains Research and Development Corp., 2011).

MAINTAINING A LOW TEMPERATURE IN WINTER

Costs aeration in winter of cereals are much lower, which brings a very high gain. When the weather is very cold, humidity is very low and the ventilation in the cold did remove some of the moisture content of the grain. Grains are a good thermal insulator, and after aeration they remain at very low long-term elevated temperature. The restart process of aeration humidity increased, the occurrence of moisture on the surface of the occasions of cold cereals, mushrooms influencing enhancing development (Grain Storage fact sheet, 2011).

CONCLUSIONS

Storage of grain in a sealed environment maintain constant temperature and moisture content.

Warehouse where wheat is stored should be fine-tuned to reduce microbiological contamination and improve grain quality.

Selected storage grains should have a low contamination level, have to be cleaned and passed through a specific selection test.

The storage can inactivate metabolic changes but we cannot permanently stop these reactions.

Advanced storage method is more convenient to use, increase the length of life, improves the organoleptic properties during storage.

Advanced storage method consumes less time processing is easier and reduce costs.

A storage which follows certain procedures will greatly reduce the risk of degradation while wheat and reduce harmful mycotoxins development of people and animals.

Mold growth in the storage of grain is a special issue because of the harmful consequences of mycotoxins on human health.

Thermal insulation properties and hygroscopicity grain causes the aeration process is carried out in an appropriate manner, to prevent the growth of grain moisture and condensation.

REFERENCES

Adetunji, M. O. (2007). Economics of Maize Storage Techniques by Fanners in Kwara State, Nigeria. *Pakistan Journal of Social Sciences*, 4(3), 442-45. https://doi.org/10.1080/10454440802537280. Design of silos to control mold growth (2019).

Edde, P. A. (2012*).* A review of the biology and control of Rhyzoperthadominica (F.) the lesser grain borer. *Journal of Stored Products Research*, 48: 1-18.

Foy, C., & Wafula, M. (2016). *Scaling up of Hermetic Bag Technology (PICS) in Kenya: Review of successful scaling of agricultural technologies*. United States Agency for International Development. Retrieved from https://www.agrilinks.org/sites/default/files/resource/files/BFS%20Scaling%20Review%20-%20Kenya%20Report%20REVISED%20508%2011-16-16.pdf.

Gaceu L. (2017). Aspects regarding electrochemical detection of the antioxidant activity for subcritical extracts from *Pleurotus ostreatus*, *Journal of EcoAgriTourism*, Vol. 13, No. 1, ISSN 1844-8577, pp. 53-57, ref.12.

Gaceu L. (2017).Comparative study regarding the antioxidant activity of subcritical extracts from *Vitis semen*, Mustard and *Polygonum cuspidatum*, *Journal of EcoAgriTourism*, Vol. 13, No. 2, ISSN 1844-857, pp. 48-52, ref.12.

Grain storage fact sheet (2011). *Grains Research and Development Corporation*. Available at: https://agridrydryers.com/wpcontent/uploads/2019/01/Dealing-withHigh-Moisture-Grain.pdf.

Grains Research and Development Corp. (2011). Stored Grain Project. Aerating sored grain. Cooling or drying for quality control. A Grains Industry Guide. Available at: https://www.graintec.com.au/media/34545/ Aerating%20stored%20grain%20%20A%20Grains%20Industry%20Guide.pdf.

Jayas, D.S. (2012). Storing Grains for Food Security and Sustainability. *Agricultural Research*, 1:21-24.

K. Kim, J. Kim, C. Lee, S. Noh, and M. Kim (2006). "Simple instrument for moisture measurement in grain by free-space microwave transmission", *Trans. ASABE*, vol. 49, no. 4, pp. 1089-1093.

Kang'ethe, E. (2011). Situation analysis: *Improving food safety in the maize value chain in Kenya*. Report prepared for FAO. College of Agriculture and Veterinary Science, University of Nairobi, Nairobi. Retrieved from http://www.fao.org/fileadmin/user_upload/agns/pdf/WORKING_PAPER_AFLATOXIN_REPORTDJ 10thOctober.pdf.

Kumar, D., Kalita, P. (2017). Reducing postharvest losses during storage of grain crops to strengthen food security in developing countries. *Foods*, 6(1), 8.

Maina, A. W., Wagacha, J. M., Mwaura, F. B., Muthomi, J. W., &Woloshuk, C. P. (2016). Postharvest practices of maize farmers in Kaiti District, Kenya and the impact of hermetic storage on populations of Aspergillus spp. and aflatoxin contamination. *Journal of Food Research*, 5(6), 53. https://doi.org/10.5539/jfr.v5n6p53.

MG Trade Engineering. *Silos, Dryers*, *Flat storage facilities, Seed installations, Feed mills*. Available at: https://www.mgtrade.ro/en/.

Nash, J., Peña, O., Galford, G., Gurwick, N., Pirolli, G., White, J., &Wollenberg, E. (2017). Reducing food loss in agricultural development projects through value chain efficiency. *CCAFS Working Paper*, No. 204. Retrieved from https://ccafs.cgiar.org/ publications/ reducing-food-loss-agricultural-development-projects-thr ough-value-chain-efficiency#.W84afvZRdpk

Navarro, S. (2012). The use of modified and controlled atmospheres for the disinfestation of stored products. *Journal of Pest Science*, 81:1-24.

Nduku, T. M., De Groote, H., &Nzuma, J. M. (2013). Comparative analysis of maize storage structures in Kenya. *4th Conference of the African Association of Agricultural Economists (AAAE)*. Retrieved from https://pdfs.semanticscholar.org/4097/4684765e2cbed f51180c96466c873d4718ec.pdf.

Nicolopoulou-Stamati, P., Maipas, S., Kotampasi, C., Stamatis, P., & Hens, L. (2016). Chemical pesticides and human health: The urgent need for a new concept in agriculture. *Frontiers in Public Health*, 4, 148. https://doi.org/10.3389/fpubh.2016.00148.

Njoroge, A. W., Affognon, H. D., Mutungi, C. M., Manono, J., Lamuka, P. O., & Murdock, L. L. (2014). Triple bag hermetic storage delivers a lethal punch to Prostephanustruncatus (Horn) (Coleoptera: Bostrichidae) in stored maize. *Journal of Stored Products Research*, 58, 12-19. https://doi.org/10.1016/j.jspr.2014.02.005.

Olsson, J., Börjesson, T., Lundstedt, T., Schnürer, J. (2000). Volatiles for mycological quality grading of barley grains: determinations using gas chromatography–mass spectrometry and electronic nose. *Int. J. Food Microbiol*., 59(3), 167-178.

Owens G. (2003). *Cereals processing technology*, Woodhead Publishing Limited Abington Hall, Abington Cambridge, England, 238 pp., ISBN 1 85573 561 X.

Paliwal, J., Wang, W., Symons, S.J., Karunakaran, C. (2004). Insect species and infestation level determination in stored wheat using near-infrared spectroscopy. *Can. Biosyst. Eng*., 46, 7.17-7.24.

Thermal and mechanical analysis of grain storage silos under forced aeration conditions using advanced modelling methods (2019).

W. Wang and Y. Dai (2011*). A grain moisture detecting system based on capacitive sensor*, *Int. J. Digital Content Technol. Appl.*, vol. 5, no. 3, pp. 203–209, Mar.

Williams, S. B., Baributsa, D., &Woloshuk, C. (2014). Assessing Purdue Improved Crop Storage (PICS) bags to mitigate fungal growth and aflatoxin contamination. *Journal of Stored Products Research*, 59, 190-196. https://doi.org/10.1016/ j.jspr.2014.08.003.

Wi-Wheat: Contact-free Wheat Moisture Detection with Commodity WiFi (2018).

Wllkin, D. R , Armitage, D M., Cogan, P. M. and Thomas, K. P. (1990). *Integrated pest control strategy for stored grain*. Project Report No. 24, 87 pp. Home-Grown Cereals Authority, London, UK.

Y. Yang, J. Wang, C. Wang et al. (2000). Study on on-line measurement of grain moisture content by neutron gauge, *Trans. Chinese Society of Agricultural Engineering*, vol. 16, no. 5, pp. 99-101.

Yang, Y.; L. T. Wilson; F. H. Arthur; J. Wang and C. Jia. (2017). Regional analysis of bin aeration as an alternative to insecticidal control for post-harvest management of *Sitophilus oryzae* (L.) and *Rhyzopertha dominica* (F.). *Ecological Modelling* 359, 165-181.

Z. Liu, et al. (2015). Research on online moisture detector in grain drying process based on V/F conversion, *Hindawi Math. Problems Eng*., vol. (2015), p. Article ID 565764.

Zhang, H., Wang, J. (2007). Detection of age and insect damage incurred by wheat, with an electronic nose. J. *Stored Prod. Res*. 43(4), 489-495.

Zhang, H.,Wang, J., Ye, S.,Chang, M. (2012). Applicationof electronic nose andstatistical analysis to predict quality indices of peach. *Food Bioprocess Technol*., 5(1), 65-72.

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BIOGENIC AMINE ANALYSIS IN FRESH MEATS AND MEAT MEALS USED AS RAW MATERIALS FOR DRY PET FOOD PRODUCTION

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Abstract

Fresh meats and meat meals are important components for the production of dry pet food. Both of these raw materials are by-products of meat processing generated during the production of food for human consumption. Being by-products, they can be more subject to contamination and proliferation of microorganisms which degrade the organic component and lead to the development of degradation products such as biogenic amines. Biogenic amines are nitrogen compounds produced by microbial decarboxylation of amino acids, thus being very present in foods rich in certain amino acids. The ingestion of foods containing a large amount of biogenic amines can cause intoxication and harmful consequences for the body. The increase in the presence of biogenic amines in food can be attributed to direct contamination by microorganisms or to inappropriate storage conditions of the food. In fact, to prevent the formation of biogenic amines, it is needed to respect the proper times and methods for the conservation of the raw materials used.
This study analyses the possible presence of biogenic amines in the raw materials used for dry pet food production through the use of mass spectrometry, capable of identifying molecules present in small quantities.
The results show how meat meals have higher concentration of biogenic amines compared to fresh meats, suggesting that the proliferation of microorganisms and the consequent formation of these nitrogen compounds in meat meals is probably due to the low quality of the raw materials used and to their inadequate storage conditions.

Key words*: biogenic amines, pet food, fresh meats & meat meals, toxicological aspects, raw material quality.*

INTRODUCTION

Amine compounds are molecules naturally present in living organisms and therefore in food as a consequence of metabolic processes. This kind of amines are chemicals that occur naturally, generated by bacteria degrading the amino acid component. They are classified in three categories, according to their chemical structure: Aromatic amines (Histamine, 2-Phenethylamine, Tryptamine and Tyramine), Aliphatic amines (Cadaverine and Putrescine) and Aliphatic polyamines (Agmatine, Spermidine and Spermine) (Figure 1).
These compounds can be labelled as "biogenic amines" (Bardózc, 1995; Smith, 1980), and are formed by the decarboxylation of amino acids or by the amination or transamination of aldehydes and ketones by specific microbial enzymatic pathways (Learey et al., 2018; Shalaby, 1996; Suzzi & Torriani, 2015).
The formation of biogenic amines is strictly dependent on the content of proteins and free amino acids in the food. In the case of pet food, whose ingredients are mainly represented by meat processing by-products and therefore particularly rich in protein, they are often characterized by the presence of high concentrations of biogenic amines (den Brinker et al., 2003; Learey et al., 2018).
In fact, if on the one hand, the presence of biogenic amines depends on the type of microorganisms present in food and their growth, on the other hand, it is also strictly dependent on factors associated with raw materials such as meat composition, pH and

handling conditions; all these factors influence the availability of free amino acids subject to the microbial decarboxylation reaction (Ruiz-Capillas & Jimenez-Colmenero, 2005).

Biogenic amines can then represent a valid indicator for evaluating the freshness of pet food and the possible microbial contamination it can undergo (Learey et al., 2018). The raw materials used for the production of dry pet food are mostly composed of animal by-products that can be subject to the formation of biogenic amines due to transport and handling processes, during which a series of reactions mediated by proteolytic enzymes of microbial derivation can lead to the formation of free amino acids possibly undergoing decarboxylation reactions. This process continues until the ingredients are subject to high temperature stages, such as extrusion, which deactivate the proteolytic enzymes. Heat treatments cease the action of enzymes but do not destroy biogenic amines, which are instead stable to heat. Hence, the concentration of formed amines will not be reduced during processing and may even increase during storage phases, due to further microbial contaminations (Radosevich, 2006; ten Brink et al., 1990). The majority of dry pet food nowadays on the market is produced starting from two different kinds of raw materials: meat meals and fresh meats (Thompson, 2008). Fresh meats are obtained as waste of the meat intended for human consumption, while meat meals derive from meat by-product processing according to the Regulation (EC) No 1069/2009 of the European Parliament and of the Council of 21/10/2009. These meals are mainly used by pet food manufacturers to supply protein sources in order to prepare pet kibbles; however, the intensive industrial process they undergo may cause the onset of raw material degradation which could foster microbial processes leading to the formation of biogenic amines (Camire et al., 1990; Lankhorst et al., 2007; Piergiovanni & Limbo, 2010; Rokey, 2010; Singh et al., 2007; Tran et al., 2008; Williams et al., 2006).

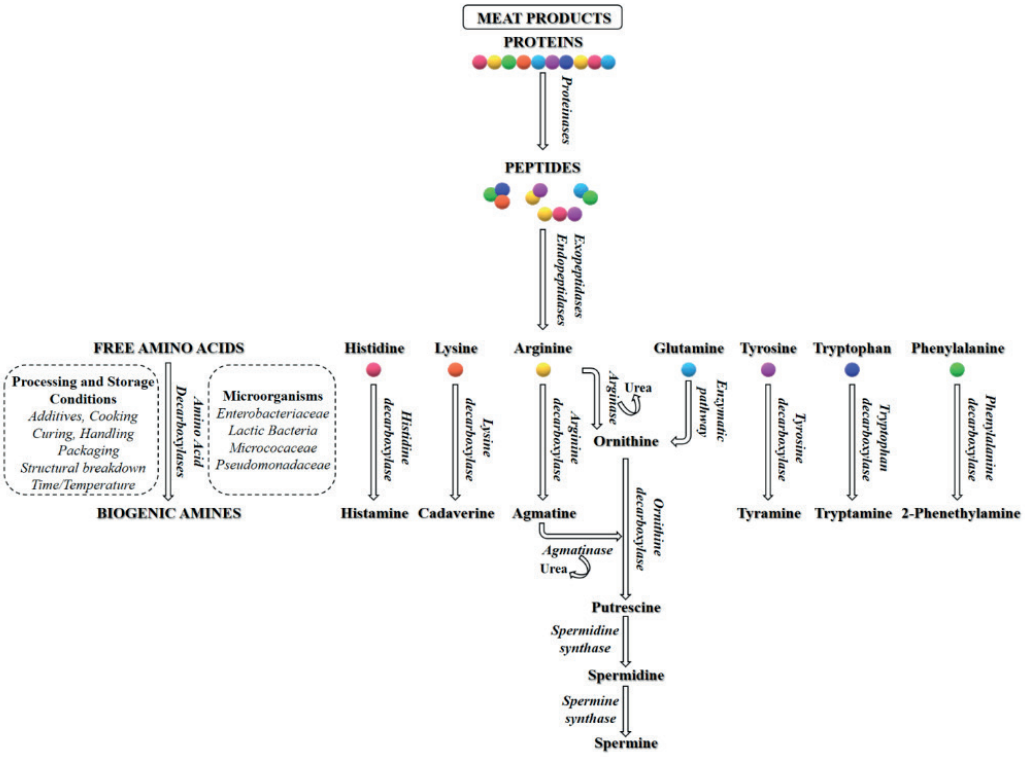

Figure 1. Representation of the factors influencing the formation of biogenic amines

A possible way to limit the formation of biogenic amines in pet food could therefore be represented by a series of measures aimed at reducing microbial contamination and the subsequent degradation of the protein component during the storage and handling of raw materials used for pet food production. It has been shown that in sterile meat there are no biogenic amines, while their concentration increases proportionally in parallel with the development of microbial flora (Bardózc, 1995; Slemr & Beyermann, 1985). Although some biogenic amines, such as Putrescine and Cadaverine, are naturally present at low concentrations in meat products following some reactions of cellular metabolism, the pool of biogenic amines in the final product is very often far greater than the quantities naturally present. It is therefore necessary to control all those factors and processes that can promote the formation of free amino acids and microbial growth, in order to limit the presence of these amines in the final product. It is thus clear how the storage and handling of raw materials can greatly influence the concentration of biogenic amines. For instance, as reported above, it has been seen that heating processes of raw material can significantly reduce the concentration of biogenic amines, by determining the inactivation of the microbial decarboxylases; however, the presence of these substances in the final product is directly dependent on the quality of the different raw materials used which may have already developed a large amount of these amines. In fact, high concentration of biogenic amines in processed meats or meat by-products indicates low quality of the starting raw materials (Bover-Cid et al., 2001; Paulsen et al., 1997; Ruiz-Capillas & Jimenez-Colmenero, 2005). Among biogenic amines, utmost importance is placed on Histamine, responsible for allergic reaction (Kovacova-Hanuskova et al., 2015; Maintz & Novak, 2007; Taylor & Eitenmiller, 1986; White, 1990), Cadaverine, Tyramine, Tryptamine, 2-Phenethylamine, Putrescine, Spermidine and Spermine, which all can have toxic effects on the body in different ways (del Rio et al., 2019; Learey et al., 2018; Lewis, 1998; Til et al., 1997). To date, no guidelines have been drawn regarding the threshold levels for biogenic amines present in pet food, although numerous studies have been carried out showing that high concentrations of some biogenic amines, *e.g.* histamine, induce adverse effects in animals (Bjeldanes et al., 1978; Blonz & Olcott, 1978; Privitera et al., 1969). Adult animals are usually able to detoxify biogenic amines at low concentrations, while puppies may develop harmful effects following the daily intake of even small quantities of these substances (Radosevich, 2006).

The aim of this work is to evaluate the presence of biogenic amines in the raw materials used for dry pet food production. An analysis of the different samples by LC/MS–QTOF (Liquid Chromatography/Mass Spectrometry - Quadrupole Time Of Flight) was carried out in order to quantify the possible presence of biogenic amines.

MATERIALS AND METHODS

Raw Materials

The raw materials used in this study are listed in Table 1 and they consist of: chicken fresh meat for companion animal food, 10 batches from pet food manufacturers (Italy), chicken meat meal for companion animal food, 10 batches from pet food manufacturers (Italy); pork fresh meat for companion animal food, 10 batches from pet food manufacturers (Italy), pork meat meal for companion animal food, 10 batches from pet food manufacturers (Italy); salmon fresh meat for companion animal food, 10 batches from pet food manufacturers (Italy), salmon meat meal for companion animal food, 10 batches from pet food manufacturers (Italy).

Table 1. List of raw materials used in this study

Raw Materials		
Chicken	Fresh meat for companion animal food	10 batches from pet food manufacturers
	Meat meal for companion animal food	10 batches from pet food manufacturers
Pork	Fresh meat for companion animal food	10 batches from pet food manufacturers
	Meat meal for companion animal food	10 batches from pet food manufacturers
Salmon	Fresh meat for companion animal food	10 batches from pet food manufacturers
	Meat meal for companion animal food	10 batches from pet food manufacturers

Determination of Moisture content

Food moisture was calculated according to the method described by da Silva et al. (2018). Briefly, an exact amount of raw material (40 g) was dried in oven (Termaks TS 8136) at 90°C

for 6 hours, then it was cooled down at room temperature in a desiccator containing silica gel. Samples were then weighed using OHAUS™ Analytical Balance (Pioneer™) until a stable weight was reached. Water content was calculated as the difference between initial and final weight.

Sample preparation

A quantity corresponding to 100 mg of each dry sample was carefully weighed in an Eppendorf tube and 1 mL of Methanol containing 2.5 µg/mL of Phenylglycine as Internal Standard was added. Tubes were shaken 20 minutes at 1500 rpm at room temperature in a Thermomixer (T-Shaker Thermomixers, EuroClone). The tubes were then centrifuged at 3300 × g for 10 minutes (Eppendorf™ 5415D Centrifuge) and the supernatant transferred into a vial. An amount corresponding to 0.5 µL of each sample was injected into the LC/MS-QTOF system (Agilent™ 1290/Agilent™ 6530).

Determination of Biogenic Amines

The Ion Pairing Chromatography (IPC) method was used to achieve a wide separation of polar metabolite classes with 150 × 2.1 mm, 3 µm ACME Amide C18 column (Phase Analytical Technology, LLC) thermostated at 50°C. The separation of biogenic amines was achieved using a flow of 0.35 mL/min of a binary gradient of 0.3% heptafluorobutyric acid in water (solvent A) and 0.1% formic acid in Methanol (solvent B). Initial condition was 2% of B for 2 minutes followed by a gradient from 2 to 80% of B in 5 minutes, and a final isocratic step of 8 minutes.

The spectrometer operated in high resolution full scan mode monitoring positive ions. The quantitative data were obtained by external calibration in the range 0.05-2.5 µg/mL of a homemade mix of each biogenic amine in pure Methanol.

Statistical analysis

Data shown in this study, regarding the analysis of the content of biogenic amines of the raw materials used for dry pet food production, are reported as mean values of the ten analysed batches (Table 1) ± standard error of the mean (SEM). The t-Student test was used to investigate the significance of the different biogenic amine content in meat meals and fresh meats. The level of significance for the data was set at $p < 0.05$. All statistical tests were done using GraphPad Prism 6.00 for Windows (GraphPad Software, Inc., San Diego, CA).

RESULTS AND DISCUSSIONS

The presence of biogenic amines in the raw materials was evaluated through LC/MS-QTOF. This analysis was performed following the method explained in the Materials and Methods section.

Prior to the assessment of biogenic amines by LC/MS-QTOF, the moisture level was evaluated for each raw material. The results shown in Figure 2 reveal how, as expected, fresh meats exhibit higher water content compared to meat meals.

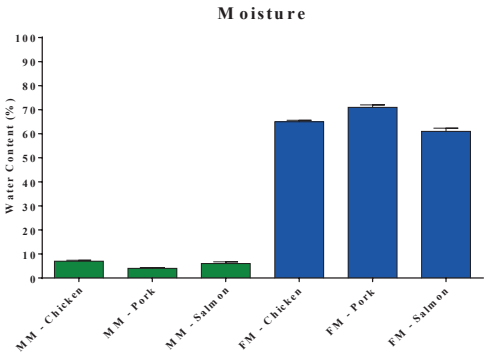

Figure 2. Water content in chicken, pork and salmon meat meal (MM) and fresh meat (FM) for companion animal food determined by stable weight reaching after oven-drying at 90°C for 6 hours, data are reported as mean ± SEM, n = 10

The humidity level in fresh meats ranges from about 60% in the case of salmon to 70% in the case of pork, whereas a water content lower than 10% is peculiar to all meat meals. This feature is the result of the high temperature treatment and dry processes used for the preparation of meat meals.

Subsequently, Mass Spectrometry analysis was performed on the same amount of dry sample for each category of raw material in order to evaluate the content of biogenic amines. The previously obtained data regarding moisture allowed to calculate the quantity of wet sample that had to be taken in order to reach the same dry quantity for each class of raw material. The

content of biogenic amines was expressed in mg/kg of dry sample.
The first biogenic amines analysed was Histamine. This amine derives from the decarboxylation of the amino acid Histidine, and its intake is involved with the development of allergic phenomena (Ruiz-Capillas & Jimenez-Colmenero, 2005). The results shown in Figure 3 display how fresh meats has a significantly lower quantity of histamine compared with meat meals, and this happened for all the raw materials analysed.

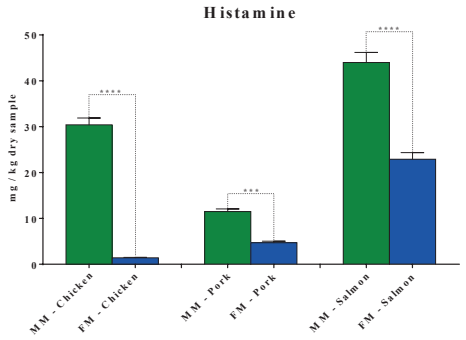

Figure 3. Histamine content in chicken, pork and salmon meat meal (MM) and fresh meat (FM) for companion animal food determined by LC/MS-QTOF and normalized for water content (expressed as mg of Histamine per kg of dry sample). Data are reported as mean ± SEM, n = 10; ***$p < 0.001$, ****$p < 0.0001$

The amount of Histamine in the chicken fresh meats, for all the batches analysed, is about twenty times less than in meat meals; as for pork and salmon fresh meats, the quantity of Histidine is about halved with respect to the corresponding meat meals, with the highest concentrations being recorded for the salmon. These results highlight how pet food products made with meat meals may contain more Histamine and therefore be more harmful to pets, which may thus undergo toxic and allergic reactions (Bjeldanes et al., 1978; Blonz & Olcott, 1978; del Rio et al., 2017; Kovacova-Hanuskova et al., 2015; Lewis, 1998; Linares et al., 2016; Maintz & Novak, 2007; Privitera et al., 1969; Taylor & Eitenmiller, 1986; White, 1990). Subsequently the concentration of Cadaverine, a biogenic amine deriving from the decarboxylation of the amino acid Lysine (Ruiz-Capillas & Jimenez-Colmenero, 2005), was evaluated. The results show that the concentration of Cadaverine in chicken fresh meats is more than three times smaller than in meat meals; while in pork fresh meats it is less than half as compared to its content in meat meals. In salmon fresh meat was found a concentration of Cadaverine four times lower than in the corresponding meat meal, which also showed the highest concentration among those analysed. (Figure 4). In general, all the fresh meats analysed show a statistically significant lower content of Cadaverine compared to meat meals.

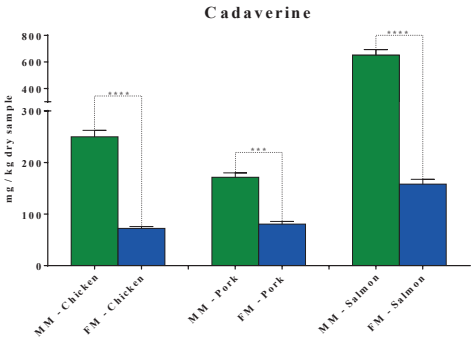

Figure 4. Cadaverine content in chicken, pork and salmon meat meal (MM) and fresh meat (FM) for companion animal food determined by LC/MS-QTOF and normalized for water content (expressed as mg of Cadaverine per kg of dry sample). Data are reported as mean ± SEM, n = 10; ***$p < 0.001$, ****$p < 0.0001$

These values therefore show how meat meals have probably gone through more intense microbial processes, particularly lysine decarboxylation processes, than fresh meats, with the consequent formation of Cadaverine, a potentially toxic amine for organism (del Rio et al., 2019; Lewis, 1998; Til et al., 1997).
The concentration of Tyramine, a biogenic amine deriving from the decarboxylation of the amino acid Tyrosine (Ruiz-Capillas & Jimenez-Colmenero, 2005), was then evaluated. In Figure 5 it is shown how the concentration of Tyramine in chicken fresh meats is more than five times smaller than in meat meals; while it becomes half in the case of pork and more than three times lower in the case of salmon samples. The highest concentrations of Tyramine are peculiar to chicken and salmon meat meals, both being more than double when compared to pork meat meals. Again, all the fresh meats analysed show a statistically significant lower content of Tyramine compared to meat meals.

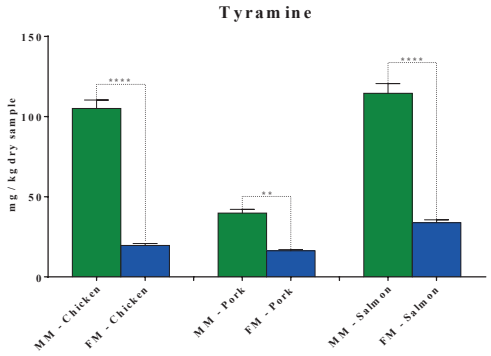

Figure 5. Tyramine content in chicken, pork and salmon meat meal (MM) and fresh meat (FM) for companion animal food determined by LC/MS-QTOF and normalized for water content (expressed as mg of Tyramine per kg of dry sample). Data are reported as mean ± SEM, n = 10; $^{**}p < 0.01$, $^{****}p < 0.0001$

These findings therefore confirm what was previously seen for the other biogenic amines, corroborating the hypothesis that meat meals have probably gone through intense microbial processes, resulting in more efficient Tyrosine decarboxylation reactions. This leads to the formation of Tyramine, a toxic compound for the body (Lewis, 1998; Til et al., 1997), which has been shown by recent studies to be particularly impactful to intestinal cells (del Rio et al., 2017; Linares et al., 2016).

Afterwards the concentration of Tryptamine, deriving from the decarboxylation of the amino acid Tryptophan (Ruiz-Capillas & Jimenez-Colmenero, 2005), was evaluated. Again, the concentration of this biogenic amine in fresh meats is significantly lower than in meat meals for all the raw materials analysed. In particular, for chicken fresh meats the concentration is about twenty times lower; while in the other samples the concentration is less than half as compared to that found in meat meals (Figure 6). As is the case with the other biogenic amines previously analysed, the highest concentrations are found in chicken and salmon meat meals. These findings again underline how fresh meats contain less biogenic amines in comparison with meat meals, being therefore confidently better in terms of quality and thus less harmful for pets.

The exposure to Tryptamine, which is formed following unwanted microbial degradation processes, is indeed responsible for toxicity phenomena such as serotonergic neurotoxicity and hallucinations, mediated by agonism at the 5HT1A and 5HT2A receptors (Tittarelli et al., 2015).

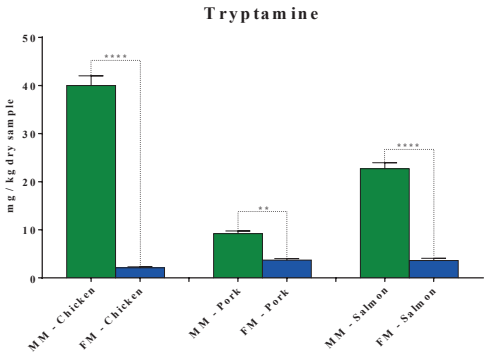

Figure 6. Tryptamine content in chicken, pork and salmon meat meal (MM) and fresh meat (FM) for companion animal food determined by LC/MS-QTOF and normalized for water content (expressed as mg of Tryptamine per kg of dry sample). Data are reported as mean ± SEM, n = 10; $^{**}p < 0.01$, ****p < 0.0001

The concentration of 2-Phenethylamine, a biogenic amine deriving from the decarboxylation of the amino acid Phenylalanine (Ruiz-Capillas & Jimenez-Colmenero, 2005), was also tested. Even in this case, evidence emerges of how fresh meats are qualitatively better, as they show a statistically significantly lower content of this biogenic amine compared to meat meals (Figure 7).

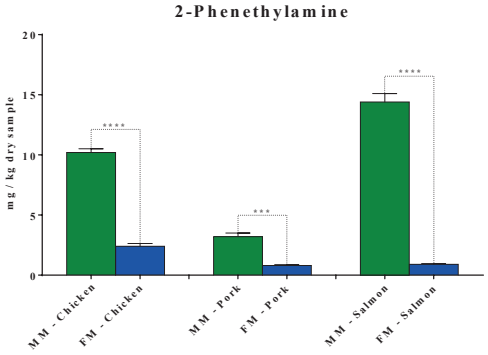

Figure 7. 2-Phenethylamine content in chicken, pork and salmon meat meal (MM) and fresh meat (FM) for companion animal food determined by LC/MS-QTOF and normalized for water content (expressed as mg of 2-Phenethylamine per kg of dry sample). Data are reported as mean ± SEM, n = 10, $^{***}p < 0.001$, $^{****}p < 0.0001$

2-Phenethylamine formed as a result of unwanted microbial degradation processes was found to be four times less concentrated in chicken and pork fresh meats as compared to the corresponding meat meals, but the largest difference was found for salmon samples, whose fresh meats contain 2-Phenethylamine in a concentration around fourteen times lower. Similarly to what seen before, the highest concentrations were observed for chicken and salmon meat meals.

The intake of this biogenic amine is toxic to the body, in that studies have shown that 2-Phenethylamine itself or its derivatives can accumulate in the kidneys and induce nephrotoxicity phenomena (Mossoba et al., 2016).

Finally, the microbial degradation pathways of Arginine and Glutamine amino acids were analysed. Although starting from different compounds, these pathways then converge to a common route. Arginine can initially be decarboxylated by Arginine decarboxylase, obtaining a biogenic amine called Agmatine (Galgano et al., 2012; Ruiz-Capillas & Jimenez-Colmenero, 2005), which is not directly responsible for toxicity problems, but can be further degraded to form another biogenic amine called Putrescine, responsible for toxicity phenomena (De Vera et al., 1992; del Rio et al., 2019; Lewis, 1998; Til et al., 1997). This amine, following the transfer of the propylamine group from S-adenosylmethioninamine, can subsequently be converted initially into Spermidine and then into Spermine (Ruiz-Capillas & Jimenez-Colmenero, 2005), secondary biogenic amines which can be toxic to the nervous system and give rise to disorders such as emaciation, aggressiveness, convulsions and paralysis phenomena (Til et al., 1997).

Another route for Arginine degradation is attributed to the action of other microbial enzymes, Arginase, which initially catalyse a hydrolysis reaction with the formation of Ornithine (Ruiz-Capillas & Jimenez-Colmenero, 2005), a non-proteinogenic amino acid. The latter is subsequently decarboxylated by Ornithine decarboxylase with the formation of Putrescine, which can then undergo the pathway described above.

The formation of the intermediate amino acid Ornithine can also be obtained starting from another amino acid, *i.e.* Glutamine, which is first converted into Glutamate and then in Pyrroline-5-carboxylate before the formation of Ornithine (Jones, 1985).

The results shown in Figure 8 indicate that Agmatine has a concentration about sixteen times lower in chicken fresh meats and six times lower in pork fresh meats compared to their meat meals. The greatest difference is recorded for salmon samples, whose fresh meats exhibit almost two hundred times smaller quantity of Agmatine with respect to salmon meat meals. The latter were also found to have the highest concentrations among the different meat meals analysed. In general, the highest Agmatine content in all the meat meals analysed is always statistically significant ($p < 0.0001$) when compared with the respective fresh meats.

As for Ornithine, which is produced from both Arginine and Glutamine, the same trend is always recorded, characterized by a statistically significant higher biogenic amine content in meat meals compared to fresh meats. All fresh meats tested have a quantity of Ornithine corresponding to about half of what is measured in meat meals, with chicken meat meals showing the highest concentration.

Putrescine, another meeting point between the two pathways of amino acid degradation, is significantly less concentrated in all the fresh meats analysed compared to the meat meals, in particular for chicken and salmon fresh meats, where the greatest differences were found: salmon fresh meats indeed possess a quantity of Putrescine about twenty times lower than meat meals. Again the highest concentrations found were those in chicken and salmon meat meals.

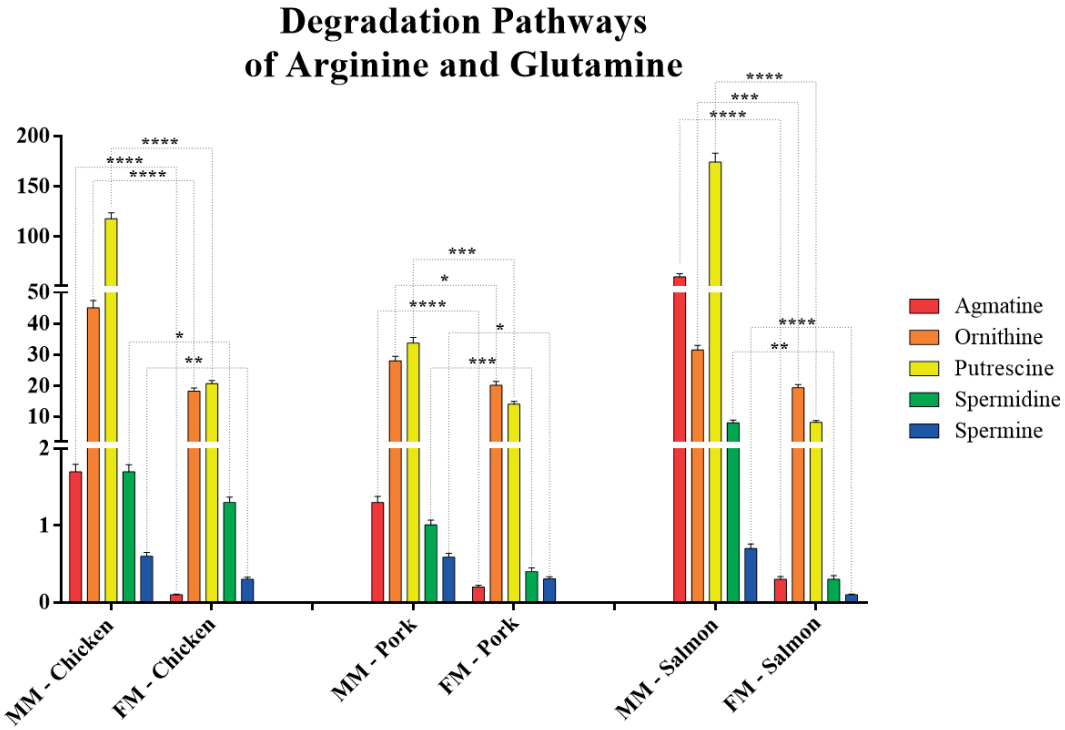

Figure 8. Agmatine, Ornithine, Putrescine, Spermidine and Spermine content in chicken, pork and salmon meat meal (MM) and fresh meat (FM) for companion animal food determined by LC/MS-QTOF and normalized for water content (expressed as mg of biogenic amine per kg of dry sample). Data are reported as mean ± SEM, n = 10; *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$, ****$p < 0.0001$

The fact that the biogenic amines belonging to the Arginine and Glutamine degradation pathways are more represented in the meat meals instead of in fresh meats implies that almost certainly meat meals are qualitatively lower from this point of view, potentially more harmful to pets and probably inadequately stored.

All of the findings listed above could be justified by the fact that meat meals are produced through intensive industrial processing that may cause the partial degradation of raw materials (Camire et al., 1990; Lankhorst et al., 2007; Piergiovanni & Limbo, 2010; Rokey, 2010; Singh et al., 2007; Tran et al., 2008; Williams et al., 2006). The degraded protein material could be the substrate of microorganisms, which, following decarboxylation reactions, can lead to the formation of biogenic amines, which are toxic to the organism, especially for puppies (Bjeldanes et al., 1978; Blonz & Olcott, 1978; Learey et al., 2018; Privitera et al., 1969; Radosevich, 2006).

In this study, it has been shown how there are significant differences in the content of biogenic amines of different raw materials used for dry pet food production. Fresh meats appear to contain lower quantity of biogenic amines, probably thanks to the fact that, since they are generated as meat by-products intended for human consumption, they are not produced with intense industrial processes such as thermals or mechanicals. By doing so, fresh meats result less degraded, better preserved and more protected from the action of microorganisms.

In fact, as they are less degraded, the substrates for the microbial decarboxylating enzymes are only available to a lesser extent, and the decarboxylases cannot thus efficiently convert the amino acids into biogenic amines. As mentioned before, this is supposedly due to the fact that fresh meats are probably qualitatively better and more adequately stored than meat meals, reducing the range of action for microbial decarboxylases.

All of these aspects can heavily influence the quality of the final products and could help the manufacturer companies to understand which

raw materials are the best choice for making healthier dry pet food.

CONCLUSIONS

This study revealed the different concentration levels of biogenic amines between fresh meats and meat meals.

Meat meals, for all the categories of raw materials analysed, *i.e.* chicken, pork and salmon, showed a higher concentration of biogenic amines compared to fresh meats, and this is the case for all the decarboxylation products analysed.

Therefore, these results suggest that meat meals, regardless of the raw material considered, go intensely through microbial degradation processes, probably as a result of both the aggressive industrial processes they undergo and the incorrect handling and storage conditions.

In conclusion, this study has disclosed that fresh meats, for all the categories taken into account, are qualitatively better from a toxicological point of view, being less subject to microbial degradation processes, and therefore probably better preserved than meat meals.

These findings allow us to confidently state that fresh meats, for all the categories analysed, can be considered the best choice as raw materials for dry pet food production.

REFERENCES

Bardócz, S. (1995). Polyamines in food and their consequences for food quality and human health. *Trends in Food Science & Technology*, 6(10), 341-346.

Bjeldanes, L. F., Schutz, D. E., & Morris, M. M. (1978). On the aetiology of scombroid poisoning: cadaverine potentiation of histamine toxicity in the guinea-pig. *Food and Cosmetics toxicology*, 16(2), 157-159.

Blonz, E. R., & Olcott, H. S. (1978). Effects of orally ingested histamine and/or commercially canned spoiled skipjack tuna on pigs, cats, dogs and rabbits. *Comparative Biochemistry and Physiology C*, 61, 161-163.

Bover-Cid, S., Izquierdo-Pulido, M., & Vidal-Carou, M. C. (2001). Effectiveness of a Lactobacillus sakei starter culture in the reduction of biogenic amine accumulation as a function of the raw material quality. *Journal of Food Protection*, 64(3), 367-373.

Camire, M. E., Camire, A., & Krumhar, K. (1990). Chemical and nutritional changes in foods during extrusion. *Critical Reviews in Food Science & Nutrition*, 29(1), 35-57.

da Silva Carneiro, J., Nogueira, R. M., Martins, M. A., de Souza Valladão, D. M., & Pires, E. M. (2018). The oven-drying method for determination of water content in Brazil nut. *Bioscience Journal*, 34(3).

De Vera, N., Serratosa, J., Artigas, F., & Martínez, E. (1992). Toxic effects of putrescine in rat brain: Polyamines can be involved in the action of excitotoxins. *Amino Acids*, 3(3), 261-269.

del Rio, B., Redruello, B., Linares, D. M., Ladero, V., Fernandez, M., Martin, M. C., Ruas-Madiedo, P., & Alvarez, M. A. (2017). The dietary biogenic amines tyramine and histamine show synergistic toxicity towards intestinal cells in culture. *Food chemistry*, 218, 249-255.

del Rio, B., Redruello, B., Linares, D. M., Ladero, V., Ruas-Madiedo, P., Fernandez, M., Martin, M. C., & Alvarez, M. A. (2019). The biogenic amines putrescine and cadaverine show in vitro cytotoxicity at concentrations that can be found in foods. *Scientific reports*, 9(1), 1-7.

den Brinker, C. A., Rayner, C. J., Kerr, M. G., & Bryden, W. L. (2003). Biogenic amines in Australian animal by-product meals. *Australian Journal of Experimental Agriculture*, 43(2), 113-119.

Galgano, F., Caruso, M., Condelli, N., & Favati, F. (2012). Focused review: agmatine in fermented foods. *Frontiers in microbiology*, 3, 199.

Jones, M. E. (1985). Conversion of glutamate to ornithine and proline: pyrroline-5-carboxylate, a possible modulator of arginine requirements. *The Journal of nutrition*, 115(4), 509-515.

Kovacova-Hanuskova, E., Buday, T., Gavliakova, S., & Plevkova, J. (2015). Histamine, histamine intoxication and intolerance. *Allergologia et immunopathologia*, 43(5), 498-506.

Lankhorst, C., Tran, Q. D., Havenaar, R., Hendriks, W. H., & Van der Poel, A. F. B. (2007). The effect of extrusion on the nutritional value of canine diets as assessed by in vitro indicators. *Animal feed science and technology*, 138(3-4), 285-297.

Learey, J. J., Crawford-Clark, S., Bowen, B. J., Barrow, C. J., & Adcock, J. L. (2018). Detection of biogenic amines in pet food ingredients by RP-HPLC with automated dansyl chloride derivatization. *Journal of separation science*, 41(24), 4430-4436.

Lewis, R. A. (1998). *Lewis' dictionary of toxicology*. CRC press.

Linares, D. M., del Rio, B., Redruello, B., Ladero, V., Martin, M. C., Fernandez, M., Ruas-Madiedo, P., & Alvarez, M. A. (2016). Comparative analysis of the in vitro cytotoxicity of the dietary biogenic amines tyramine and histamine. *Food chemistry*, 197, 658-663.

Maintz, L., & Novak, N. (2007). Histamine and histamine intolerance. *The American journal of clinical nutrition*, 85(5), 1185-1196.

Mossoba, M. E., Vohra, S. N., Wiesenfeld, P. L., & Sprando, R. L. (2016). Nephrotoxicity of combining 2-phenethylamine and N, N-dimethyl-β-phenethylamine. *Applied In Vitro Toxicology*, 2(1), 49-55.

Paulsen, P., Bauer, F., & Vali, S. (1997). Biogenic, amines in fermented sausage. 1. Methods for the

determination of biogenic amines. *Fleischwirtschaft* (Frankfurt), 77(5), 450-452.

Piergiovanni, L., & Limbo, S. (2010). *Food packaging: Materiali, tecnologie e soluzioni.* Springer Science & Business Media.

Privitera, P. J., Loggie, J. M., & Gaffney, T. E. (1969). A comparison of the cardiovascular effects of biogenic amines and their precursors in newborn and adult dogs. *Journal of Pharmacology and Experimental Therapeutics*, 166(2), 293-298.

Radosevich, J. (2006). *Analyzing amines: Evaluating potential toxicity in petfood ingredients.* Petfood Industry. August.

Rokey, G. J., Plattner, B., & Souza, E. M. D. (2010). Feed extrusion process description. *Revista Brasileira de Zootecnia*, 39, 510-518.

Ruiz-Capillas, C., & Jimenez-Colmenero, F. (2005). Biogenic amines in meat and meat products. *Critical Reviews in food Scince and Nutrition*, 44(7-8), 489-599.

Shalaby, A. R. (1996). Significance of biogenic amines to food safety and human health. *Food research international*, 29(7), 675-690.

Singh, S., Gamlath, S., & Wakeling, L. (2007). Nutritional aspects of food extrusion: a review. *International Journal of Food Science & Technology*, 42(8), 916-929.

Slemr, J., & Beyermann, K. (1985). Concentration profiles of diamines in fresh and aerobically stored pork and beef. *Journal of Agricultural and Food Chemistry*, 33(3), 336-339.

Smith, T. A. (1981). Amines in food. *Food Chemistry*, 6(3), 169-200.

Suzzi, G., & Torriani, S. (2015). Biogenic amines in foods. *Frontiers in microbiology*, 6, 472.

Taylor, S. L., & Eitenmiller, R. R. (1986). Histamine food poisoning: toxicology and clinical aspects. *CRC Critical Reviews in Toxicology*, 17(2), 91-128.

ten Brink, B., Damink, C., Joosten, H. M. L. J., & In't Veld, J. H. (1990). Occurrence and formation of biologically active amines in foods. *International journal of food microbiology*, 11(1), 73-84.

Thompson, A. (2008). Ingredients: where pet food starts. *Topics in companion animal medicine*, 23(3), 127-132.

Til, H. P., Falke, H. E., Prinsen, M. K., & Willems, M. I. (1997). Acute and subacute toxicity of tyramine, spermidine, spermine, putrescine and cadaverine in rats. *Food and Chemical Toxicology*, 35(3-4), 337-348.

Tittarelli, R., Mannocchi, G., Pantano, F., & Saverio Romolo, F. (2015). Recreational use, analysis and toxicity of tryptamines. *Current neuropharmacology*, 13(1), 26-46.

Tran, Q. D., Hendriks, W. H., & van der Poel, A. F. (2008). Effects of extrusion processing on nutrients in dry pet food. *Journal of the Science of Food and Agriculture*, 88(9), 1487-1493.

White, M. V. (1990). The role of histamine in allergic diseases. *Journal of allergy and clinical immunology*, 86(4), 599-605.

Williams, P. A., Hodgkinson, S. M., Rutherfurd, S. M., & Hendriks, W. H. (2006). Lysine content in canine diets can be severely heat damaged. *The Journal of nutrition*, 136(7), 1998S-2000S.

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PHYTO-FEED ADDITIVES PRODUCTION: TECHNOLOGICAL ASPECTS AND BIOLOGICAL VALUE

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Abstract

The results of the qualitative assessment of the final products of the technology include: mowing of vegetative plants, such as alfalfa, amaranth, clover, pea-oat mixture; grinding; wet fractionation with obtaining marc and juice; thermal coagulation, filtration of juice, drying of the chloroplast fraction by spraying or in a vibro-boiling layer.In the process of transformation of vegetative plants, biologically active phyto-feed additives are obtained as the final product. During the production of which the processed phytomaterials were influenced by regime processing parameters related to temperature, processing of time on certain stages, acidity, etc. A computational method developed in the paper for determining the biological value of phyto-feed additives is implemented taking into account the importance of protein efficacy ratio.

Key words*: phyto-feed additives, protein quality, heat treatment, solubility, protein digestibility, process parameters*.

INTRODUCTION

The technological chain of protein feed production - processing of the initial mass-feed additive includes a number of complex interrelated processes that affect its final result (the quality of the feed produced, its quantity and energy consumption). The process of mechanical dehydration of plant mass (wet fractionation) can be considered as one of the main processes (Colas et al., 2013). Finally the process flow is divided into two kinds of the production - juice and pulp. The structural and functional analysis of the technology processes made it possible to draw preliminary conclusions. Each process of this complex technological system has input, output parameters and control actions. All these components are closely interrelated and determine the functioning of the process. The interconnection of processes in such a technological system is associated with a significant impact on the final result in case of serious violations in one of them. The technology for processing pomace includes several processes: drying by thermal methods or active ventilation, mixing with other feed components and briquetting of the mixture, production of haylage or silage feed for the laying of the pulp deposited using the methods of canning. Juice processing includes a number of complex processes that have a greater impact on the quality and quantity of the final product-biologically active feed additives. These processes include three stage: coagulation of proteins in juice plants (alfalfa, amaranth etc.), separation of the coagulate into a chloroplast paste-like fraction and serum, spray drying or in a vibro-boiling layer, depending on the production requirements. Classification of processes by their functional purpose, means of implementation, types of operations performed, nature, mode and level of regulation, give possibility to more accurately represent this complex technological structure of relationships. The intermediate type of feed material is the raw material for the next phase of the resource cycle. The end result of the entire technological system is the product. The processes associated with the heat effect on the processed product determine both the quantitative and qualitative side of the feed produced, related to the absorption of nutrients (supplements) by the animal and poultry body.

A large number of factors affect the quality of the final product in the technological complex of production of biologically active feed additives from green plants. The main factors are the time

spent on mowing raw materials before processing, the conditions for processing them in technological flows, the nature of impacts and the degree of oxidation by the working bodies of machines, the sanitary condition of the product, and the modes of heat treatment of feed raw materials (Kalli et al., 2018). The conditions of heat treatment of products have a significant impact on their quality (Pęksa & Miedziank, 2014). It depends not only on temperature and exposure time, but also on the residual content of phenols and carbohydrates in the material being processed (Seczyk et al., 2019; Rong et al., 2013). The leaf protein concentrate (LPC) can be separated from deproteinised juice (DPJ) by filtration through cotton cloth (Badar & Kulkarni, 2011). It was that the process of Green Crop Fractionation (GCF) can be employed for the preparation of feed grade pressed crop residue (PCR) and food grade leaf protein concentrate (Rathor, 2016). Toxic constituents like nitrates and oxalates, accumulated in the foliages of several plant species, were generally removed in the DPJ and as a result of which the protein concentrate (PC) and LPC contained safer levels of these toxic elements in view of their value as either feed or food (Sayeed & Gogle, 2002). In this way green foliage can be fractionated mechanically into three fractions: (i) fibrous pressed crop, (ii) leaf protein concentrate and (iii) deproteinised juice. Leaf concentrate is an extremely nutritious human food, containing approximately 50% (dry weight) high quality protein, together with numerous micronutrients, principally b-carotene, vitamins B_6, B_9, E and K, plus iron, calcium and magnesium The green juice is a raw material for high quality fodder proteins, cosmetic proteins, human nutrition or platform chemicals like lactic acid and lysine (Arlabosse et al., 2011). Pumpkin and amaranth leaves recorded the highest protein yield (10.5-11.75%). The protein contents in the leaves of sweet potato, cowpea, cabbage and sugar been were much lower (Ghaly & Alkoaik, 2010). The data of chemical analysis and calculation of the total amount of exchange energy in the feed, cannot fully give reliable information about its quality. Biologically active phyto-feed additive and by-products of technology might have high digestion by the animal as well as low one. This fact is the main qualitative criterion of obtaining feed, taking into account the influence of technological processing regimes onto feed quality indicators. It is necessary to choose such regimes in technological processes, which at the final stage of the whole chain would guarantee saving biologically active nutrients (amino acids, vitamins, minerals) and high digestibility of feed additives as well. The most important components in the feed are protein, fats, fiber, fate soluble vitamins, so the influence of technological regimes on the quality of feed can be assessed by the degree of protein digestion by animals. Biologically active feed additives are rich in carotene, vitamins, and protein. The amino acid composition is similar in composition to the amino acids of animal origin. Technological equipment and innovative solutions, their interconnections in processes, operating parameters of equipment for the production of biologically active feed additives and the organization of the raw material base, technical means for cleaning, transportation and processing into the main products, represent a promising direction in the industry of industrial feed production with effective use in poultry and animal husbandry.

MATERIALS AND METHODS

The methodology for obtaining biologically active phyto-feed additives is based on the fact that the plant cell protein is contained in all intracellular formations - the cytoplasm, chloroplast, mitochondria, and nucleus. The most valuable part of the intracellular protein is ribulose-1.5-biphosphate carboxylase.

Intracellular protein of green plants is a rich source of protein and biologically active additives. The essence of the technology consists in mowing and grinding plants, pressing green juice by pressing methods, coagulation for the formation of chloroplast and cytoplasmic flakes, separating them as a paste by filtration or centrifugation. Green mass extracts are a by-product of this technology. The main components of pomace are fiber and serum after filtration.

The second stage of fractionation provides the production of green mass of seeded herbs, biologically active additives in the form of juice, paste, dry concentrate. These products are full-fledged substitutes for animal proteins and soy

meal and are used as a dietary supplement in the feeding diets of poultry, piglets and calves.

The processing of green juice and the use of the press residue ensure the complete preservation of the biological crop. The stages of processing include fractionation of leaf-stem biomass, the production of paste drying and production of dry biologically active feed additives for poultry diets of various breeds and ages, young poultry, piglets, calves, sheep and goats, broilers and breeding poultry. The kinetics of protein-carbon-phenol complexes formation foresee that the effect of heating on processed products should be determined by a comprehensive study of a scope of biochemical indicators of products obtained under different conditions. One of the main factors determining the quality of the protein is its digestibility in the gastrointestinal tract. It is well known that the correlation between the amino acid composition of the protein and its biological value occurs only if there is a sufficient rate of proteins digestibility by digestive enzymes.

From the practice of using feed in animal husbandry, negative correlation is considered the level of digestibility of dry matter in the diet, animal and amount of its consumption, especially highly productive ones. For example, dairy cattle need to be feed by fodders with a digestibility of dry matter at least 65%. In addition, the main indicator of the feed diets usefulness is the balance in essential nutrients. Hence, there are high demands to industrial technologies, equipment and management in feed production, in order to obtain feed with high exchangeable energy. The amount of metabolizable energy in the feed can be determined by the following regression equations: for pigs:

ME = 20.85 dp + 36.63 df + 14.27 dfb + 16.95 dnfe; for fowl:

ME = 17.84 dp + 39.78 df + 17.71 dfb + 17.71 dnfe; for cattle:

ME = 17.6 dp + 31.23 df + 13.63 dfb + 14.78 dnfe; where: dp - digestible protein, g;

df - digestible fat, g; dfb - digestible fiber, g;

dnfe - digestible nitrogen-free extractives, g.

The total biological value of the concentrate of phyto-feed can be determined by a calculation method based on the value of the protein efficiency ratio (PER), determined from the change in body weight of experimental animals (Bhilave et al., 2012). PER takes into account the ratio of weight gain by animals to the amount of edible protein when feeding standardized animals with standardized rations. The calculated method for determining the PER index considers the content of essential amino acids in a protein and its digestibility *in vitro*. Computed PER (C-PER), takes into account the digestibility of protein and casein, the content of essential amino acids and the score of each essential amino acid in the studied protein and casein. C-PER is calculated using the equations:

$$z = (SPC) \cdot 2.94 \cdot \frac{2.5}{2.94} \qquad (1)$$

$$C - PER = -2.1074 + 2.8525 \cdot z - 0.4030 z^2$$

A qualimetry method is quite useful (Kuznetsov et al., 2019). It allows determining the quality characteristics of products based on the assessment of each one. Also it takes into account the weight ratio of the properties of the product. The mathematical model proposed of the qualimetric method is following:

$$K = M_a \sum_{i=1}^{i=l} m_{ai} K_{ai} + M_в \sum_{i=l+1}^{i=p} m_{вi} K_{вi} + M_c \sum_{i=p+1}^{i=q} m_{ci} K_{ci} + Md \sum_{i=q+1}^{i=n} m_{di} K_{di}, \quad (2)$$

where: n - features, characterizing the quality of products; M_a, $M_в$, M_c, M_d - the relative weight of each group of properties characterizing quality, $M_a + M_в + M_c + M_d = 1$ m_{ai}, $m_{вi}$, m_{ci}, m_{di} - the relative weight of each i-th property for each group of properties.

$$\sum_{i=1}^{l} m_{ai} = 1; \sum_{i=l+1}^{p} m_{вi} = 1; \sum_{i=p+1}^{q} m_{ci} = 1; \sum_{i=q+1}^{n} m_{di} = 1 \qquad (3)$$

RESULTS AND DISCUSSIONS

The study of processes on stationary hydraulic presses in perforated cylinders, on auger mechanical presses was part of the task of experimental research on the fractionation of the green mass of alfalfa into juice and pomace. Also, the analysis of the process of juice separation under different modes of influence on the mass and finding dependencies that characterize this process was carried out. Program of studies provided that: the determination of the yield of green mass, the impact of interest remove moisture depending on the pressure, exposure time, initial moisture

content, volume of the processed mixture, removing plots of socket data a certain amount of weight depending on the application, stable load repeated impact: determine the impact of contact area and the magnitude, time pressure squeezing on the magnitude and intensity of the impact of the juice.

The results of the experiments showed that there is a close relationship between the initial humidity of the green mass of alfalfa and amaranth and the effectiveness of the dehydration process. As the mass humidity increases, the pressing capacity for juice and pulp increases, and the energy intensity decreases on the contrary. It is characteristic that a sharp increase in energy intensity is observed when a mass with humidity below 70% is dehydrated. Weak protein coagulation, the formation of small flakes (10-20 microns) is noted already at the acidity of the juice pH 5.6. Acidification of the solution to a pH of 4.05 contributes to more active protein deposition, the formation of a clear precipitate of coagulated chloroplasts. Increasing the heating temperature reduces the time required for protein coagulation. As a starting material, alfalfa juice was used with an initial temperature of 20 ... 25°C, a dry matter content of 11% and 3.2% protein. The steam pressure varied within $1.5 \cdot 10^5 - 6 \cdot 10^5 Pa$. The temperature of the heating steam (direct contact with the juice) was 140 ... 155°C, the steam consumption within 0.17 ... 0.23 kg/kg. The temperature of the coagulated juice varied within 45 … 90°C.The obtained data showed that for complete coagulation of proteins in the juice, it is necessary to extract the already coagulated juice for 50 ... 90 seconds. During this time, proteins are aggregated into larger a structural compound, which positively affects the separation by any means - centrifugation, filtration, and sedimentation. The analysis of the obtained experimental data showed that the operating modes of the centrifuge that satisfy the qualitative separation of the suspension into paste and brown juice are: - by the separation factor in the range of 1250 ... 1360; - by the second feed of the suspension -0.32 ... 0.35 l/s. Positive results on separation efficiency and changes in precipitation humidity are not achieved even when the feed is reduced to 0.12 l/s (for separation factors F_r = 420 and 698). When the separation factor increases to 903 ... 1360, the separation quality increases dramatically, the humidity of the chloroplast paste decreases, and the separation efficiency increases to 88.5%.Experimental studies of drying biologically active phyto-feed additives in a vibro-fluidized layer were conducted in a laboratory setting, simulating the camera vibro-dryers and to determine: the frequency and amplitude of vibration; the speed and temperature of the coolant; the drying time, the thickness of the dried layer. Chloroplast paste with a moisture content of 62 ... 36% was granulated through spinners with a diameter of 3 mm. The speed of the coolant varied within 1.4 ... 2.5 m/s, the amplitude of vibrations 5 ... 10 mm, the frequency of vibrations of the working cup 400 ... 700 fluctuations/min, the thickness of the granule layer varied within 20 ... 120 mm, the temperature of the heat carrier from 50 to 140°C. The results showed that with increasing time of heat treatment above 80°C and a process time more than 30 minutes - loss of carotene increase by 50%, and with increasing treatment temperature the losses increase even with a decrease in drying time. A short-term increase in the coolant temperature to 140°C at the initial moment of drying provides a sharp decrease in moisture to 420 g/kg, and in the future the drying intensity decreases. Determined time point, at which begins the growth temperature of the dried granules, the ingredients for a coolant temperature of 90 ... 140°C - 6 ... 9 min. Main time of removing moisture from the pellets is 20 ... 21 min, further - final drying. The modes that do not overheat the granules are determined, and the loss of carotene, protein and vitamins in the product is minimal. The found product characteristics make it possible to carry out a predicted energy calculation of heat for heating the material and time ranges of drying at which irreversible denaturation of protein does not occur. Various effects of coolant temperatures, both in magnitude and duration, allowed us to determine the nature of fermentation of leaf protein proteins with pepsin and trypsin, which can be used to judge the assimilation of the obtained products by the animal body. The qualimetry method is associated with experimental ones for estimating the values of M and m, which is associated with a considerable degree of subjectivity. It should be

emphasized that the considered methods, including the C-PER index, do not take into account some of the important features of the kinetics of protein hydrolysis by enzymes, as well as the solubility of biologically active phyto-feed additives. The total calculated indicator of the protein feeds quality should be described by the equation:

$$Q_{pgc} = \left(m_1 \frac{C - PER_{sample}}{C - PER_{standard}} + m_2 \frac{1}{n} \sum_{i=1}^{n} \frac{V^{sample}}{V_n^{standard}} \right) \varphi , \quad (4)$$

In equation (4), the parameter V takes into account the vitamin amount in the feed:

$$V = \frac{1}{n} \sum_{i=1}^{n} \frac{n_{sample}}{n_{standard}} , \quad (5)$$

where $n_{sample.}$ and $n_{standard}$ - the amount of vitamins in the sample and in the standard, respectively;

φ - factor taking into account the sanitary and hygienic indicators of the feed;

$$\varphi = \prod_{i-1}^{z} \varphi_i ,$$

where $\varphi_i =$ { 0 at concentration of PC* ≤ δ ; 1 at concentration of PC ≥ δ }

z - number of accepted safety criteria;

*PC - permissible concentration;

$C\text{-}PER_{sample}$, $C\text{-}PER_{standard}$ - calculated indicator that takes into account the digestibility of protein in the feed, the content of essential amino acids, respectively, in the sample and standard;

$V_n^{sample}, V_n^{standard}$ - vitamin content in the sample and standard;

m_1, m_2 - coefficients of significance.

The hydrolysis of protein in the body of an animal can be described by the following equation:

$$p = a + b\left(1 - exp^{-ct}\right), \quad (6)$$

where ρ - the amount of hydrolytic material in a certain period of time (t);

a, b, c - constant in exponential equation.

Constant a - represents the rapidly digestible fraction; c - rate constant of hydrolysis fraction per 1 hour; b - the amount of material hydrolyzed in a specific period of time.

Since the criterion for evaluating the production modes of all processes of the technological system is the quality of the product produced, it is necessary to know the dynamics of the decomposition of dietary supplements, which must be obtained experimentally. In this case, the dynamics of the hydrolysis rate of the phyto-feed additives makes it possible to evaluate the effect of temperature regimes on the chloroplast proteins for dietary supplements. At the same time, the rate of digestion, as already noted, is an important indicator that determines the possible quota to substitute animal-derived proteins for plant ones in animal diets. Taking into account the determination of the availability of amino acids (N_i) to be attacked by enzymes, such as pepsin and trypsin, the amount of digested protein $S^0{}_{t_k}$ during time (t_k) can be described by the following formula:

$$S^{\circ}_{(t_k)} = \min_{1 \le n \le 8} \left(\frac{A_n}{F_n} \right) K_1 \cdot K_2 \sum_{i=1}^{20} \frac{A_i N_i}{100} \int_0^{\tau_K} \left[1 - exp\left\{ -\alpha_i \left(\tau - \tau_i \right) \right\} \right] \cdot d\tau , \quad (7)$$

Where: K_1 - protein content in a biologically active phytonutrient additive;

K_2 - protein digestibility;

A_n - essential amino acids content, in ideal protein (FAO);

A_i - amino acids content;

α_i - characteristic time;

τ - time;

t_i - the time of appearance of the amino acid in the hydrolyzate.

But this equation does not take into account the vitamin content of the feed and the kinetics of protein solubility, depending on the processing temperature. These parameters are included into generalized quality indicator K. The value of K will vary depending on the dynamics of the breakdown, changes in the amino-acids composition. All this is able to characterize the regimes under which the biologically active phyto-feed additive is produced. In this regard, taking into account the vitamin content in the product and the influence of the temperature parameters on the final product, the quality of the produced biologically active additive, should be described by the system of equations:

$$\begin{cases} K = \left[m_1 S(T,\tau) + m_2 V(T,r) \right] \varphi \\ S(T,\tau) = S^{\circ} exp\left(-\lambda_1(T)\tau \right) \\ V(T,\tau) = V^{\circ} exp\left(-\lambda_2(T)\tau \right) \end{cases} \quad (8)$$

where:
m_1, m_2 - the relative weight of the amino acid and vitamin portions in the feed;
$S°(t_к)$ - the amount of digested protein determined by equation (7);
T - temperature;
T - time;
V^o, $V(\tau)$ - the content of vitamins in the original feed and by the time τ after the start of drying, mg per kg;
$\lambda_1(T)$, $\lambda_2(T)$ - constants characterizing the breakdown of protein and vitamins at certain temperature.
The temperature dependence of the decay constant λ_1 (T) and λ_2 (T) in the simplest case is determined by the Arrhenius law:

$$\lambda_1(T) = \lambda_1^{\circ} exp\left(-\frac{E}{RT}\right)$$
$$\lambda_2(T) = \lambda_2^{\circ} exp\left(-\frac{E}{RT}\right) \quad (9)$$

where: E - activation energy of 600 J/mol;
R - universal gas constant equal to 8.314 J/(K mol);
T - temperature in °K.
The rate of isothermal denaturation can be described by the equation:

$$\frac{dx}{d\tau} = -K''(1-x) \quad (10)$$

where: x - degree of denaturation of the feed, calculated by the change in solubility;
K″ - denaturation rate constant.

CONCLUSIONS

The proposed method for assessing the quality of produced bioactive phyto-feed additives from green plants allows predicting the impact of thermal effects (heating the raw mass before processing, modes of coagulation, filtration and drying of additives, etc.) on changes in protein vitamins, biologically active components, content, in the final product. It could be used to predict the digestibility of phyto-feed additives by animals.

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REFERENCES

Arlabosse, P., Blanc, M., Kerfaï, S., Fernandez, A. (2011). Production of green juice with an intensive thermo-mechanical fractionation process. Part I: Effects of processing conditions on the dewatering kinetics. *Chemical Engineering Journal*, Elsevier, 168, 586-592.

Badar, K.V. and Kulkarni, A. U. (2011). LPC is novel source of protein for human health and nutrition: A review. *Current Botany*, 2(1):05-07.

Bhilave, M.P., Bhosale, S.V., Nadaf, S.B. (2012). Protein Efficiency Ratio (PER) of Ctenopharengedon idella Fed on Soyabean Formualted Feed. *Biological Forum - An International Journal*, 4(1):79-81.

Colas, D., Doumeng, C., Pontalier, P.Y. & Rigal, L. (2013). Green crop fractionation by twin-screw extrusion: Influence of the screw profile on alfalfa (Medicago sativa) dehydration and protein extraction. *Chemical Engineering and Processing: Process Intensification,* Vol. 72, p. 1-9.

Ghaly, A.E. and Alkoaik, F.N. (2010). Extraction of Protein from Common Plant Leaves for Use as Human Food. *American Journal of Applied Sciences,* 7(3): 331-342.

Kalli, E., Lappa, I., Bouchagier, P., Tarantilis, P.A., Skotti, E. (2018). Novel application and industrial exploitation of winery by-products. *Bioresources and Bioprocess,* 5, 46.

Kuznetsov, A.V., Vitchuk, N.A., Antsev, Yu., Sabinina, A.L. (2019). Application of qualimetry methods for quality assessment of production processes at components suppliers enterprises. Quality Management and Reliability of Technical Systems. *IOP Conf. Series: Materials Science and Engineering* 666.

Pęksa, A. and Miedziank, J. (2014). Amino Acid Composition of Enzymatically Hydrolysed Potato Protein Preparations. *Czech J. Food Sci,* Vol. 32, no. 3: 265–272

Rathor, B. M. (2016). Analysis of leaf protein concentrate (LPC) from green foliages of leguminous crop plants. *Research Journal of Life Sciences, Bioinformatics, Pharmaceutical and Chemical Sciences,* 2(3), no. 204, 7 p.

Rong, H., Yu, C.Q., Li, Z.H., Shimojo, M. & Shao, T. (2013). Evaluation of fermentation dynamics and structural carbohydrate degradation of Napiergrass ensiled with additives of urea and molasses. *Pak Vet J*, 33, 374-377.

Sayyed, I.U. and Gogle, D.P. (2002). In: *Plant Resource Development*. (Mungikar, A.M. & Bhuktar, A.S. Eds.) Saraswati Printing Press, Aurangabad, 229-236.

Seczyk, L., Swieca, M., Kapusta, I., Gawlik-Dziki, U. (2019). Protein-Phenolic Interactions as a Factor Affecting the Physicochemical Properties of White Bean Proteins. *Molecules*, 24, 408.

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BIOCHEMICAL APPROACHES FOR GOAT MILK YOGURT PRODUCTION

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Abstract

Goat milk production is a dynamic and growing industry that is fundamental to the wellbeing of hundreds of millions of people worldwide and is an important part of the economy in many countries. In this research goat milk samples, obtained from the individual farms were investigated to chemical composition and physico-chemical properties. The strains of valuable native lactic bacteria of the species Streptococcus thermophilus and Lactobacillus bulgaricus were selected with stable technological characteristics for the fermentation of goat milk. The scheme for preparing starter cultures and recommendations regarding the use of consortia of symbiotic cultures for the production of goat milk yogurt have been developed. Starter cultures were obtained for the manufacture of goat milk yogurt, with biotechnological properties characteristic for fermented dairy products.

Key words*: goat milk, native lactic acid bacteria, yogurt production.*

INTRODUCTION

Goat milk production is a dynamic and growing industry that is fundamental to the wellbeing of hundreds of millions of people worldwide and is an important part of the economy in many countries (Li X.Y. et al., 2020; Wang Y. et al., 2019). Goat milk has a promising source of protein, vitamins, minerals and fatty acids (Chen X. et al., 2020; Asresie A. et al., 2014; Beshkova D.M. et al, 2011). Goat milk is known for its better digestibility, lower allergenic potential due to the low content of lactose, as well as the presence of health-promoting compounds (Verruck S. et al., 2019; Hassan F.A.M. et al., 2014; Paz N.F. et al., 2014). From goat milk usually is obtained butter, yogurt, sour milk etc. (Picon A. et al., 2019; Shori A.B. et al., 2015). Fermented dairy products have delicious sensory properties, fine consistency and pleasant specific taste (Muelas R. et al., 2018). Especially fermented goat milk products have significant commercial potential, large destination and multiple health benefits for population (Yurchenko S. et al., 2018; Innocente N. et al., 2016). Considering the importance of fermented dairy products from goat milk, which are in demand on internal and external markets, elaboration of the technological process for manufacturing of the products is necessary (Saha B.N.P. et al., 2016; Serhan M. et al., 2016).

According to sanitary-epidemiological safety, the goats have a much lower disease risk, they do not suffer from brucellosis, tuberculosis and other diseases that affect cattle. Goats farming have an important food potential, which must be exploited at industrial level.

At the international level, the proposed topic carries researches on the preparation and optimization of goat milk yogurt technology and manufacture (Caleja C. et al., 2016; Garcia V. et al., 2014). In order to develop the industrial level of goat's farming, obtaining the high quality goat's milk products, is necessary to determine the milk quality properties and the harmlessness as a raw material (Zhao X. et al., 2020; Sun Y. et al., 2020; Sousa Y.R.F. et al., 2019; Zhu Z. et al., 2019).

Currently, the theoretical and applied research is insufficient for an objective assessment of the

effect of the constituent components of goat milk on the quality of products, which hinders the use of goat milk when creating new generation food products of high biological and nutritional value, including the functional area (Deshwal G.K. et al., 2020; Feng C. et al., 2019; Verruck S. et al., 2019; Yangilar F., 2013).

The purpose of the research was the selection of lactic acid bacteria to determine the most promising combinations for use in the preparation of starter cultures for yoghurt manufacture.

MATERIALS AND METHODS

Fat content was performed in accordance with ISO 2446: 2008. Concentrated sulfuric acid was used as the main reagent, which converts insoluble calcium salts of milk into soluble sulfuric acid casein compound. The latter dramatically reduces the amount of adsorption of fat globules and thereby contributes to their merger. The butyrometer was mounted on a stand and 10 ml of H_2SO_4 was poured. Slightly tilting the device, acid was carefully poured over its wall to 11 ml of a well-mixed product (20°C). To avoid mixing the product with H_2SO_4, 1 ml of isoamyl alcohol was added. Butyrometer was taken by the neck and, holding the cork, shaken several times until a homogeneous mass without flakes was obtained in the tube. After that, the butyrometer was placed (holding the stopper down) for 5 minutes in a water bath at 65-70°C. Then, the fat released on the butyrometer was counted on a scale.

Protein content was performed in accordance with ISO 8968-1: 2014. The method included the quantitative determination of nitrogen in the samples under study. The nitrogen contained in the test samples was heated with concentrated sulfuric acid in the presence of catalysts and transferred to ammonium sulphate, the sample itself was completely destroyed. Ammonia was squeezed out of ammonium sulphate with concentrated base, which was distilled into a receiver with a certain volume of titrated acid. Measuring the amount of acid remaining in the receiver after the end of the distillation, the amount of ammonia in the sample was calculated and, therefore, the amount of fixed nitrogen in the sample under study. Calculations are made according to the formula:

$$WN = \frac{1,4007 \cdot (Vs - Vb) \cdot Mr}{m}, \quad [\%]$$

where:

WN - the mass fraction of nitrogen in the sample [%];

Vs - the volume of hydrochloric acid (accurate to 0.05 ml) [ml];

Vb - the volume of hydrochloric acid (with an accuracy of 0.05 ml) [ml];

Mr - the molecular weight of hydrochloric acid;

m - the mass of the test portion (with an accuracy of 0.1 mg) [mg];

1.4007 - coefficient of calculation for the expression of the nitrogen content in the sample [%].

Lactose content of the tested samples was determined in accordance with the standard method ISO 22662: 2007, which provides high performance liquid chromatography (HPLC) with refractometric detection. 10 ml of a clear sample obtained after centrifugation was measured. This sample was filtered through a 0.45 μm membrane. Through a cartridge for filtering C18 was loaded with 10 ml of ethanol and 10 ml of distilled water. Then the sample was passed through a C18 cartridge. After each filtration, the cartridge was washed with 10 ml of ethanol, and then with 10 ml of distilled water. Then 1 ml of a standard solution of lactose, previously prepared at a concentration of 2.4%, was measured and injected into the injection device. When the device lever was moved 90°, the quaternary pump sucked 10 μl of the solution and switched it into the acetonitrile phase. Further, the separation of water and lactose occurred depending on the retention time. The refractometric detector determined the value of the peak areas of water and lactose, then the information was transferred to the software database. The analysis time was 12 minutes. Therefore, 1 ml of the sample was taken for analysis (clear phase after centrifuging the milk) and subjected to qualitative and quantitative analysis in the same way as the standard solution.

Free fatty acid content was determined on a Hewlett-Packard chromatograph (model 5890, Palo Alto, CA, USA), with a flame ionization detector (FID) and connected to a ChemStation computer (Hewlett-Packard, Palo Alto, CA,

USA). This method allows you to set the mass fraction of fatty acids to their total content in triglycerides. The separation of fatty acids was carried out depending on the chain length and the degree of their unsaturation, by analogy with their closest standards. The mass fraction of each acid was calculated on the basis of the obtained chromatogram over the areas of the peaks using a standard graph.

Physico-chemical properties of the goat milk was recorded using the EKOMILK Total Bulteh 2000 automatic milk analyzer. All the determinations were performed in duplicate.

Statistical analysis of the results was carried out by least square method with application of Microsoft Office Excel program. Differences were considered statistically significant if probability was greater than 95% ($q < 5\%$). All assays were performed at room temperature, 20 ± 1ºC. Experimental results are represented according to standard rules.

RESULTS AND DISCUSSIONS

Analysis of the chemical composition and physico-chemical properties of goat milk

In this study, a comparative analysis of the fat, protein, fat and ash content of the goat milk was made. Table 1 presents experimental data of goat milk evaluation.

Table 1. Chemical composition and physico-chemical properties of goat milk

No	Characteristics	Values
1	Fat mass fraction, %	3.58 ± 0.19
2	Mass fraction of dry degreased substance (DDS), %	9.32 ± 0.5
3	Protein mass fraction, %	4.1 ± 0.11
4	Protein mass fraction, % (Kjeldahl method)	4.28 ± 0.03
5	Lactose mass fraction,%	4.4±0.2
6	Cryoscopic temperature, ºC	- 0.530
7	Density, g/cm^3	1.031 ± 0.0028
8	pH	6.5 ± 0.70

In goat milk according to ISO 8968-1: 2014 was determined the mass fraction of proteins (%), which constituted 4.28 ± 0.03. The fatty acid composition of goat milk was studied using gas-liquid chromatography. Experimental data showed the content of 23 fatty acids, represented by saturated, monounsaturated and polyunsaturated fatty acids. It is known that the chemical composition of milk differs depending on season, feeding, age, lactation period. The high content of skimmed dry matter in goat's milk indicates that it is better for technological processing.

Development of starter culture strains for goat milk yogurt production

Goat milk products with valuable nutritional properties for humans can be positioned as a healthy diet product. The technology of dairy acid products involves the fermentation of milk with pure cultures or bacterial consortia containing different strains of lactic bacteria. In the manufacture of yogurt, it is recommended to apply EPZ-producing strains in starter cultures, which provide sensory and rheological characteristics, that are necessary for the finished products without the use of food additives. When developing lactic acid bacteria consortia for starter cultures, it is important to consider the relationship between strains and possible changes in microflora during the subsequent cultivation of dairy products. By combining different species of lactic acid bacteria and regulating the fermentation temperature, it is possible to obtain a product with the desired taste and aroma, texture and dietary properties. In our previous studies isolation and selection of valuable lactic acid bacteria strains for the goat milk fermentation has been performed (Popovici C. et al., 2019). Starter cultures have been prepared according to the proposed technology (Figure 1). In the next step, the research was intent on the association between *Streptococcus thermophilus* strains within the species. In order to create combinations between strains of the same species, the strains selected by *Streptococcus thermophilus* were gradually associated in a 1:1 ratio, their compatibility at the level of acidifying and coagulating action being studied. The strains were inoculated into milk (20-30 ml). After incubation, until the clot was obtained, the obtained combinations were re-seeded twice in sterile skimmed milk. Were selected associations, which have demonstrated intensive acidogenesis action - within 5 hours,

with the formation of a homogeneous, dense, creamy or viscous coagulation with moderate filant.

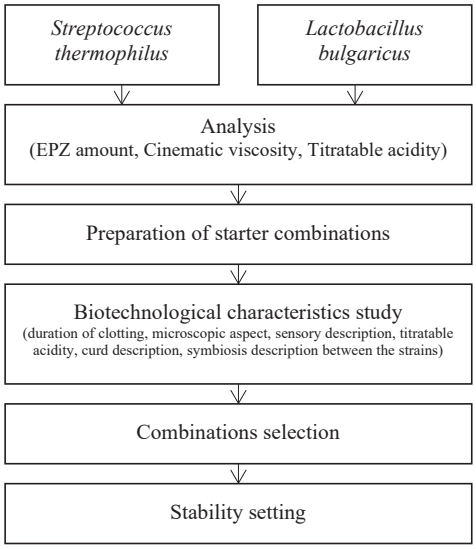

Figure 1. Steps of starter culture preparation for goat milk yogurt production

Fermented dairy products are very popular all over the world for their specific properties and beneficial effect on the human body. A crucial role in their manufacture is played by the biochemical processes caused by starter cultures. Therefore, the quality of dairy products depends on the quality of the starter cultures used in their production, which, in turn, is determined by the characteristics of the microorganisms within the starter culture.

The starter crops for the manufacture of yogurt should be made of *Streptococcus thermophilus* and *Lactobacillus bulgaricus* species. Therefore, in the next stage, associations formed of *lactobacilli* and *thermophilic streptococci* were created and studied. Based on the associations formed within the species, 3 combinations of of *Streptococcus thermophilus* and *Lactobacillus bulgaricus* species were created, of which two EPZ starter cultures and one starter culture without EPZ as a control culture, which were investigated according to biotechnological clues. The results of the investigations are shown in Table 2.

Table 2. The associations description of native strains for goat milk yoghurt

No	Characteristics	Association I	Association II	Association III
1	Duration of clotting, hours	3.5±0.5	3.5±0.5	4.0±0.5
2	Titratable acidity, °T	112±2	118±1	98±2
3	Viscosity, cSt	43.97±1.3	70.25±1.72	47.49±1.3
4	Amount of EPZ, mg/100 ml	58.43±1.9	106.51±1.0	0
5	Appearance of the clot	O, V, F, D, fz	O, V, F, D, fz	O, D, nV, fz

Note: O - homogeneous, V - viscous, nV - non - viscous, F - filamentous, D - dense, fz - without removing the whey.

Based on the data obtained from the formation of associations between the species *Streptococcus thermophilus* and *Lactobacillus bulgaricus* in starter cultures for the production of goat milk yogurt, it is obvious that the starter culture consisting of 2 *Streptococcus thermophilus* strains CNMN-LB-50 + *Lactobacillus bulgaricus* CNMN-LB-42 has a higher viscosity and faster clotting time than the starter culture consisting of 3 *Streptococcus thermophilus* strains CNMN-LB-50 + *Streptococcus thermophilus* CNMN-79 + *Lactobacillus bulgaricus* CNMN-LB-42.

In all varieties, the elimination of the whey was not detected, the titratable acidity was within the limits and contained 98-118°T. Therefore, the associations formed correspond to the requirements stipulated for the starter cultures intended for the manufacture of fermented dairy products. Also, the elaborated starter cultures were examined microscopically to determine the ratio between *Streptococcus thermophilus* and *Lactobacillus bulgaricus* cultures. The results are shown in Figure 2.

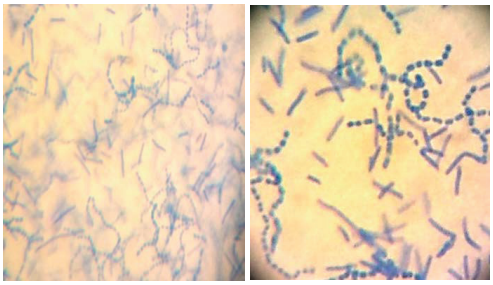

Figure 2. Microscopic aspect of the formed associations

The starter culture compounds were tested for the technological characteristics and symbiotic nature in the case of multicomponent cultures in order to select the ones with the highest

prospects for use in the manufacture of fermented dairy products. The cultures were inoculated in goat milk within 5% amount. At the initial stage, the acidogenesis activity of the native starter cultures was analyzed. The acidification activity of the starter cultures for 8 hours of fermentation at temperatures of 40 ± 1 °C, it was found that the decrease of the acidity of the starter had a 5 hours of fermentation and values equal to 4.58 ± 0.02 - 4.31 ± 0.03 pH units. After 5 hours of fermentation, when the clot is already formed, the decrease of the activity of the starter culture continues in parallel with some small deviations. After 8 hours of fermentation the active acidity has the following values: the pH of the cultures YO1 - 4.17 ± 0.05, YO2 - 4.15 ± 0.02, YO3 - 4.28 ± 0.01, which after 8 hours they have not changed fundamentally, from where we can conclude that starter cultures will not cause the deterioration of the finished product during storage.

CONCLUSIONS

In the presented research, the chemical composition and physico-chemical properties of goat milk were determined. The strains of valuable native lactic bacteria of the species *Streptococcus thermophilus* and *Lactobacillus bulgaricus* were selected with stable technological characteristics for the fermentation of goat milk, corresponding to the requirements for lactic bacteria intended for the yogurt production. The scheme for preparing starter cultures and recommendations regarding the use of consortia of symbiotic cultures for the goat milk yoghurt production have been developed. Starter cultures have been obtained for the goat milk yogurt production with biotechnological properties characteristic for fermented dairy products.

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REFERENCES

Asresie, A., Adugna, M. (2014). Bioactive Properties of Goat Milk: It's Hypoallergenic, Nutritional and Therapeutic Significance: A Review. *Global Journal of Animal Scientific Research*, 2(4), 315-320.

Beshkova, D.M., Simova, E.D., Dimitrov, Zh.P., Simov, Zh.I. (2011). Enhancing Proteolytic Activity, Nutritional and physiological benefits of caprine yogurt. *Proceedings of IDF International Symposium on Sheep, Goat and other non-Cow Milk*, Athens, Greece, 154-156.

Caleja, C., Barros, L., Antonio, A.L., Carocho, M., Oliveira, M.B.P.P., Ferreira, I.C.F.R. (2016). Fortification of yogurts with different antioxidant preservatives: A comparative study between natural and synthetic additives. *Food Chemistry*, 210, 262-268.

Chen, X., Li, X.Y., Zhao, X., Qin, Y.S., Wang, C.F. (2020). Proteomics and microstructure profiling of goat milk protein after homogenization. *Journal of Dairy Science*, 102(5), 3839-3850.

Deshwal, G.K., Ameta, R., Sharma, H., Singh, A.K. et al. (2020). Effect of ultrafiltration and fat content on chemical, functional, textural and sensory characteristics of goat milk-based Halloumi type cheese. *LWT*, 126, 109341.

Feng, C., Wang, B., Zhao, A., Wei, L., Zhang, F. (2019). Quality characteristics and antioxidant activities of goat milk yougurt with added jujube pulp. *Food Chemistry*, 277, 238-254.

Garcia, V., Rovira, S., Boutoial, K., Lopez, M.B. (2014). Improvements in goat milk quality: A review. *Small Ruminant Research*, 121(1), 51-57.

Hassan, F.A.M., Abbas, H.M., Abd El-Gawad, M.A.M., Enab, A.K. (2014). Goats dairy products as a potentially functional food. *Life Science Journal*, 11(9s), 648-657.

Innocente, N., Biasutti, M., Rita, F., Brichese, R., Comi, G., Iacumin, L. (2016). Effect of indigenous Lactobacillus rhamnosus isolated from bovine milk on microbiological characteristics and aromatic profile of traditional yogurt. *LWT-Food Science and Technology*, 66, 158-164.

ISO 2446:2008 (IDF 226:2008). Milk - Determination of fat content.

ISO 8968-1:2014 (IDF 20-1:2014). Milk and milk products - Determination of nitrogen content.

ISO 22662:2007 (IDF 198:2007). Milk and milk products - Determination of lactose content by high-performance liquid chromatography.

Li, X.Y., Cheng, M., Li, J., Zhao, X., Wang C.F. (2020). Change in the structural and functional properties of goat milk protein due to pH and heat. *Journal of Dairy Science*, 103(2), 1337-1351.

Muelas, R., Martí de Olives, A., Romero, G., Díaz, J.R., Sendra, E. (2018). Evaluation of individual lactic acid bacteria for the fermentation of goat milk: Quality parameters. *LWT*, 98, 506-514.

Paz, N.F., Oliveira, E.G., Kairuz, M.S.N., Ramon, A.N. (2014). Characterization of goat milk and potentially

symbiotic non-fat yogurt. *Food Science and Technology (Campinas)*, 34(3), 629-635.

Picon, A., López-Pérez, O., Torres, E., Garde, S., Nuñez, M. (2019). Contribution of autochthonous lactic acid bacteria to the typical flavour of raw goat milk cheeses. *International Journal of Food Microbiology*, 299, 8-22.

Popovici, C., Tita, M.A., Cartasev, A., Brinza, R., Bogdan, N. (2019). Isolation and characterization of lactic acid bacteria strains from goat milk for potential use in the production of yougurt. *Journal of EcoAgriTourism*, 15(39), 55-66.

Popovici, C., Tita, M.A., Brinza, R. (2018). Sensory evaluation and rheological behavoir of yogurts prepared from goat milk. *Proceedings of the 57th Annual Science Conference of Ruse University "New Industries, Digital Economy, Society-Protections of the Future"*, 57, 351-360.

Saha, B.N.P., Vasiljevica, T., McKechnieb, S., Donkor, O.N. (2016). Physicochemical, textural and rheological properties of probiotic yogurt fortified with fibre-rich pineapple peel powder during refrigerated storage. *LWT - Food Science and Technology*, 65, 978-986.

Serhan, M., Mattar, J., Debs, L. (2016). Concentrated yogurt (Labneh) made of a mixture of goats' and cows' milk: Physicochemical, microbiological and sensory analysis. *Small Ruminant Research*, 138, 46-52.

Shori, A.B., Baba, A.S. (2015). Survival of Bifidobacterium bifidum in cow- and camel milk yogurts enriched with *Cinnamomum verum* and *Allium sativum*. *Journal of the Association of Arab Universities for Basic and Applied Sciences*, 18, 7-11.

Sousa, Y.R.F., Medeiros, L.B., Pintado, M.M.E., Queiroga, R.C.R.E. (2019). Goat milk oligosaccharides: Composition, analytical methods and bioactive and nutritional properties. *Trends in Food Science & Technology*, 92, 152-161.

Sun, Y., Wang, C., Sun, X., Jiang, S., Guo, M. (2020). Characterization of the milk fat globule membrane proteome in colostrums and mature milk of Xinong Saanen goats. *Journal of Dairy Science*, 103(4), 3017-3024.

Verruck, S., Dantas, A., Prudencio, E.S. (2019). Functionality of the components from goat's milk, recent advances for functional dairy products development and its implications on human health. *Journal of Functional Foods*, 52, 243-257.

Wang, Y., Eastwood, B., Yang, Z., Campo, L., Hemar, Y. (2019). Rheological and structural characterization of acidified skim milks and infant formulae made from cow and goat milk. *Food Hydrocolloids*, 96, 161-170.

Yangilar, F. (2013). As a Potentially Functional Food: Goats' Milk and Products. *Journal of Food and Nutrition Research*, 1(4), 68-81.

Yurchenko, S., Sats, A., Tatar, V., Kaart, T., Jõudu, I. (2018). Fatty acid profile of milk from Saanen and Swedish Landrace goats. *Food Chemistry*, 254, 326-332.

Zhao, X., Cheng, M., Zhang, X., Li, X., Wang, C. (2020). The effect of heat treatment on the microstructure and functional properties of whey protein from goat milk. *Journal of Dairy Science*, 103(2), 1289-1302.

Zhu, Z., Zhu, X., Kong, F., Guo, W. (2019). Quantitatively determining the total bacterial count of raw goat milk using dielectric spectra. *Journal of Dairy Science*, 102(9), 7895-7903.

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RADIOFREQUENCY TREATMENT TO CONTROL MICROBIAL FOOD SPOILAGE ISSUES - A CRITICAL REVIEW

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Abstract

Food spoilage may be defined as a process or few changes that could lead to an undesirable or unacceptable product for consumption. Food stability may be hampered by the contamination of a large diversity of microbial spoilers, from prokaryotes (Gram-negative and Gram-positive bacteria) to unicellular (yeasts) and multicellular eukaryotes (moulds). Effective preventive measures and innovative preservation methods have been intensively studied with the aim of food spoilage reduction and food shelf life improvement. The primary focus of food preservation has been on controlling microbial populations, with a specific emphasis on pathogenic microorganisms. In the last years, innovative processes and technologies have been applied in food industry as non-conventional preservative hurdles, which also respond to consumer demand for safe minimally processed food. This paper studies the effect of radiofrequency (RF) treatment on food spoilage microorganisms.

Key words*: food spoilage, radiofrequency, antimicrobial.*

INTRODUCTION

In recent years, food spoilage has been a growing problem in the food industry, mainly because of the limitation for the use of preservatives as a conservation method, in order to apply mild conservation techniques as a direct response to the consumers' requirements. Microorganisms that are present in food products continue to be a problem both for maintaining the quality of the food products and also regarding safety aspects. Therefore, food safety is a constant concern for the food industry (Deliu et al., 2013). Taking these matters into consideration, studies have been made in order to better understand the spoilage microorganisms and their phenotypic development and genetic makeup (Sanders et al., 2015). Unwanted organoleptic changes in food products may occur because of the food decomposition, which is a metabolic process that takes place inside the food matrix. These organoleptic changes consist in appearance, taste and consistency alterations of the food product, and even though generally they do not cause food borne illnesses, they affect the retail value of the food products. Some microorganisms that are involved in the food decomposition process may cause food poisoning (Shekarforoush et al., 2015).

Microbial food spoilage is characterised by random contamination with microorganisms which may lead to uncontrolled changes to the food product. The nature and character of a contaminant are not known before the food-microorganism contact happens. Consequently, in food preservation and prevention of spoilage, it is desired to account for all possible contaminants. In practice this is not feasible as many different species and strains from very different sources may cause spoilage, even in a selective environment based on a defined food formulation. This problem drives the need to better characterise possible contaminants and to describe the microbial biodiversity. Such knowledge may help to define better the boundaries for preservation of a particular food category, which is of relevance to consumers demanding minimally preserved, high quality and yet reliable food products. The importance of assessing biodiversity has been well appreciated for food pathogens (Lianou & Koutsoumanis, 2013).

The constant study and evaluation of the processing time, heating uniformity and quality influence over the final treated food product is

performed due to the fact that these factors derive from the food heating processing methods. Another very important factor that is being evaluated is the energy efficiency prediction. There are several technologies (physical, chemical, and biological) used to control or reduce microorganisms in foods. Apart from the traditional processes such as thermal processing, novel technologies such as high-pressure processing, pulsed electric field processing, irradiation, ultraviolet light, ozone, others are also being developed (Deliu et al., 2013; Bermudez-Aguirre and Barbosa-Canovas, 2010).

The most common electromagnetic fields applied in the food industry are microwaves at 915 and 2450 MHz and radio frequency at a nominal 27 MHz (Brunkhorst et al., 2016). Radio-frequency (RF) heating treatment is more preferred by the food industry because it uses a longer wavelength electromagnetic energy that microwaves heating (MW). RF heating systems are primarily composed of two parts: a generator and an applicator. The generator supplies radiofrequency power, with free running oscillating systems being the most common generators used in food processing industries. The applicator consists of electrode plates that send electromagnetic currents to the products through field applicators (Rowe, 2018).

Radio-frequency (RF) heating treatment does not affect the air surrounding the product, only targeting the product itself. The overheating and over-dehydration of the treated products surface is being avoided by equalizing moisture throughout the product because the interior of the product gets hot faster than the surface, RF treatment bringing the moisture from inside outward (Orsat & Raghavan, 2014). Because of the above mentioned, the RF treatment can be applied in many applications of the food industry like drying of food products. Due to the good penetration depth, heat homogeneity and more stable control of the product temperature, in recent years, there has been an increased interest in radio-frequency thermal drying (Altemimi et al., 2019).

This paper studies the effect of radiofrequency (RF) treatment applied in food products, with special emphasis on the effect on the microorganisms involved in food spoilage.

INACTIVATION MECHANISM OF RF

Radiofrequency is a part of the electromagnetic spectrum with frequency in the range of 30-300 MHz. It is an indirect electro heating technique where initially, the electrical energy is transformed to electromagnetic radiation and slowly released as heat into the desired food sample depending on the dielectric characteristics of the food product (Rifna et al., 2019). The RF heating is achieved by using electric resistance heating and dipole heating resulted from the movement of the dissolved ions in the treated food product (Awuah et al., 2005).

Effectiveness of RF treatments on microorganism inactivation relies on few key factors such as temperature, frequency, effect of sample depth, sample moisture, capacity of equipment, microbial targets. The microbial decontamination using radiofrequency heating is based on the principle that when the heat is developed at a faster rate within the microbial cell than in another medium, the cells get thermally destroyed at a low heating rate (Rifna et al., 2019). As a consequence of exposing bacteria to RF radiations, injury may take place, which causes the leakage of some intracellular components such as ATP, nucleic acids, and proteins. This leakage leads to imbalance of the energy system, the enzymatic activity, and finally to cell death (Roohinejad et al., 2018).

The transmembrane potential is artificially increased by the exposure of a cell to an electric field that creates a charge which is stored on the cell membrane. If the transmembrane potential level is high enough and maintained for a long enough period of time, a significant increase in the cell membrane permeability to macromolecules and ions will appear.

The membranes of cells will reseal when the induced permeabilization level is moderate, and so the cell will be viable minutes after field delivery. The cells will die if the permeabilization level is made with prolonged and very high field (González-Sosa et al., 2014).

RF TREATMENT APPLICATIONS IN FOOD INDUSTRY

Lately, many studies were focused on the effect of dielectric heating processing over a large variety of food products on the microbial and pest reduction effects, such as poultry, meat, eggs and egg by-products, fish, fresh or canned fruits and vegetables, soy milk, jam, cakes, pasta, breads, spices, starch and ready to eat meals (Mitelut et al., 2011; Orsat & Raghavan, 2014).

Some examples are summarized in Table 1 and further described in more detail.

Villa-Rojas et al. (2017) studied RF heating of organic wheat flour, and evaluated *Enterococcus faecium* as a surrogate for RF inactivation of *Salmonella*. *Salmonella* reduction of 7 log was achieved at 0.45 and 0.65 a_w at room temperature, while 5 and 3 log reductions were reached for *Salmonella* and *E. faecium*, respectively, at 0.25 a_w. These data suggest that RF heating has potential as an inactivation treatment for *Salmonella*, and that *E. faecium* is a feasible surrogate to validate the efficacy of RF treatments.

Radiofrequency (RF) heating was evaluated by Chen et al. (2019) as a novel spice decontamination technology of cumin seeds. For the sample treatment a RF system of 6kW 27.12 MHz was used and two microbial strains, *Salmonella enterica* and *Enterococcus faecium* were inoculated in the cumin seeds samples in order to study the microbial change after the RF treatment. The treated samples displayed different results: *E. faecium* strain showed higher survival rate than *Salmonella* in all three batches of cumin samples, thus concluding that *E. faecium* can be used as a surrogate of *Salmonella* in the RF treatment process. In terms of quality of the treated cumin samples, no significant differences were recorded (Chen et al., 2019).

A study performed by Kou et al. (2018) analysed the use of non-thermal processing inactivation over some microorganisms with the help of RF treatments. Two types of strains, *Escherichia coli* and *Staphylococcus aureus,* that are present in apple juice and mash potatoes were treated with RF and conventional heating treatments. In order to compare their efficiency over the inactivation of the tested microorganisms, a radio-frequency treatment system that operates at 27.12 MHz frequency was designed. This system is comprised of a thermal heating block that emulates the desired heating temperature, uniformity and rate.

The survival rates of the *Escherichia coli* and *Staphylococcus aureus* strains present in the analysed food samples were similar as well for the heating block treatments as well for the radio-frequency treatments. A 1 log CFU/ml absolute difference of survival populations was observed. The results showed that the microorganism inactivation was observed only at the energy of 27.12 MHz obtained by the RF system (Kou et al., 2018).

The radio-frequency (RF) heating efficiency to inactivate *Salmonella typhimurium* and *Escherichia coli* O157: H7 on black and red pepper spice was investigated by Kim et al. (2012). A 27.12 MHz RF heating system consisted of two parallel plate electrodes was used, with the sample being placed between them. Two types of pepper samples, black peppers (whole and ground) and red peppers, were inoculated with *S. typhimurium* and *E. coli* O157:H7 strains and then treated with RF energy for different periods of time: 50 seconds for black peppers and 40 seconds for red peppers. Colour changes that occurred during the RF treatment of the samples were evaluated. The results showed that the RF heating treatment applied for 50 seconds to the black peppers resulted in 2.80 to 4.29 log CFU/g reductions of *S. typhimurium* and *E. coli* O157:H7. As for the red pepper samples that were treated with RF for 40 seconds, the pathogens were reduced by 3.38 log CFU/g to more than 5 log CFU/g (below the detection limit) without affecting the colour quality change (Kim et al., 2012).

In 2019, Wei et al. started a study that concerned the application of RF processing for pasteurization of black pepper that has been ground. At a radio-frequency treatment time of 130 seconds more than 5.98 log CFU/g reduction for *Salmonella* spp. was obtained, as for the *E. faecium* strain a reduction of 3.89 log CFU/g was obtained. Higher thermal resistance of *E. faecium* strain indicated its suitability as surrogate for *Salmonella* spp. during RF heating of ground black pepper (Wei et al., 2019).

Table 1. Effect of RF treatment applied in different food products

Product	Effect of RF treatment	Reference
Organic wheat flour	reduction of *Salmonella* and *E. faecium* in tested samples	Villa-Rojas et al., 2017
Cumin seeds	*E. faecium* strain showed higher survival rate than *Salmonella* in cumin samples	Chen et al., 2019
Black and red pepper	reduction of *S. typhimurium* and *E. coli* O157:H7 colour of samples was not affected	Kim et al., 2012
Black pepper	reduction of *Salmonella* spp. and *E. faecium* in tested samples	Wei et al., 2019
Peaches and nectarines	RF treatment effective to control *Monilinia fructicola* in tested samples	Casals et al., 2010
Cooked carrots	combined sterilization effect of ZnO nanoparticles with RF treatments prolonged the shelf life of cooked carrots up to 60 days, and reduced the loss of hardness, carotenoids and colour difference value of tested sample	Xu et al., 2017
In-shell walnuts	100% mortality rate for the instar navel orange worm larvae	Wang et al., 2007
Milk	inactivating of *Listeria* and *E. coli* in tested samples	Awuah et al., 2005
Soybean milk	reduction of *Bacillus subtilis* spores in tested samples	Uemura et al., 2010
Orange juice	reduction of *Escherichia coli* K12 in tested samples	Geveke et al., 2007
Pre-packaged bread Loaf	reduction of *Penicillium citrinum* spores shelf-life was prolonged by 28 ± 2 days	Liu et al., 2010
Peanut butter Cracker sandwiches	reduction of *S. typhimurium* and *E. coli* O157:H7 in both creamy and chunky peanut butter	Ha et al., 2013
Ham	improvement of shelf life of repacked hams by reducing the bacterial load and moisture loss	Orsat et al., 2004
Ground beef	heating to 55°C reduced *E. coli* in homogenates containing 2.5% potassium bicarbonate, 0.5% citric acid, and blends of citric acid and potassium bicarbonate at varying concentrations (0.5% citric acid and 1.5% bicarbonate)	Nagaraj et al., 2016

The growth control of brown mould in peaches and nectarines with the help of RF heating was studied by Casals et al. (2010). The samples were inoculated with *Monilinia fructicola* or with natural *Monilinia* spp. Inoculum. The results showed that a RF treatment at 27.12 MHz, with 17 mm distance between the fruit samples and upper electrode and an exposure time set at 18 min was selected as effective conditions to control brown moulds development in peaches without affecting fruit quality (Casals et al., 2010).

Effects of ZnO nanoparticles combined with RF heating on the sterilization and product quality attributes (hardness, colour, carotenoids and microstructure) of cooked carrots were investigated by Xu et al. (2017). The results of this study demonstrated that the combined sterilization effect of ZnO nanoparticles with RF treatments was greater than ZnO nanoparticles or RF heating treatment alone and prolonged the shelf life of cooked carrots up to 60 days. RF heating 20 mm/20 min (plate spacing/RF heating time) combining ZnO nanoparticles reduced the loss of hardness, carotenoids and colour difference value (ΔE) of cooked carrots (Xu et al., 2017).

A study concerning the use RF treatment as an alternative method to chemical fumigation in order to control postharvest insect contamination of in-shell walnuts was carried out by Wang et al. (2007). The RF treatment process was made possible by using a 25 kW, 27 MHz RF processing technique to achieve surface temperature of 60°C of the walnut samples. The minimum temperature has to be 52°C and the minimum treatment time needs to be 5 min. The results showed a mortality rate of 100% for the instar navel orange worm larvae, which is the most heat tolerant target pest, which is present in both air-dried and unwashed walnuts. The 100% mortality rate was achieved over a relatively wide range of walnut moisture contents (3-7.5%).

Awuah et al. (2005) used a 2 kW, 27.12 MHz RF heater, for the evaluation of the effectiveness of RF heating in inactivating surrogates of both *Listeria* and *Escherichia coli* cells in milk under continuous flow conditions. Depending on product residence time and RF power level, RF heating was found to be capable of inactivating both *Listeria* and *E. coli* in milk, with *E. coli* being the most heat sensitive. For a total residence time of 55.5 s (i.e., 29.5 and 26 s in the

applicator and holding tube, respectively), up to 5- and 7-log reductions were found for heating *Listeria* and *E. coli*, respectively at 1200 W, and an applicator tube exit temperature of approximately 65°C (Awuah et al., 2005).

Uemura et al. (2010) developed an equipment for soybean milk pasteurization using RF flash heating (RF-FH). The soybean milk samples were exposed to an electrode with its surface covered in a 50 μm thick Teflon film and a 28 MHz RF-FH energy was applied. The exposure time was 0.4s and the treatment temperature was at 115°C which lead to a four-logarithm-order reduction of *Bacillus subtilis* spores in the soybean milk sample. In the same study, the soybean milk samples were processed with of RF-FH and conventional processing in order to obtain tofu. The results of the study revealed that the tofu produced using RF-FH processing had higher gel strength than the tofu made using conventional heating (Uemura et al., 2010).

Geveke et al. (2002) performed a study on non-thermal inactivation of microorganisms contained in some liquid samples. The liquid samples were treated with RF energy and in the same time heat was removed in order to control the temperature while in order to minimize localized heating the turbulent flow was maintained. Electric field strength of approximately 0.5 kV/cm was obtained from an 18 MHz RF processor, and then applied to the liquid samples. No non-thermal effects of the RF treatment energy were observed in yeast in apple cider, beer, tomato juice, liquid whole egg, deionized water, *Escherichia coli* K-12 and *Listeria innocua*. Also, there were no synergistic effects of RF energy related with heat. The low temperature effects of RF energy at 18 MHz and 0.5 kV/cm were due to heat (Geveke et al., 2002).

The effect of radio frequency electric fields (RFEF) on the inactivation of *E. coli* in apple and orange juice was investigated by Geveke et al. (2007). Pulp-free orange juice samples were processed using an 80 kW RFEF pasteurizer at flow rates of 1.0 l/min and 1.4 l/min. The orange juice samples inoculated with *Escherichia coli* K12 were treated with electric field strengths of 15 kV/cm and 20 kV/cm at three different range frequencies: 21, 30, and 40 kHz. A reduction of 3.3 log of *Escherichia coli* K12 was observed at the orange juice samples compared to the control sample, at an outlet temperature of 65°C. It was shown that by increasing the temperature and time of the RF treatment and decreasing the frequency, enhanced the inactivation levels. The variation of the electric field strength over the conditions used in the experiments had no effect on the inactivation of the *Escherichia coli* K12. After the RFEF thermal processing of the samples, no enzymatic browning or diminution of the ascorbic acid concentrations were observed. The voltage and the current used in the experiments resulted in an electric energy of 180 J/ml (Geveke et al., 2007).

A study concerning the applications of radio frequency (RF) treatment combined with conventional hot air treatment used to provide uniform heating for control of mould in pre-packaged bread loaf samples was done by Liu et al. (2010). In order to conduct the experiments a 6 kW, 27.12 MHz RF system was used. The treatment parameters used in the experiments were selected in a way that they did not influence the bread quality and based on minimum time–temperature conditions that were required for 4-log reduction of *Penicillium citrinum* spores. The core and periphery of bread loafs samples were subjected to heat with approximately the same heating rate during the combined hot air and RF treatments, thus the maximum difference of heating temperatures was less than 5°C in one bread slice. The overall differences in sample qualities between RF treated bread samples and control samples were not significant, the water activity and moisture contents of RF treated samples increased in the beginning of the treatment and then decreased, compared to the untreated samples. The firmness values showed an increasement during the storage of the both types of samples, treated and untreated. In order to control the *P. citrinum* spores, because of greater heating uniformity, a lower mean product temperature and a shorter holding were used with combined RF and hot air treatment in order to compare with the conventional heating alone. A reduction of 4-log of *P. citrinum* spores was obtained when heating bread samples at about 58°C. The shelf-life was prolonged by 28 ± 2 days for the treated white bread samples at room temperature (23°C) (Liu et al., 2010).

Ha et al. (2013) investigated the efficacy of RF heating to inactivate *S. typhimurium* and *E. coli*

O157:H7 in peanut butter cracker sandwiches. The treatment consists of a 27.12 MHz RF heating system with a maximum time of exposure of 90s. After treatment, a log reduction (CFU/g) of 4.29 was registered for *S. typhimurium* and 4.39 for *E. coli* O157:H7 in the case of creamy peanut butter. Regarding chunky peanut butter, the treatment led to a log reduction of 4.55 for *S. typhimurium* and 5.32 for *E. coli* O157:H7 (Ha et al., 2013).

Orsat et al. (2004) studied the radio-frequency heating at 27.12 MHz for the pasteurization of ham samples repacked in plastic films. The samples were brought to internal temperatures of 75°C and 85°C in 5 min and maintained at those temperatures for an additional 5 min. The ham samples were vacuum-packed in three different plastic films and stored at 4°C for 1 to 28 days. The study indicates that RF heating, combined with suitable packaging system, can lead to an improvement of the shelf life of repacked hams by reducing the bacterial load, moisture loss and maintaining an overall greater product sensory quality and acceptance (Orsat et al., 2004).

Nagaraj et al. (2016), investigate the inactivation of *E. coli* in ground beef homogenate using RF treatment both individually and in combination with antimicrobial agents. The RF heating of inoculated beef homogenate without antimicrobial agents to end-point temperatures of 50°C and 55°C resulted in 0.84 log reduction, respectively 0.94 log reductions. Adding antimicrobials (such as potassium, sodium, and ammonium bicarbonates (0.5 and 1.5%); potassium lactate (2.5%); citric acid (0.5%); and a blend of citric acid (0.5%) and potassium bicarbonate (1.5%) to the beef homogenates before RF treatment did not inactivate *E. coli*. Heating to 55°C reduced *E. coli* more than 5 log (CFU/mL) in homogenates containing 2.5% potassium bicarbonate, 0.5% citric acid, and blends of citric acid and potassium bicarbonate at varying concentrations (0.5% citric acid and 1.5% bicarbonate) (Nagaraj et al., 2016).

A simulation of RF heating was performed using a finite element shell for microbial decontamination of egg shell immersed in deionized water by Lau et al. (2017). In all the investigated configurations, concentrated heating occurred in the yolk. After 20 min of RF heating, some configurations reached about 60°C in the yolk. The cooling effect of the water together with lower electric field intensity in the egg caused the focused heating in the yolk. Extrapolation of the model demonstrated that a RF heating process (10.5 kV at top electrode) followed by a hot water immersion process can achieve a minimum of 3 log reductions of *Salmonella* in the yolk within 37 min (Lau et al., 2017).

CONCLUSIONS

Nowadays, these novel processing techniques are being intensively tested to be used in food industry in order to improve the safety and quality of the food products that we eat, as they are capable of inactivating microorganisms, promoting chemical reactions, changing cell permeability, and even inactivating enzymes. Due to major advantages offered by RF treatment, including the possibility to fast penetration up to 20 cm or more into food for more uniform and efficient heating and limited negative side effects (reduced food quality or objectionable sensory perception), RF technology has considerable potential to replace traditional (water and steam) and microwave heating for food processing.

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REFERENCES

Altemimi, A., Aziz, S. N., Al-HiIphy, A. R. S., Lakhssassi, N., Watson, D. G., Ibrahim, S. A. (2019). Critical review of radio-frequency (RF) heating applications in food processing. *Food Quality and Safety*, 3, 81–91.

Awuah, G. B., Ramaswamy, H. S., Economides, A., Mallikarjunan, K. (2005). Inactivation of *Escherichia coli* K-12 and *Listeria innocua* in milk using radio frequency (RF) heating. *Innovative Food Science and Emerging Technologies*, 6, 396–402.

Bermúdez-Aguirre, D., Barbosa-Cánovas, G. V. (2012). Disinfection of selected vegetables under nonthermal treatments: Chlorine, acid citric, ultraviolet light and ozone. *Food Control*, 29(1), 82–90.

Brunkhorst, C., Ciotti, D., Fredd, E., Wilson, J. R., Geveke, D. J., Kozempel, M. (2016). Development of process equipment to separate nonthermal and thermal effects of RF energy on microorganisms, *Journal of*

Microwave Power and Electromagnetic Energy, 35(1), 44–50.

Casals, C., Viñas, I., Landl, A., Picouet, P., Torresa, R., Usalla, J. (2010). Application of radio frequency heating to control brown rot on peaches and nectarines. *Postharvest Biology and Technology*, 58, 218–224.

Chen, L., Wei, X., Irmak, S., Chaves, B. D., Subbiah, J. (2019). Inactivation of *Salmonella enterica* and *Enterococcus faecium* NRRL B-2354 in cumin seeds by radiofrequency heating. *Food Control*, 103, 59–69.

Deliu, I., Giosanu, D., Stanescu, C. (2013). The microwaves effects on liquid foods, *Scientific Bulletin. Series F. Biotechnologies*, XVII, 208–211.

Geveke, D. J., Brunkhorst, C., Fan, X. (2007). Radio frequency electric fields processing of orange juice. *Innovative Food Science and Emerging Technologies*, 8(4), 549–554.

Geveke, D. J., Kozempela, M., Scullena, O. J., Brunkhorst, C. (2002). Radio frequency energy effects on microorganisms in foods. *Innovative Food Science and Emerging Technologies*, 3(2), 133–138.

González-Sosa, J., Ruiz-Vargas, A., Arias, G., Ivorra, A. (2014). Fast flow-through non-thermal pasteurization using constant radiofrequency electric fields. *Innovative Food Science and Emerging Technologies*, 22, 116–123.

Ha, J. W., Kim, S. Y., Ryu, S. R., Kang, D. H. (2013). Inactivation of *Salmonella enterica* serovar *Typhimurium* and *Escherichia coli* O157:H7 in peanut butter cracker sandwiches by radio-frequency heating. *Food Microbiology*, 34, 145–150.

Kim, S. Y., Sagong, H. G., Choi, S. H., Ryu, S., Kang, D. H. (2012). Radio-frequency heating to inactivate *Salmonella typhimurium* and *Escherichia coli* O157:H7 on black and red pepper spice. *International Journal of Food Microbiology*, 153, 171–175.

Kou, X., Li, R., Hou, L., Zhang, L., Wang, S. (2018). Identifying possible non-thermal effects of radio frequency energy on inactivating food microorganisms. *International Journal of Food Microbiology*, 269, 89–97.

Lau, S. K., Thippareddi, H., Subbiah, J. (2017). Radiofrequency Heating for Enhancing Microbial Safety of Shell Eggs Immersed in Deionized Water, *Journal of Food Science*, 1–11.

Lianou, A., Koutsoumanis, K. P. (2013). Strain variability of the behavior of foodborne bacterial pathogens: a review. *International Journal of Food Microbiology*, 167(3), 310–321.

Liu, Y., Tang, J., Mao, Z., Mah, J. H., Jiao, S., Wang, S. (2010). Quality and mold control of enriched white bread by combined radio frequency and hot air treatment. *Journal of Food Engineering*, 104, 492–498.

Mitelut, A., Popa, M., Geicu, M., Niculita, P., Vatuiu, D., Vatuiu, I., Gilea, B., Balint, R., Cramariuc, R. (2011). Ohmic treatment for microbial inhibition in meat and meat products. *Romanian Biotechnological Letters*, 16(1), 149–152.

Nagaraj, G., Purohit, A., Harrison, M., Singh, R., Hung, Y. C., Mohan, A. (2019). Radiofrequency pasteurization of inoculated ground beef homogenate. *Food Control*, 59, 59–67.

Orsat, V., Raghavan, G. S. V. (2014). Radio-Frequency Processing. *Emerging Technologies for Food Processing (Second Edition)*, 385–398.

Orsat, V., Bai, J., Raghavam, G. S. V., Smith, J. P. (2004). Radiofrequency heating of ham to enhance shelf-life in vacuum packaging. *Journal of Food Engineering*, 27, 267–283.

Rifna, E. L., Singh, S. K., Chakraborty, S., Dwivedi, M. (2019). Effect of thermal and non-thermal techniques for microbial safety in food powder: Recent advances. *Food Research International*, 126, 108654.

Roohinejad, S., Koubaa, M., Sant'Ana, A. S., Greiner, R. (2018). 4. Mechanisms of Microbial Inactivation by Emerging Technologies. *Innovative Technologies for Food Preservation*, *Elsevier Inc*., 111–132.

Rowe, R. (2018). Radiofrequency Heating, BSEN 3240.

Sanders, J. W., Oomes, S. J. C. M., Membré, J. -M., Wegkamp, A., Wels, M. (2015). Biodiversity of spoilage lactobacilli: Phenotypic characterization. *Food Microbiology*, 45, 34–44.

Shekarforoush, S. S., Basiri, S., Ebrahimnejad, H., Hosseinzadeh, S. (2015). Effect of chitosan on spoilage bacteria, *Escherichia coli* and *Listeria monocytogenes* in cured chicken meat. *International Journal of Biological Macromolecules*, 76, 303–309.

Uemura, K., Takahashi, C., Kobayashi, I. (2010). Inactivation of *Bacillus subtilis* spores in soybean milk by radio-frequency flash heating. *Journal of Food Engineering*, 100, 622–626.

Villa-Rojas, R., Zhu, M. J., Marks, B. P., Tang, J. (2017). Radiofrequency inactivation of *Salmonella Enteritidis* PT 30 and *Enterococcus faecium* in wheat flour at different water activities. *Biosystems Engineering*, 156, 7–16.

Wang, S., Monzon, M., Johnson, J. A., Mitcham, E. J., Tang, J. (2007). Industrial- scale radio frequency treatments for insect control in walnuts. II. Insect mortality and product quality. *Postharvest Biology and Technology*, 45(2), 247–253.

Wei, X., Lau, S. K., Stratton, J., Irmak, S., Subbiah, J. (2019). Radiofrequency pasteurization process for inactivation of *Salmonella* spp. and *Enterococcus faecium* NRRL B-2354 on ground black pepper. *Food Microbiology*, 82, 388–397.

Xu, J., Zhang, M., Bhandari, B., Kachele, R. (2017). ZnO nanoparticles combined radio frequency heating: A novel method to control microorganism and improve product quality of prepared carrots, *Innovative Food Science and Emerging Technologies*, 44, 46–53.

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ASSESSMENT OF ACRYLAMIDE CONTENT IN SOME FOODSTUFFS ON THE ROMANIA MARKET

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Abstract

The paper aimed to present the evaluation of acrylamide (AA) content in foodstuffs during 2018-2019 periods on the Romania market. It is based on the data provided by the National Sanitary Veterinary and Food Safety Authority (ANSVSA). The acrylamide levels were analyzed within ANSVSA by high performance liquid chromatography coupled with a diode array detector (HPLC-UV). The number of foodstuffs samples analyzed in Romania at the national level by ANSVSA was 138 from which 50 were analyzed in 2018 and 88 in 2019. From the total of foodstuffs samples analyzed only in one foodstuff sample was detected the AA level in 2018 and in sixteen foodstuffs samples was detected the AA in 2019.

Key words*: acrylamide, food safety, risk policy.*

INTRODUCTION

Acrylamide (AA) is a food-borne chemical as a result of cooking practices and especially in foodstuffs with high-carbohydrate content which are processed by high temperature (>120°C) (Ghalebi et al., 2019; Pratama & Jacxsens, 2019). Researchers from national food safety authorities, from academia medium, and from food industry have sought to better understand the mechanisms of acrylamide formation and to find ways to minimize its formation in foodstuffs. Maillard reaction, is the main mechanism of acrylamide formation in foodstuffs containing carbohydrates and amino acids, in particular asparagines, but it can be formed and through other pathways, such as the reaction between aspartic acid and reducing sugars, thermal degradation of amino acids and proteins, by the conversion of acrolein, acrylic acid, wheat gluten or by de-amination of 3-aminopropionamide, a result of asparagine enzymatic decarboxylation, as well as decarboxylation and deamination of asparagines (Nachi et al., 2018; Michalak et al*.,* 2019; Maan et al., 2020).

Acrylamide is found in many types of foodstuffs, including breakfast cereals, bakery products, roasted coffee, cappuccino powder, cookies, crackers, snacks, biscuits, wafers, frenchfries, fried potatoes, and potato chips (Ghalebi et al., 2019; Mousa et al*.,* 2019; Kang et al., 2020; Mesías, et al*.,* 2020; Schouten et al*.,*2020; Wang et al., 2020). For example, acrylamide concentration in potato chips fried in sunflower oil ranged from 525 to 722 µg/kg (Mekawi et al*.,* 2019). It is not found in protein-rich material such as meat and fish and in boiled products (Pratama & Jacxsens, 2019). Acrylamide has also been found in the environment, cosmetics, drinkingwater, as well as cigarette smoke (Tölgyesi & Sharma, 2020).

The toxicity of acrylamide has been acknowledged long ago, since 2002, when it was first reported that acrylamide can be found in foodstuffs by Tareke et al. (Murray et al., 2020). Human exposure to acrylamide may have toxicological effects (neurotoxicity, genotoxicity, carcinogenicity, and reproductive toxicity), and acrylamide has been classified as carcinogenic by the International Agency for Research on Cancer in the 2A group (probably carcinogenic in humans) (Fernandes et al*.,* 2019). Acrylamide is found in various tissues of the body such as liver, kidney, brain, heart and even breast milk (Ghalebi et al., 2019). Several studies using human cells have shown that chronic low-level exposure to acrylamide can lead to progressive degeneration of the peripheral and central nervous systems characterized by cognitive and motor abnormalities (Murray et al., 2020; Wang et al., 2020).

Therefore, many efforts have been made to decrease content of acrylamide in foodstuffs and develop analytical method for the determination of acrylamide. Among the methods of reducing the level of acrylamide in foods may be following: the use of the enzyme asparaginase, which converts asparagine to aspartic acid (Anese et al., 2011); the use of extracts with antioxidant properties (Miśkiewicz et al., 2019); the use of fermentation processes performed with lactic acid bacteria (LAB) for bread manufacturing (Bartkiene et al., 2011); manipulating processing conditions such as time and temperature of the heating process, and including certain preheating treatments such as soaking and blanching(Yıldız et al., 2017); the addition of different phenol standards and olive leaf extract (OLE) during the sterilization and baking process; the identification of potato varieties low in the acrylamide precursors asparagine and reducing sugars that vary with variety, nitrogen (N) fertilizer applications, and other agronomic factors (Johnson et al., 2019; Yu et al., 2019; Fernández et al., 2020; Martín-Vertedor et al., 2020; Rifai & Saleh, 2020).

European Commission started monitoring of acrylamide levels in processed foods since 2007 (Ghalebi et al., 2019). Acrylamide can be analysed using the following methods: gas or liquid chromatography (GC or LC); high performance liquid chromatography mass spectrometry (HPLC-MS), high performance liquid chromatography tandem mass spectrometry (HPLC-MS/MS), the bromination, xanthydrol, and silylation; ultra-performance liquid chromatography (UPLC); liquid chromatography-high resolution mass spectrometry (LC-HRMS); electrochemical biosensor for acrylamide detection based on hemoglobin entrapped in ionic liquid-carbon and other (Fernandes et al., 2019; Michalak et al., 2019; Wawrzyniak et al., 2019; Desmarchelier et al., 2020; Li et al., 2020; Tölgyesi & Sharma, 2020).

The determination of AA levels in different foodstuffs from Romania market was performed by the National Sanitary Veterinary and Food Safety Authority (A.N.S.V.S.A.) by using HPLC-UV method. The aim of this study was to analyze the data, regarding the level of AA in some foodstuffs and the distribution of in Romania, provided by ANSVSA in 2018 and 2019.

MATERIALS AND METHODS

Samples

A total of 138 foodstuffs samples of various types were collected from different counties from Romania market in order to determine their acrylamide level (AA). The foodstuffs samples were analyzed by the National Sanitary Veterinary and Food Safety Authority (A.N.S.V.S.A.). The foodstuffs samples were analyzed in the 2018 and 2019 years namely 50 samples in 2018 and 88 in 2019.

Laboratory tests were performed within the ANSVSA for foodstuffs samples that come from retail-supermarkets, hypermarkets units, specialized stores. The samples were collected by the inspectors of the veterinary sanitary directions and for the food safety from 7 counties in 2018 (Timis, Constanta, Mures, Iasi, Suceava, Prahova, Dolj) and from 16 counties in 2019 (Alba, Constanta, Suceava, Cluj, Timis, Iasi, Mures, Prahova, Bucharest, Dolj, Neamt, Braila, Arges, Vrancea, Bacau, Gorj). The samples were collected under the Surveillance and Control Program in the food safety of non-animal origin field, approved by Order A.N.S.V.S.A. no. 35/2016, with subsequent modifications, or within the self-control program of the production units.

The distribution of the 50 foodstuffs samples analyzed through it AA content by counties in 2018 are shown in Table 1.

Table 1. The AA level in foodstuffs samples analyzed in 2018 from Romania market

Category	Foodstuffs tested	Counties
Biscuits	4	Dolj (1) Iasi (1) Timis (2)
Fooddishes	1	Iasi (1)
Breakfast cereals	11	Constanta (1) Dolj (2) Iasi (2) Mures (2) Timis (2) Suceava (1) Prahova (1)
Roast coffee	9	Constanta (1) Dolj (1) Iasi (1) Mures (4) Prahova(1) Timis (1)
French fries	6	Constanta (1) Dolj (2) Iasi (1) Timis (1) Prahova(1)
Potato crisps	5	Constanta (1)
Food products based on potato	5	Dolj (1) Iasi (1) Prahova(1) Timis (1) Dolj (1) Prahova(1) Timis (2) Iasi(1)
Bread	4	Dolj (1) Iasi (1) Prahova (1) Timis (1)
Bakery products	3	Constanta(3)
Cocoa products	1	Prahova (1)
Confectionary products	1	Dolj (1)

The 88 foodstuffs samples analyzed for it AA content in 2019 are shown in Table 2 in which are presented the category of products analyzed and the numbers of collected samples from each Romania county.

Table 2. The AA level in foodstuffs samples analyzed in 2019 from Romania market

Category	Foodstuffs tested	Counties
Biscuits	6	Dolj (1) Mures (3) Timis (2)
Fooddishes	5	Constanta (1)
		Cluj (1) Alba (1) Bucuresti (1) Prahova (1)
Breakfast cereals	8	Iasi (1) Dolj (1)
		Constanta (1) Cluj (1) Bucuresti (1)
		Suceava (1) Prahova (1) Timis (1)
Roast coffee	18	Iasi (2) Dolj (1) Constanta (1)
		Cluj (2) Alba (1)
		Bucuresti (3) Mures (3)
		Arges (2) Prahova (2)
		Timis(1)
French fries	9	Iasi (1) Cluj (1) Gorj (5) Timis (2)
Potato crisps	8	Iasi (1), Dolj (1) Constanta(1) Cluj (1) Prahova(1) Bacau (1) Timis (2)
Bread	15	Iasi (1) Dolj (1)
		Constanta(1) Cluj (1)
		Braila (3) Bucuresti (2) Vrancea (2) Bacau (2) Timis (2)
Bakery products	7	Iasi (1) Bucuresti (5) Prahova (1)
Cocoa products	1 3	Dolj (1) Iasi (2) Constanta (1)
Confectionary products	8	Bucuresti (2) Bacau (2) Braila (2) Neamt (2)

Acrylamide analysis

The acrylamide levels from the food products were analyzed by high performance liquid chromatography coupled with a diode array detector (HPLC-UV). The method showed a good sensitivity: LOD and LOQ were 20 and 50 μg kg^{-1} for coffee, potato and potatoes products and 20 and 25 μg kg^{-1} for cereals, bakery products and bread respectively. The method used was previously described by Wang et al. (2013).

RESULTS AND DISCUSSIONS

The results obtained for the samples analyzed were reported to the guidance levels for acrylamide based on the EFSA monitoring data which came into force on April 11, 2018 admitted by the European Commission (EC) Recommendation no. 2158/2017 established values (Raffan and Halford, 2019).

The benchmark level which were set by EC (2017/2158/EU) for different foodstuffs products are shown in Table 3.

Table 3. Benchmark level for AA in foodstuffs set by the EC (2017/2158/EU)

Food	Benchmark level [μg/kg]
French fries	500
Potato crisps	750
Soft bread (wheat)	50
Soft bread (other)	100
Breakfast cereals: bran products, whole grain cereals, gun puffed grain	300
Breakfast cereals: wheat and rye based	300
Breakfast cereals: maize, oat, spelt, barley and rice based	150
Biscuits	350
Crackers	400
Crispbread	350
Gingerbread	800
Cereal-based baby foods	40
Baby foods (not cereal based) without prunes	-
Baby foods (not cereal based) with prunes	-
Biscuits and rusks for infants and young children	150
Roast coffee	400
Instant coffee	850
Coffee substitute (cereal-based)	500
Coffee substitute (chicory)	4000

Benchmarks means the performance indicators used to verify the effectiveness of mitigation measures and build on experience and the presence of the substance in comprehensive food categories. The reference cannot be used directly as a reference to assess whether a product can be placed on the market or not.

If the reference levels are exceeded, the operators in the sector should review the mitigation measures applied without delay and adjust processes and controls in order to achieve levels of acrylamide as low as possible below the established reference levels.

The foodstuffs category samples analyzed in 2018 are shown in Table 4.

The samples were classified into 11 categories, based on population consumtion and the risk degree establsihed by ANSVSA within the Surveillance and Control Program in the food safety of non-animal origin field.

According to the ANSVSA data in 2018 only in one foodstuff sample the AA was detected. The foodstuff sample in which AA was detected was a roast coffee sample of which value was under the recommandated values set by EC (2017/2158/EU) as it may be seen from Table 3. So, according to the evaluated data in 2018, the foodstuffs samples analyzed from the Romania market, within the surveillance and control program in the field of food safety of non-animal origin were in agreement with the EC recommendations level for AA values.

Table 4. The AA level in foodstuffs products in 2018

Category	Samples	Level of AA (μg/kg)	Benchmark level 2017 (μg/kg)
Biscuits	4	Undetectable	350
Fooddishes	1	Undetectable	500
Breakfast cereals	11	Undetectable	300
Roast coffee	8	Undetectable	400
	1	225	
French fries	6	Undetectable	500
Potato crisps	5	Undetectable	750
Food products based on potato	5	Undetectable	500
Bread	4	Undetectable	50
Bakery products	3	Undetectable	50
Cocoa products	1	Undetectable	350
Confectionary products	1	Undetectable	350

In 2019 were evaluated by ANSVSA 88 foodstuffs samples which were classified into 10 categories as it may be seen from the Table 5.

Table 5. The AA level in foodstuffs products in 2019

Category	Samples	Level of AA (µg/kg)	Benchmark level 2017 (µg/kg)
Biscuits	6	Undetectabe	350
Fooddishes	4	Undetectabe	500
	1	< 50	
Breakfast	6	Undetectabe	300
cereals	1	32.77	
	1	172.92	
Roast coffee	12	Undetectabe	400
	1	130.89	
	1	79.88	
	1	97.88	
	1	177.56	
	1	243	
	1	< 50	
French fries	7	undetectabe	500
	2	< 50	
Potato crisps	8	undetectabe	750
Bread	12	undetectabe	50
	2	< 25	
	1	25.6	
Bakery	6	undetectabe	50
products	1	25.39	
Cocoa	3	undetectabe	350
products	1 (cocoa)	264.1	
Confectionar	8	undetectabe	350
y products			

As it may be seen from the data analyzed in 2019 by ANSVSA for foodstuffs samples from the Romania market it may be noticed that no foods samples exceeded the AA level recommended by EC (2017/2158/EU) which are shown in Table 3. Only for 16 foodstuffs samples from 88 ones the AA level was detected. Therefore, AA was detected in 18.18% from the total of foodstuffs samples analyzed. From those, the highest AA level was recorded for a cocoa sample. For the foodstuffs categories analyzed the most samples in which was detected AA was roast coffee, namely from 18 samples analyzed in 6 samples AA was detected. From the 6 roast coffee samples analyzed in which AA wasdetected 4 was in a roasted coffee beans form. If we compared the number of foodstuffs samples analyzed in 2018 year compared to 2019 year from the AA level point of view, depending of the category product type, we noticed increased of the samples number analyzed in 2019 compared to 2018 year. In the Table 6 is shown the ratio level increased for the foodstuffs category samples analyzed in 2018 year compared to the 2019 year.

Table 6. Comparative study between the foodstuffs category samples analyzed in 2018 and 2019 years

Category	Samples 2019	Samples 2018	Ratio increase 2019/2018
Biscuits	6	4	1.5
Fooddishes	5	1	5.00
Breakfast cereals	8	11	0.72
Roastcoffe	18	9	2.00
Frenchfries	9	6	1.5
Potatocrisps	8	5	1.6
Bread	15	4	3.7
Bakeryproducts	7	3	2.3
Cacao products	4	1	4.0
Confectionary products	8	1	8.0

CONCLUSIONS

This study compiles the presence of AA from different foodstuffs from the Romania market in order to monitor the evolution of AA presence in different foodstuffs from the last two years, namely, 2018 and 2019. The study has also a descriptive purpose in order to analyze the number of foodstuffs samples analyzed from Romania market from its AA level content. According to the analyzed data the number of analyzed samples was almost twice higher in 2019 compared to 2018 year. If in the year 2018 the foodstuffs collected for analysis were only from 7 counties from the Romania country, in the 2019 year the number of counties from which were collected foodstuffs samples for analysis has been almost doubled reaching to 16. From the total of 138 samples analyzed in 2018 and 2019, acrylamide was detected in 17 samples. The highest AA levels were found in a cocoa product for which was detected a value of 264.1 µg/kg. From the category of foodstuffs samples analyzed the AA was detected the most in roast coffee samples. However, for no foodstuffs sample analyzed on Romania market the AA level did not exceeded the recommended level set by EC (2017/2158/EU). The results show that in Romania a widespread potential risk from AA for public health occurred in cocoa and coffee products. The study supplied important information's for AA level from different foodstuffs from Romania market for food policy makers, public health experts and consumers.

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REFERENCES

Anese, M., Quarta, B., Frias, J. (2011). Modelling the effect of asparaginase in reducing acrylamide formation in biscuits, *Food Chemistry*, 126, 435–440.

Bartkiene, E., Jakobsone, I., Juodeikiene, G. (2013). Vidmantiene, D., Pugajeva, I., Bartkevics, V., Study on the reduction of acrylamide in mixed rye bread by fermentation with bacteriocin-like inhibitory substances producing lactic acid bacteria in combination with *Aspergillus niger* glucoamylase, *Food Control*, 30, 35-40.

Desmarchelier, A., Hamel, J., & Delatour, T. (2020). Sources of overestimation in the analysis of acrylamide-in coffee by liquid chromatography mass spectrometry. *Journal of Chromatography A*, 1610, 460566.

Fernandes, C. L., Carvalho, D. O., & Guido, L. F. (2019). Determination of Acrylamide in Biscuits by High-Resolution Orbitrap Mass Spectrometry: A Novel Application. *Foods*, 8(12), 597.

Ghalebi, M., Hamidi, S., & Nemati, M. (2019). High-performance liquid chromatography determination of acrylamide after its extraction from potato chips. *Pharmaceutical Sciences*, 25(4), 338-344.

Johnson, A. M., Porter, G., &Camire, M. E. (2019). Low-Acrylamide French Fry Acceptance: A Pilot Study. *Journal of food science*, 84(12), 3717-3725.

Kang, D. E., Lee, H. U., Davaatseren, M., & Chung, M. S. (2020). Comparison of acrylamide and furan concentrations, antioxidant activities, and volatile profiles in cold or hot brew coffees. *Food Science and Biotechnology*, 29(1), 141-148.

Li, N., Liu, X., Zhu, J., Zhou, B., Jing, J., Wang, A., ... & Guo, S. (2020). Simple and sensitive detection of acrylamide based on hemoglobin immobilization in carbon ionic liquid paste electrode. *Food Control*, 109, 106764.

Maan, A. A., Anjum, M. A., Khan, M. K. I., Nazir, A., Saeed, F., Afzaal, M., & Aadil, R. M. (2020). Acrylamide Formation and different mitigation strategies during food processing - A review. *Food Reviews International*, 1-18

Martillanes, S., & Martín-Vertedor, D. (2020). Evaluation of phenolics and acrylamide and their bioavailability in high hydrostatic pressure treated and fried table olives. *Journal of Food Processing and Preservation*, e14384.

Martín-Vertedor, D., Fernández, A., Hernández, A., Arias-Calderón, R., Delgado-Adámez, J., & Pérez-Nevado, F. (2020). Acrylamide reduction after phenols addition to Californian-style black olives. *Food Control*, 108, 106888.

Mekawi, E. M., Sharoba, A. M., & Ramadan, M. F. (2019). Reduction of acrylamide formation in potato chips during deep-frying in sunflower oil using pomegranate peel nanoparticles extract. *Journal of Food Measurement and Characterization*, 13(4), 3298-3306.

Mesías, M., Delgado-Andrade, C., Holgado, F., & Morales, F. J. (2020). Impact of the consumer cooking practices on acrylamide formation during the preparation of French fries in Spanish households. *Food Additives & Contaminants: Part A*, 37(2), 254-266.

Michalak, J., Czarnowska-Kujawska, M., &Gujska, E. (2019). Acrylamide and Thermal-Processing Indexes in Market-Purchased Food. *International Journal of Environmental Research and Public Health*, 16(23), 4724.

Miśkiewicz, K., Nebesny, E., Rosicka-Kaczmarek, J., Żyżelewicz, D., Budryn, G. (2018). The effects of baking conditions on acrylamide content in shortcrust cookies with added freeze-dried aqueous rosemary extract, *Journal of Food Science and Technology*, 55(10),4184–4196.

Mousa, R. M. (2019). Simultaneous mitigation of 4 (5)-methylimidazole, acrylamide, and 5-hydroxymethylfurfural in ammonia biscuits by supplementing with food hydrocolloids. *Food Science & Nutrition*, 7(12), 3912-3921.

Murray, S. M., Waddell, B. M., & Wu, C. W. (2020). Neuron-specific toxicity of chronic acrylamide exposure in *C. elegans. Neurotoxicology and teratology*, 77, 106848.

Nachi, I., Fhoula, I., Smida, I., Taher, I. B., Chouaibi, M., Jaunbergs, J., ... & Hassouna, M. (2018). Assessment of lactic acid bacteria application for the reduction of acrylamide formation in bread. *LWT*, 92, 435-441.

Pratama, Y., & Jacxsens, L. (2019). Quantitative Risk Assessment of Acrylamide in Indonesian Deep Fried Fritters as Street Food Products. *Current Research in Nutrition and Food Science Journal*, *7*(3), 662-669.

Rifai, L., & Saleh, F. A. (2020). A Review on Acrylamide in Food: Occurrence, Toxicity, and Mitigation Strategies. *International Journal of Toxicology*, doi.org/10.1177/1091581820902405.

Tölgyesi, Á., & Sharma, V. K. (2020). Determination of acrylamide in gingerbread and other food samples by HILIC-MS/MS: A dilute-and-shoot method. *Journal of Chromatography B*, 1136, 121933.

Schouten, M. A., Tappi, S., & Romani, S. (2020). Acrylamide in coffee: formation and possible mitigation strategies - a review. *Critical Reviews in Food Science and Nutrition*, 1-15.

Wang, B., Guerrette, Z., Whittaker, M. H., & Ator, J. (2020). Derivation of a No significant risk level (NSRL) for acrylamide. *Toxicology Letters*, 320, 103-108.

Wang, H., Feng, F., Guo, Y., Shuang, S., & Choi, M. M. F. (2013). HPLC-UV quantitative analysis of acrylamide in baked and deep-fried Chinese foods. *Jorunal of Food Composition and Analysis,* 31, 7-11.

Wawrzyniak, R., & Jasiewicz, B. (2019). Straightforward and rapid determination of acrylamide in coffee beans by means of HS-SPME/GC-MS. *Food chemistry*, 301, 125264.

Yıldız, H. G.,Palazoğlu, T. K., Miran, W., Kocadağlı, T., Gökmen, V. (2017). Evolution of surface temperature and its relationship with acrylamide formation during conventional and vacuum-combined baking of cookies, *Journal of Food Engineering,* 197, 17-23.

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SUGARS CONTENT AND PHYSICOCHEMICAL PARAMETERS OF ROMANIAN RAPE HONEY

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Abstract

The aim of this study was to evaluate the physicochemical characteristics of rape honey collected from different regions of Romania. Five rape honey samples were selected to determine their physicochemical parameters, sugar content and antioxidant activity, including total polyphenols content and total flavonoids content. The results of the melissopalynological analysis showed that all honey samples have a percentage of pollen grains Brassica napus well above the minimum of 45%, which is necessary to classify the samples as monofloral honey. The results obtained for rape honey samples indicated a low electrical conductivity (118-173 μS/cm), a pH between 4.03-4.24 and a free acidity between 12.37-25.54%. The limit set for HMF content was exceeded by a sample of rape honey (48.6 mg HMF/kg) and the moisture content varied between 17.30-19.12% and was not exceeded the moisture content set by Codex Alimentarius (20%). Fructose, glucose, sucrose, maltose, turanose, trehalose, melezitinose, and raffinose were identified and quantified in all samples. Results were submitted to analysis of variance (ANOVA) using Statgraphics Centurion XVIII software trial version.

Key words*: authentication, honey, physicochemical properties.*

INTRODUCTION

In recent years, rape crops have grown considerably in Romania because the demand for rape oil, which is used for food and biodiesel, and for animal feed has increased. The large number of rape crops has also led to an increase in the production of rape honey.Rape honey has a light amber color, is sourish-sweet, and has a slightly fermented, musty aroma of rape flower (Siegmund et al., 2017; Wang et al., 2014). The aroma and taste of honey are given by the content in volatile etheric oils and by other volatile components existing in honey in very low concentrations. Rape honey has a discreet aroma, but the intensity of the aroma varies depending on the degree of freshness (Mărghitaș, 2005).

Rape honey has high glucose content and crystallizes very quickly with small crystals but this does not adversely affect its quality (Persano Oddo et al., 2004). Rape honey has pollen that contains 24% crude protein and amino acids such as methionine, threonine and valine.

The existing honey varieties have different composition depending on the source of origin (plants and environmental conditions). The properties of honey are thus influenced by the nectar/pollen originating from the plant, moisture content, aroma, color, or sugar content (da Silva et al., 2016). Honey has more sugars in its composition than any other product of animal origin and the sugar content depends largely on the origin of honey (botanical and geographical) and is influenced by processing, storage and climate (Xu et al., 2019; Da Silva et al., 2016).

Honey is a healthy food that is widely consumed both for its sweet taste and its high nutritional and medical importance (Khan, et al., 2018). Honey is renowned for its antimicrobial properties, properties that are attributed to phenolic compounds derived from honey (Estevinho et al., 2008). Studies have shown that honey has a broad spectrum of bioactive activity such as: anti-inflammatory, anti-carcinogenic, analgesic, antithrombotic activity. Natural honey also has the ability to reduce the risk of cardiovascular disease, and honey consumption may also be associated with a decrease in body weight (Yaghoobi et al., 2008). The authenticity of honey implies both aspects of its geographical and botanical origin and the fact that honey must be entirely pure, with no adulteration with any type of sugar syrup (Xu et al., 2019). The demand for monofloral honey has increased considerably in the last years and for this reason it is necessary to be able to determine some

parameters that contribute to the authentication of the origin of honey that include the botanical and geographical source (Da Silva et al., 2016). Honey authentication is possible by combining the determination of physicochemical parameters with the melissopalynological analysis (Oroian et al., 2015). In recent years, new analytical methodologies have been used to determine the botanical origin including chromatographic, molecular and biological spectroscopic methods (Siddiqui et al., 2017; Escriche et al., 2017).

The purpose of this work was to determine for Romanian rape honey the physicochemical parameters (melissopalynological analysis, moisture, color, pH, free acidity, electrical conductivity), hydroxymethylfurfural content, antioxidant activity-DPPH radical scavenging activity, total polyphenols content, total flavonoids content and sugar content.

MATERIALS AND METHODS

Samples

Five samples of rape honey from 2017 and 2018 were purchased from different regions of Romania. The honey samples were liquefied at 50°C and homogenized prior to analysis.

Melissopalynological analysis

A mixture of 10 grams of honey and 40 mL of distilled water was prepared and then centrifuged at 4500 rpm for 15 minutes. The resulting residue was mixed again with water and subjected to centrifugation for another 15 minutes.The sediment collected from centrifugation by removing the supernatant was spread on a microscopic lamella and the pollen grains were counted with a Motic microscope (Motic, China) at ×40 magnification (Louveaux & Vorwohl, 1970).

Physicochemical analysis

The color of the honey samples was analyzed by two methods, namely using a portable chromameter, CR-400 (Konica Minolta, Japan) and a Pfund photometer HI 96785 (Hanna Instrumets, USA).

Using the analytical methods proposed by the International Honey Commission, the physicochemical parameters were determined, as follows: moisture content (using Abbe refractometer, Leica Mark II Plus), pH (using a pH meter Mettler Toledo FiveGo, Mettler Toledo, USA), free acidity (using TITROLINE easy, Schott Instruments, Germany) and electrical conductivity (using a portable conductometer HQ14d, HACH, USA).

The hydroxymethylfurfural (HMF) content was determined using the White method **(**White, 1978) and a UV-VIS-NIR SCHIMADZU UV-3600 spectrophotometer (Schimadzu Corporation, Japan).

To identify the total polyphenols content, total flavonoids content and to determine the antioxidant activity, the preparation of the samples was done according to the method proposed by Biesaga & Pyrzyńska (2013), as follows: 1 g of honey sample was extracted with 5 mL of 40% methanol/acidified water (v/v, pH = 2 adjusted with HCl). The honey samples were homogenized with a magnetic stirrer for 15 min.

To identify the total polyphenols content 0.2 mL of extract was mixed with 2mL of Folin - Ciocalteu 1:10 and 1.8 mL Na_2CO_3, 7.5% (w/v). After incubation in the dark the absorbance of the reaction mixture was measured at 750 nm.

To identify the total flavonoids content 5 mL of extract obtained according to the method proposed by Biesaga & Pyrzyńska (2013) were mixed with 300 µL of $NaNO_2$ 5% (w/v) and 300 µL of $AlCl_3$ (w/v). After 5 minutes in the dark the samples were mixed with 2 mL of NaOH 1 N and after another 6 minutes in the dark the absorbance of all samples was measured at 510 nm.

The DPPH (1,1-diphenyl-2-picrylhydrazyl) radical scavenging activity of honey samples was determined as described by Brand-Williams et al. (1995). 35 µL of honey sample were mixed with 250 µL of DPPH. The absorbance of the solution was measured at 515 nm. The results were expressed as % DPPH using the formula in Eq. 1:

$$\% \text{ DPPH} = \left(A_0 - \frac{A_1}{A_0} \right) \times 100 , \quad (1)$$

where A_0 is the DPPH absorbance, A_1 is the sample absorbance.

The UV-NIR HR4000CG-UV-NIR spectrometer (Ocean Optics, USA) and UV-NIR HR4000CG-UV-NIR spectrometer (Ocean Optics, USA) were used to measure the absorbance.

Determination of sugars composition

To determine the sugar content, the honey samples (5 g of each sample) were dissolved in distilled water (40 mL), mixed with 25 mL of methanol (in a 100 mL volumetric flask) and then brought to volume with distilled water. The samples obtained as described above were analyzed with a HPLC instrument (Schimadzu, Kyoto, Japan). This instrument was equipped with a LC-20 AD liquid chromatograph, SIL-20A auto sampler, CTO-20AC column oven, and RID-10A refractive index detector. The separation was performed on a Phenomenex Luna® Omega 3 µm SUGAR 100 Å HPLC Column 150 x 4.6 mm. Before being injected into the HPLC instrument, the samples were filtered through 0.45 µm PTFE membrane filters. The sample volume injection was 10 µL. The flow rate was 1.3 mL/min and the mobile phase was acetonitrile: water (80:20, v/v); the temperature of the column and detector was 30 °C.

RESULTS AND DISCUSSIONS

Melissopalynological analysis

Melissopalynological analysis is the method that has been used over the years to identify the botanical and geographical sources of honey (Soria et al., 2004). Pollen analysis provides valuable information regarding the organoleptic properties of honey and its composition (Von der Ohe et al., 2004). A percentage of 45% pollen granules present in honey samples indicates that honey is authentic and thus its botanical origin is known (Siddiqui et al., 2017). In the rape honey samples analyzed in the present study the percentage of pollen grains had a maximum value of 73% and a minimum of 53%. The results of the melissopalynological analysis indicate that the 5 samples of rape honey were authentic. Rajs et al. (2017) analyzed 21 samples of Croatian rape honey and reported that the percentage of pollen varied between 60 and 98%.

Color

An important feature for the market is the color of honey. Color is the main feature evaluated by consumers as an attribute of quality (Dominguez & Centurion, 2015). Honey color provides information about the quality of honey, the source from which it comes and also about the particularities of production (Tuberoso et al., 2014). The specific color of the rape honey that was analyzed in this study was extra light amber because the values on the Pfund scale varied between 18.81 and 40.09 mm Pfund (Table 1). Wang et al. (2014) studied ten samples of rape honey from Shaanxi, China and reported a color variation ranging from 20.5 to 25.0 mm Pfund.

pH

The organic acids in the composition of honey are responsible for the pH values between 3.5 and 5.5 and offer protection in case of microbial attacks. If the pH value increases above 7.2 then microorganisms have an environment favorable for development, and as a result the pH can be considered an indicator of microbial growth (Da Silva et al., 2016).

The analyzed rape honey samples had a pH value between 4.03 and 4.24. The pH of the rape honey samples investigated by Wang et al. (2014) was between 3.83 and 4.25. Tomczyk et al. (2019) analyzed in their study the rape honey from Poland and Slovakia and reported a pH value of 3.88 (Polish honey) and 3.61 (Slovak honey).

Free acidity

Free acidity is an indicator of the freshness of honey. When honey begins to deteriorate, the process of fermentation of sugars into organic acids appears and the result is the increase of free acidity (Da Silva et al., 2016). The rape honey samples analyzed in this study had a free acidity between 12.37 and 25.54 meq/kg (Table 1). According to the Council Directive (2001) the maximum limit for free acidity is 50 milli-equivalents of acid per 1000 grams (Codex Alimentarius, 2001) and the values of free acidity obtained in this study show that the honey samples analyzed were fresh.

Kędzierska-Matysek et al. (2016) reported for the rape honey collected from Lublin, Poland that all the samples that were analyzed were acidic (the average value was 18.71). Dinkov et al. (2016) reported free acidity values between 35-38 meq/kg when analyzing rape honey from Stara Zagora, Bulgaria.

Table 1. Physicochemical properties for Romanian rape honey. Values and standard deviation in brackets

Parameter	Sample 1	Sample 2	Sample 3	Sample 4	Sample 5	F –ratio
L*	41.68(0.60)^{c}	48.63(0.69)^{a}	43.54(0.62)^{b}	36.43(0.52)^{e}	39.29(0.56)^{d}	116.97***
h*$_{ab}$	90.83(1.29)^{b}	95.77(1.37)^{a}	83.62(1.19)^{c}	92.87(1.32)^{ab}	96.24(1.37)^{a}	30.16***
C*$_{ab}$	24.93(0.35)^{c}	26.82(0.38)^{b}	30.79(0.43)^{a}	18.32(0.26)^{e}	19.22(0.27)^{d}	450.29***
Color (mm Pfund)	27.72(0.39)^{c}	18.81(0.26)^{e}	40.09(0.57)^{a}	36.13(0.51)^{b}	21.78(0.31)^{d}	900.71***
pH	4.19(0.059)^{a}	4.24(0.06)^{a}	4.03(0.057)^{b}	4.10(0.058)^{ab}	4.20(0.06)^{a}	4.03ns
Free acidity (meq/kg)	12.37(0.17)^{e}	16.23(0.23)^{d}	25.54(0.36)^{a}	17.32(0.24)^{c}	18.83(0.26)^{b}	659.19***
EC (µS/cm)	118.89(1.69)^{e}	126.52(1.80)^{d}	173.89(2.48)^{a}	146.66(2.09)^{b}	136.12(1.94)^{c}	223.77***
Moisture (%)	17.82(0.254)^{bc}	19.12(0.273)^{a}	18.41(0.263)^{b}	17.30(0.247)^{c}	18.09(0.258)^{b}	13.73**
HMF (mg/kg)	22.67(0.323)^{b}	12.74(0.18)^{c}	48.60(0.69)^{a}	9.48(0.13)^{d}	7.40(0.10)^{e}	4414.78***
TPC (mg GAE/100 g)	12.75(0.18)^{d}	23.11(0.33)^{c}	26.70(0.38)^{b}	31.04(0.44)^{a}	23.03(0.32)^{c}	771.27***
FC (mg QE/100 g)	7.66(0.10)^{d}	7.87(0.11)^{d}	16.23(0.23)^{a}	13.24(0.18)^{b}	12.41(0.17)^{c}	928.4***
DPPH	50.59(0.72)^{cd}	52.40(0.74)^{bc}	53.01(0.75)^{b}	55.80(0.79)^{a}	49.06(0.70)^{e}	23.45**
Fructose (%)	34.87(0.49)^{b}	37.61(0.53)^{a}	35.64(0.50)^{b}	35.23(0.50)^{b}	35.09(0.50)^{b}	9.49ns
Glucose (%)	33.57(0.47)^{c}	28.25(0.40)^{e}	29.46(0.42)^{d}	36.09(0.51)^{a}	34.39(0.49)^{b}	103.94***
Sucrose (%)	0^{b}	0.62(0.008)^{a}	0^{b}	0^{b}	0^{b}	9801***
Turanose (%)	0.29(0.004)^{bc}	4.16(0.05)^{a}	0.34(0.004)^{b}	0.28(0.004)^{bc}	0.26(0.003)^{d}	8274.44***
Maltose (%)	1.37(0.01)^{c}	5.97(0.08)^{a}	1.66(0.02)^{b}	0.94(0.13)^{d}	0.89(0.01)^{d}	5384.39***
Trehalose (%)	1.49(0.021)^{c}	11.34(0.16)^{a}	1.99(0.02)^{b}	0.99(0.01)^{d}	0.911(0.013)^{d}	7233.83***
Melesitose (%)	0.90(0.01)^{c}	2.95(0.04)^{a}	1.16(0.01)^{b}	0.63(0.009)^{d}	0.60(0.008)^{d}	4016.17***
Raffinose (%)	0.19(0.002)^{b}	0^{e}	0.36(0.005)^{a}	0.11(0.001)^{c}	0.096(0.001)^{d}	4725.12***
F/G ratio	1.02(0.01)^{c}	1.31(0.01)^{a}	1.19(0.01)^{b}	0.96(0.01)^{d}	1.01(0.014)^{c}	174.47***

ns - not significant ($p > 0.05$), *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, $^{a-d}$different letters in the same row indicate significant differences between samples ($p < 0.001$)

Moisture

The moisture content of honey is dependent on the season, harvest and the degree of maturity of honey in the hive (Karabagias et al., 2014). Honey moisture can be considered a limiting factor in terms of quality and stability (El Sohaimy et al., 2015). High moisture content leads to degradation of honey due to the fermentation process, while low moisture content results in the Maillard reaction and the development of caramelization (Chirifie et al., 2006).

In this study, the moisture content ranged between 17.30 and 19.12%, which showed that the rape honey samples were in accordance with the 20% limit imposed by Codex Alimentarius (2001). In the rape honey from Croatia and Slovakia studied by Raj et al. (2017) the measured values for moisture content ranged between 16.4 and 19.4%. Oroian et al. (2018) reported for sunflower, acacia, tilia, honeydew and polyfloral honey from Romania moisture content between 14.5 and 19.8%.

Electrical conductivity

In the studied samples the electrical conductivity varied between 118.89 and 173.89 µS/cm. This parameter was recently introduced in international standards as its value provides information on the botanical origin of honey.

The electrical conductivity with a value < 500 µS/cm indicates pure floral honey, while values between 500-800 µS/cm are specific for mixed honey (Saxena et al., 2010). The electrical conductivity of honey increases directly in relation to two other important parameters, namely the acid content and the ash content (El Sohaimy et al., 2015).

Szczęsna et al. (2011) reported a lower electrical conductivity for rape honey (200 µS/cm) and Tomczyk et al. (2019) reported for the Polish rape honey a similar average value (230 µS/cm). Oroian et al. (2016) reported a low electrical conductivity for acacia honey (156.58 µS/cm) as opposed to sunflower (346.1 µS/cm) and tilia honey (549.31 µS/cm). These results indicate that light honey has a low electrical conductivity.

HMF content

The degree of freshness of honey can be indicated by the content of HMF (Wang et al., 2014). As seen in Table 1, honey samples had HMF content between a minimum of 7.40 mg/kg and a maximum of 48.60 mg/kg. The limit set for HMF content (40 mg/kg) was exceeded by a sample of rape honey. The increased content of HMF can be attributed to an overheating of the honey during processing and/or storage. Wilczyńska (2012) reported for 5-HMF content values between 0.6 and 4 mg/kg. In the study by Raj et al. (2017) none of the rape honey samples from Croatia exceeded the HMF limit allowed by law. Oroian et al. (2015) reported that all analyzed samples were fresh and had a HMF content below 40 mg/kg.

Total phenolic content and total flavonoids content

Honey is a natural product that contains many phenolic compounds, and the amount and nature of phenolic acids and flavonoids are of great interest, as these compounds are known to have different nutritional properties, as well as a potential role in treating different diseases and contributing to human health (Ciulu et al., 2016). The total phenolic content (TPC) of Romanian rape honeys ranged from 12.75 to 31.04 mg of gallic acid equivalent (GAE)/100 g of honey. Tomczyk et al. (2019) reported for rape honey values between 21 mg of gallic acid equivalent (GAE)/100 g of honey for Slovak rape honey and 25 mg of gallic acid equivalent (GAE)/100 g for Polish rape honey. Bonvehi et al. (2019) studied avocado honey and reported values for TPC content between 103 and 137 mg GAE/100 g of honey.

The flavonoids content of rape honey varied from 7.66 to 16.23 mg QE/100 g.

Ibrahimi & Hajdari, (2020) studied 100 honey samples of different botanical origins from Kosovo and determined the highest TPC content in forest honey (84.17 mg GAE/100 g) and the lowest content in acacia honey (25.76 mg GAE / 100 g).

For the same honey samples, Ibrahimi & Hajdari (2020) reported that the lowest flavonoids contents were in acacia honey (1.11 mg CE/100 g), followed by mixed honey (3.44 mg CE/100 g), while the highest flavonoids contents were reported in forest honey samples (7.51 mg CE/100 g).

DPPH assay

The antioxidant activity is directly correlated with the DPPH radical scavenging activity(Ouradi et al, 2020). In the present study the highest DPPH radical scavenging activity (Table 1) was 55.80%. In comparison, in Slovak rape honey the DPPH radical scavenging activity was 11.76% and in the Polish rape honey was 21.21% (Tomczyk et al., 2019). In thyme honey samples from Marocco the DPPH radical scavenging activity was 36.54 % (Ouradi et al, 2020).

Sugars content

Honey is a complex mixture of sugars (80%) and other components (Soares et al., 2017). Cotte et al. (2004) argued that the ratio of specific carbohydrates (fructose and glucose), as well as the amount of sugars are useful tools that can be applied to distinguish monofloral honey. The content and composition of honey sugars are influenced by botanical source, geographical origin, processing and storage conditions and climate (Escuredo et al., 2014). The viscosity, energy value, granulation and hygroscopicity of honey are influenced by the sugar content (Kamal and Klein, 2011).

In the samples of rape honey the variation of fructose content was not significant in comparison to the glucose content, which presented a significant variation (29.46-36.09%). Sucrose was identified in a single sample of rape honey, while the content of turanose was constant in 3 samples but was higher in one of the rape honey samples. All types of honey that crystallize quickly have high glucose content and the F/G ratio is about 1 (Rajs et al., 2017).

Similar results for fructose (36.35-36.39%) and glucose (29.69-32.92%) content in rape honey were reported by Tomczyk et al. (2019). Rajs et al. (2017) reported that the predominant sugars in Croatian rape honey were fructose (38.3) and glucose (36.1), and the average value of fructose/glucose (F/G) ratio was 1.1.

CONCLUSIONS

In this study were analyzed the content of pollen, the physicochemical parameters, and the sugar composition of Romanian rape honey in order to classify it as monofloral honey. The results obtained for the targeted parameters allowed the characterization of honey in terms of physicochemical properties. The high percentage of pollen grains of Brassica napus, together with the physicochemical parameters confirm that the honey samples analyzed were samples of monofloral rape honey. The values determined for the physicochemical parameters of the rape honey were in accordance with the European standars for honey.

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REFERENCES

Biesaga, M., Pyrzyńska, K. (2013). Stability of bioactive polyphenols from honey during different extraction methods. *Food chemistry*, 136(1), 46-54.

Bonvehi, J. S., Coll, F. V., Bermejo, J. O. (2019). Characterization of avocado honey (*Persea americana* Mill.) produced in Southern Spain. *Food chemistry*, 287,214-221.

Brand-Williams, W., Cuvelier, M. E., Berset, C. L. W. T. (1995). Use of a free radical method to evaluate antioxidant activity. *LWT-Food science and Technology*, 28(1), 25-30.

Chirifie, J., Zamora, M. C., Motto, A. (2006). The, correlation between water activity and % moisture in honey: Fundamental aspects and application to Argentine honeys. *Journal of Food Engineering*, 72, 287-292.

Ciulu, M., Spano, N., Pilo, M., Sanna, G. (2016). Recent advances in the analysis of phenolic compounds in unifloral honeys. *Molecules*, 21(4),

Codex Alimentarius Commission. (2001). *Codex alimentarius: fats, oils and related products* (Vol. 8). *FAO*.

Cotte, J. F., Casabianca, H., Chardon, S., Lheritier, J., Grenier-Loustalot, M. F. (2004). Chromatographic analysis of sugars applied to the characterisation of monofloral honey. *Analytical and bioanalytical chemistry*, 380(4), 698-705.

COUNCIL, E. U. (2002). Council Directive 2001/110/EC of 20 December 2001 relating tohoney, Official Journal of the EuropeanCommunities L, 10, 47-52.

Da Silva, P. M., Gauche, C., Gonzaga, L. V., Costa, A. C. O., Fett, R. (2016). Honey: Chemical composition, stability and authenticity. *Food Chemistry*, 196, 309-323.

Dinkov, D., Stratev, D., Balkanska, R., Sergelidis, D., Vashin, I. (2016). Reduction Effect of Royal Jelly and Rape Honey Alone and in Combination Against Methicillin-Resistant *Staphylococcus aureus* (MRSA)

Strains. *Journal of Bacteriology and Virology*, 46(1), 36-43.

Dominguez, M. A., Centurión, M. E. (2015). Application of digital images to determine color in honey samples from Argentina. *Microchemical Journal*, 118, 110-114.

El Sohaimy, S. A., Masry, S. H. D., Shehata, M. G. (2015). Physicochemical characteristics of honey from different origins. *Annals of Agricultural Sciences*, 60(2), 279-287.

Escriche, I., Sobrino-Gregorio, L., Conchado, A., Juan-Borrás, M. (2017). Volatile profile in the accuratelabelling of monofloral honey. The case of lavender andhoney. *Food chemistry*, 226, 61-68.

Escuredo, O., Dobre, I., Fernández-González, M., Seijo, M. C. (2014). Contribution of botanical origin and sugar composition of honeys on the crystallization phenomenon. *Food chemistry*, 149, 84-90.

Estevinho, L., Pereira, A. P., Moreira, L., Dias, L. G., Pereira, E. (2008). Antioxidant and antimicrobial effects of phenolic compounds extracts of Northeast Portugal honey. *Food and chemical toxicology*, 46(12), 3774-3779.

Ibrahimi, H., Hajdari, A. (2020). Phenolic and flavonoid content, and antioxidant activity of honey from Kosovo. *Journal of Apicultural Research*, 1-6.

Kamal, M. A., Klein, P. (2011). Determination of sugars in honey by liquid chromatography. *Saudi journal of biological sciences*, 18(1), 17-21.

Karabagias, I. K., Badeka, A., Kontakos, S., Karabournioti, S., Kontominas, M. G. (2014). Characterization and classification of *Thymus capitatus* (L.) honey according to geographical origin based on volatile compounds, physicochemical parameters and chemometrics. *Food Research International*, 55, 363-372.

Kędzierska-Matysek, M., Florek, M., Wolanciuk, A., Skałecki, P., Litwińczuk, A. (2016). Characterisation of viscosity, colour, 5-hydroxymethylfurfural content and diastase activity in raw rape honey (*Brassica napus*) at different temperatures. *Journal of food science and technology*, 53(4), 2092-2098.

Khan, S. U., Anjum, S. I., Rahman, K., Ansari, M. J., Khan, W. U., Kamal, S., ... Khan, H. U. (2018). Honey: Single food stuff comprises many drugs. *Saudi journal of biological sciences*, 25(2), 320-325.

International Honey Commission. (2002). Harmonised methods of the international honey commission. *Liebefeld, Switzerland: Swiss Bee Research Centre*.

Louveaux, J., Maurizio, A., Vorwohl, G. (1970). Commission internationale de botanique apicole de l'uisb: les méthodes de la mélisso-palynologie, *Apidologie*, 1(2), 211-227.

Mărghitaş, L. A. (2005). *Albinele şi produsele lor*, ediţia 2. Ed. Ceres, Bucureşti.

Oroian, M., Amariei, S., Rosu, A., Gutt, G. (2015). Classification of unifloral honeys using multivariate analysis. *Journal of Essential Oil Research*, 27(6), 533-544.

Oroian, M., Paduret, S., Amariei, S., Gutt, G. (2016). Chemical composition and temperature influence on honey texture properties. *Journal of food science and technology*, 53(1), 431-440.

Oroian, M., Ropciuc, S., & Paduret, S. (2018). Honey authentication using rheological and physicochemical properties. *Journal of food science and technology*, 55(12), 4711-4718.

Ouradi, H., Hanine, H., Fauconnier, M. L., Kenne, T., Rizki, H., Ennahli, S., Hssaini, L. (2020). Determination of physico-biochemical proprieties and composition in volatile constituents by solid phase micro-extraction of honey samples from different botanical and geographical origins in Morocco. *Journal of Apicultural Research*, 1-15.

Persano Oddo L, Piro R, et al. (2004). Main European unifloral honeys: descriptive sheets. *Apidologie* 35(Suppl. 1):S38–S81. doi:10.1051/apido:2004049

Rajs, B. B., Flanjak, I., Mutić, J., Vukojević, V., Đurđić, S., Primorac, L. (2017). Characterization of Croatian Rape (*Brassica sp*.) Honey by Pollen Spectrum, Physicochemical Characteristics, and Multieletriacylglycerole, CRP, and body weight coment analysis by ICP-OES. *Journal of AOAC International*, 100(4), 881-888.

Saxena, S., Gautam, S., Sharma, A. (2010). Physical, biochemical and antioxidant properties of some Indian honeys. *Food Chemistry*, 118(2), 391-397.

Siddiqui, A. J., Musharraf, S. G., Choudhary, M. I. (2017). Application of analytical methods in authentication and adulteration of honey. *Food chemistry*, 217, 687-698.

Siegmund, B., Urdl, K., Jurek, A., Leitner, E. (2017). "More than Honey": investigation on volatiles from monovarietal honeys using new analytical and sensory approaches. *Journal of agricultural and food chemistry*, 66(10), 2432-2442.

Soares, S., Amaral, J. S., Oliveira, M. B. P., Mafra, I. (2017). A comprehensive review on the main honey authentication issues: Production and origin. *Comprehensive Reviews in Food Science and Food Safety*, 16(5), 1072-1100.

Soria, A. C., González, M., De Lorenzo, C., Martınez-Castro, I., Sanz, J. (2004). Characterization of artisanal honeys from Madrid (Central Spain) on the basis of their melissopalynological, physicochemical and volatile composition data. *Food Chemistry*, 85(1), 121-130.

Szczęsna, T., Rybak-Chmielewska, H., Waś, E., Kachaniuk, K., Teper, D. (2011). Characteristics of polish unifloral honeys. I rape honey (Brassica napus L. var. oleifera Metzger). *J Apic Sci*, 55(1), 111-119.

Tomczyk, M., Tarapatskyy, M., Dżugan, M. (2019). The influence of geographical origin on honey composition studied by Polish and Slovak honeys. *Czech Journal of Food Sciences*, 37(4), 232-238.

Tuberoso, C. I. G., Jerković, I., Sarais, G., Congiu, F., Marijanović, Z., & Kuś, P. M. (2014). Color evaluation of seventeen European unifloral honey types by means of spectrophotometrically determined CIE L∗ Cab∗ hab∘ chromaticity coordinates. *Food chemistry*, 145, 284-291.

Von der Ohe, W., Oddo, L. P., Piana, M. L., Morlot, M., Martin, P. (2004). Harmonized methods of melissopalynology. *Apidologie*, 35(Suppl. 1), S18-S25.

Wang, J., Xue, X., Du, X., Cheng, N., Chen, L., Zhao, J., ... Cao, W. (2014). Identification of acacia honey adulteration with rape honey using liquid chromatography–electrochemical detection and chemometrics. *Food analytical methods*, 7(10), 2003-2012.

Wilczyńska, A. (2012). Evaluation of honey's quality in aspect of factorsaffecting its antioxidant properties. *Prace Nauk*., Polska, p. 134.

White Jr, J. W. (1978). Honey. *Advances in food research*, 24, 287-374.

Xu, J., Liu, X., Wu, B., Cao, Y. (2019). A comprehensive analysis of 13 C isotope ratios data of authentic honey types produced in China using the EA-IRMS and LC-IRMS. *Journal of Food Science and Technology*, 1-17.

Yaghoobi, N., Al-Waili, N., Ghayour-Mobarhan, M., Parizadeh, S. M. R., Abasalti, Z., Yaghoobi, Z., ... Saloom, K. Y. (2008). Natural honey and cardiovascular risk factors; effects on blood glucose, cholesterol, mpared with sucrose. *The scientific world journal*, 8, 463-469.

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FOOD CONTACT MATERIALS CONTAMINATION: A CASE STUDY OF BISPHENOL A

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Abstract

Food contact materials represent a possible source of contamination of the packaged products, through monomers or additives that can migrate from the packaging, especially the plastic ones, into the product. This type of contamination is an important problem related to the safety of food products, carrying out a lot of research in this regard. These chemical contaminants include Bisphenol A, with the highest volume produced worldwide, which is used as a plasticizer or intermediate product in obtaining polymeric materials, especially in polycarbonate material, but also in obtaining epoxy resins used for inner surface coatings of cans. The release of this compound is influenced by certain factors, among which the most important are the following: temperature, contact time and light exposure. The aim of this research article is to determine the levels of bisphenol A in different packed food and to verify compliance with the limit established by the legislation in force.

Key words*: Bisphenol A, migration, polymeric materials, food packaging.*

INTRODUCTION

In recent years, more and more studies have been carried out to detect the chemical substances present in the packaging materials, to measure their levels and to determine the effects they have on human health (Alimohammadi et al., 2014). These include dioxins, alkyl phenols, organo-chlorine pesticides and bisphenol A (BPA) which can become toxic even at very low concentrations (Alfarhani et al., 2019; Alimohammadi et al., 2014).

BPA is used as a monomer to obtain different epoxy resins used as internal coatings for food and beverage cans (Chailurkit et al., 2016; Elobeid et al., 2012), but also to obtain polycarbonate plastic used for obtaining bottles for milk, water or other food products packaging (Cao and Ji, 2013; Alfarhani et al., 2019).

BPA is an endocrine disrupting chemical that mimics the activity of hormones, altering the normal functioning of the endocrine system, leading to reproductive diseases, obesity, cancer or diabetes (Alfarhnai et al., 2019; Elobeid et al., 2012). The migration of this compound from food packaging into water samples is influenced by certain factors such as the presence of other chemical compounds (detergents, amines), water hardness, repeated use of packaging, temperature at which the packaging is exposed (direct sunlight or microwave heating), time of contact (Alimohammadi et al., 2019; Cao and Ji, 2013).

Taking into account the fact that consumption of water bottled in plastic bottles is considered the main source of BPA contamination, the aim of this study was to determine the BPA levels in still mineral water available on the Romanian market.

MATERIALS AND METHODS

Sample collection

Fourteen different samples of still water (Table 1), packed in PET bottles, were purchased from the local market in Bucharest, Romania.

Reagents

Bisphenol A (2,2 - Bis(4-hydroxyphenyl) propane) was purchased from Sigma Aldrich. Nitric acid (HNO_3) and Potassium hydroxide (KOH) were purchased from Merck.

The characteristics of water samples are given in Table 1.

Table 1. Characteristics of the examined drinking waters

Sample	Mineral composition (mg/L)	pH value
P1	Ca^{2+} (45.80), Mg^{2+} (13.00), Na^{+} (0.80), HCO^{-}_3 (193.00), NO^{-}_3 (1.40)	8.0
P2	Ca^{2+} (62.57), Mg^{2+} (28.71), Na^{+} (2.36), HCO^{-}_3 (324.49)	7.44
P3	Ca^{2+} (17.20), Mg^{2+} (3.45), Na^{+} (0.74), K^{+} (0.53), HCO^{-}_3 (324.49)	-
P4	Ca^{2+} (9.126), Mg^{2+} (2.51), Na^{+} (3.023), K^{+} (1.312), HCO^{-}_3 (42.7), NO^{-}_3 (3.93)	7.27
P5	Ca^{2+} (118), Mg^{2+} (12.5), Na^{+} (6.81), K^{+} (1.81), HCO^{-}_3 (327)	-
P6	Ca^{2+} (95.8), Mg^{2+} (14.13), Na^{+} (13.25), K^{+} (7.01), HCO^{-}_3 (357.5)	7.6
P7	Ca^{2+} (64.79), Mg^{2+} (3.224), Na^{+} (0.87), K^{+} (1.215), HCO^{-}_3 (189.1)	7.76
P8	Ca^{2+} (43.2), Mg^{2+} (6.29), Na^{+} (2.59), K^{+} (0.75), HCO^{-}_3 (161)	8.2
P9	Ca^{2+} (192), Mg^{2+} (42.4), Na^{+} (71.1), HCO^{-}_3 (831)	6.7-6.9
P10	Ca^{2+} (94), Mg^{2+} (20), Na^{+} (7.7), HCO^{-}_3 (248)	7.8
P11	Ca^{2+} (80), Mg^{2+} (26), Na^{+} (6.5), K^{+} (1), HCO^{-}_3 (360)	7.2
P12	Ca^{2+} (18), Mg^{2+} (15), Na^{+} (18), HCO^{-}_3 (153)	7.7
P13	Ca^{2+} (73.1), Mg^{2+} (8.2), Na^{+} (6.81), K^{+} (3.3), HCO^{-}_3 (237)	7.23
P14	Ca^{2+} (113), Mg^{2+} (46.85), Na^{+} (55.3), K^{+} (8.5), HCO^{-}_3 (737)	-

Equipment

Bisphenol A analysis was performed using a Jasco V500 UV/VIS Spectrophotometer with a wavelength range of 190 nm to 900 nm and provided with a deuterium lamp and a tungsten iodine lamp. The instrument is capable of wavelength scanning from 10 nm/min to 4,000 nm/min.

Sample preparation

Samples were stored for a couple of days at room temperature prior to extraction. For the BPA analysis, the samples acidified with 0.5 ml of HNO_3 were boiled for 25 minutes. After cooling, 1 ml of KOH 50% was added in order to complete reaction. If BPA is present it can be observed a yellow coloration of the solution, which is measured at 430 nm.

Preparation of standard solutions

For obtaining the calibration curve, 0.1 g of BPA was weighted in a 100 ml volumetric flask and then filled with ethanol (solution A). By diluting 100 times solution A with distilled water, solution B is obtained. The standard solutions were prepared, from solution B, according to Table 2. Figure 1 shows the calibration curve obtained (by reading the extinctions at 430 nm).

Table 2. Preparation of standard solutions

Solution B (ml)	Distilled water (ml)
1	9
2	8
3	7
4	6
5	5
6	4
7	3
8	2
9	1

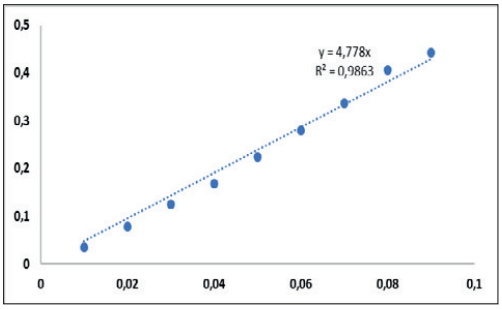

Figure 1. Calibration curve for BPA

RESULTS AND DISCUSSIONS

The results obtained for the BPA content of the analyzed samples (average of 3 parallel samples with two replicates for each one) are presented in Table 3.

BPA content ranged between < 0.1 µg/L and 1.9 µg/L. These values are higher than the values found in the literature for still mineral water bottled in PET bottles: < LOD and 0.006 µg/L (Chailurkit et al., 2016), 0.012-0.044 µg/L (Wang et al., 2020), 0.003-0.010 µg/L (Tayooka and Oshige, 2000), < LOD - 0.004 µg/L (Amiridou and Voutsa, 2011) and are affected by several factors such as mineral composition, pH, storage time and analysis method (interferences may appear at the same wavelength).

However, the values obtained are much lower than specific migration limit specified by Regulation (EU) no. 213/2018, regarding the use of BPA in materials for the inner coating of containers intended to come into contact with food and amending Regulation (EU) no. 10/2011 regarding the use of this substance in

plastics that come into contact with food, respectively 0.05 mg per kg of food (mg/kg).

Table 3. BPA concentrations in water samples

Sample	BPA concentration (μg/L)
P1	1.8 ± 0.2
P2	< 0.1
P3	< 0.1
P4	< 0.1
P5	1.1 ± 0.1
P6	< 0.1
P7	< 0.1
P8	0.7 ± 0.1
P9	0.9 ± 0.1
P10	1.8 ± 0.1
P11	< 0.1
P12	1.6 ± 0.1
P13	1.9 ± 0.2
P14	0.5 ± 0.1

CONCLUSIONS

In this article a simple and rapid method of determining bisphenol A from samples of still mineral water bottled in PET bottles was used. All the results obtained are below the maximum allowed limit, stated by Regulation (EU) no. 213/2018.

The level of BPA found in water samples packed in different polymeric bottles (PC, PVC, PE) mentioned in the literature are quite high, with maximum values found in polycarbonate bottles.

As a precautionary method, it is important to choose water packed in PET bottles, and for children it would be ideal to choose bottles labelled "BPA free".

The differences between the results of the study and the data from the specialized literature are most probably due to the experimental conditions, as well as the heterogeneity of the samples regarding the chemical composition, the pH or storage period.

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REFERENCES

Alfarhani, B. F., Al-Tameem, M., Fadhel, A. A., Hammza R. A., Kadhem M. I. (2019). Endocrine disrupting Bisphenol A detection in different water samples in Iraq. *Journal of Physics: Conference Series*, 1294(5).

Alimohammadi, M., Mehrabbegi, M., Nazmara, S., Dashtappeh M. M., Moghaddam, V. K. (2014). Quantitative assessment of Bisphenol A in multiple brands of bottled drinking water in Iran. *Iranian Journal of Toxicology*, 8(25), 1037-1040.

Amiridou, D. and Voutsa, D., 2011. Alkylphenols and phthalates in bottled waters. *Journal of Hazardous Materials*, 185(1), 281-286.

Cao, X. H., Ji, S. J. (2013). Migration study of bisphenol A from tea bottle of polycarbonate plastic to tea water. *Advanced Materials Research Vols*., 821-822, 929-932.

Chailurkit, L., Srijaruskul K., Ongphiphadhanakul B. (2016). Bisphenol A in canned carbonated drinks and plastic - bottled water from supermarkets. *Exposure and Health*, 9, 243-248.

Elobeid, M. A, Almarhoon Z. M., Virk, P. Hassan, Z. K., Omer, S. A., ElAmin, M., Daghestani, M. H., AlOlayan, E. M. (2012). Bisphenol A detection in various brands of drinking bottled water in Riyadh, Saudi Arabia using Gas Chromatography/Mass Spectrometer. *Tropical Journal of Pharmaceutical Research*, 11(3), 455-459.

Toyo'oka, T. and Oshige, Y., 2000. Determination of alkylphenols in mineral water contained in PET bottles by liquid chromatography with coulometric detection. *Analytical Sciences*, 16(10), 1071-1076.

Wang, H., Liu, Z. H., Tang, Z., Zhang, J., Yin, H., Dang, Z., Wu, P. X., Liu, Y. (2020). Bisphenol analogues in Chinese bottled water: Quantification and potential risk analysis. *Science of the Total Environment*, 713, in press.

COMMISSION REGULATION (EU) No. 10/2011 of 14 January 2011 on plastic materials and articles intended to come into contact with food.

COMMISSION REGULATION (EU) 2018/213 of 12 February 2018 on the use of bisphenol A in varnishes and coatings intended to come into contact with food and amending Regulation (EU) No 10/2011 as regards the use of that substance in plastic food contact materials.

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IDENTIFYING SYNTHETIC SWEETENERS FROM WINE BY UPLC

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Abstract

According to the wine quality and production conditions, the legislation in force states that masking defects and modifications of the wines by additions that determine changes in their taste, aroma and natural composition, can be a counterfeit product. This type of fraud is sanctioned according to the law. An example of these additives are synthetic sweeteners which, although they are forbidden, may be found in wines. Synthetic sweeteners consumption may affect the consumer health, and also the consumption of fraudulent sweetened wines can be a real danger for them. To avoid all of these inconveniences for consumers and for combating fraud in the wine sector, we can highlight the frequency of synthetic sweeteners in bottled wines with a suitable liquid chromatography method. This method establishes how to identify and quantify three of the most commonly used synthetic sweeteners (acesulfame K - E950, aspartame - E951 and saccharin - E954) that can be found in the wine matrix by ultra-performance liquid chromatography (UPLC) with UV detection. The method has selectivity, detection limit and quantification limit, linearity, precision (repeatability and reproducibility), accuracy (bias and recovery).

Key words*: wine, synthetic sweeteners, UPLC, quality, food safety.*

INTRODUCTION

Food and beverage industry has long sought to align itself with international standards on guidelines and regulations for added sugar intake from the World Health Organization. Consumers who want a sweet taste without adding energy can choose to use an artificial sweetener based on their personal taste preference and the intended use, like cooking or table top use (Chakraborty and Das., 2019). World Health Organization recommends a level up to 5-10% added sugar, fact that leads to increased use of non-caloric sweeteners (WHO, 2015). Synthetic sweeteners are in food industry since 1800's and they were often a controversial topic. The addition of non-caloric sweeteners (NNSs) to certain products raises the problem of cumulative effect of additives as well as achieving Acceptable Daily Intakes (ADIs). All over the world acesulfame K and aspartame have been the most frequently evaluated sweeteners and data from different studies showed that the intake was reduced below the level of toxicological concern. The main interest is the potential association between low/no-caloric sweeteners and population health, with special attention on changes in gut microbiota, weight management and obesity, cardiometabolic health and diabetes (Martyn et al., 2018; Reid et al., 2016; Rogers et al., 2016). Research is focused nowadays on the metabolic effects of non-nutritive sweeteners intake. The effect on gut microbiota, that can trigger glucose intolerance (Palmnas et al., 2014; Suez et al., 2014) and the interaction with novel sweet taste receptors discovered in non-taste tissues including the gut and the pancreas, which can influence insulin secretion (Jang et al., 2007; Corkey, 2012) are mentioned in many studies proving that NNSs cause metabolic disorders in human subjects (Suez et al., 2014; Jang et al., 2007; Corkey, 2012; Brown et al., 2009). As well, data from five different mammalian species (human, rats, mice, pigs, etc.) showed that NNSs can be metabolically active (Corkey, 2012; Moran et al., 2010; Mitsutomi et al., 2014). There is clearly that the old concept that

NNSs are invariable metabolically inert is no longer true (Pepino, 2015). Divergent regulations maintain a wide debate on the impact of sweeteners on food safety, consumer's health and people lifestyle (Carocho et al., 2017).

Usage of sweeteners is regulated according to their potential toxicological effects, proposed applications and the level of use in foodstuffs. The exposure of the population to these substances can be monitored by assessing the concentration of the sweeteners in the product and the consumption data of that product (WHO, 2015; D. Martyn et al. 2018). Liquid and gas chromatographic methods were developed for artificial sweeteners determination but because these compounds show low volatility, the last ones were almost abandoned. Several methods based on RP-HPLC with UV or DAD detectors have been reported for determination of aspartame, saccharin, cyclamate, acesulfame K and stevioside in different foods like beverages, canned fruits, ice cream and other dairy desserts (Shah and Jager, 2017). The main problems in developing an HPLC-UV method for NNSs are their different chemical structure and the sample matrix effect. Partially these disadvantages may be overcome when using HPLC coupled with mass spectrometry (MS/MS). Also, with this type of method higher specificity and sensitivity may be achieved. Some HPLC ESI-MS/MS methods were developed, both in negative ion mode (Zygler et al., 2011) and in multiple reaction monitoring mode (Lim et. al, 2013).

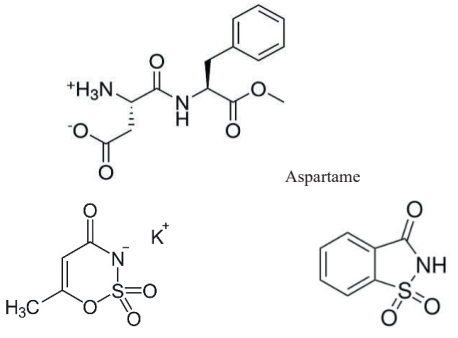

Figure 1. Chemical structure of three artificial sweeteners analysed in this study

European legislation regulates the utilization of nine artificial sweeteners in specified foodstuffs. The most commonly NNSs in foods are: acesulfame K (E950), aspartame (E951) and saccharin and its Na and Ca salts (E954) (Figure 1).

The aim of present study was to find a reliable method for analysing acesulfame K, aspartame, and saccharin in wine using UPLC-UV and to validate it. Method parameters were assessed as follows: selectivity, linearity, sensitivity, accuracy, repeatability, reproducibility, limits of detection, limits of quantification, linear range and recoveries. After validation, the method was used to analyse these three artificial sweeteners in 20 wine samples.

MATERIALS AND METHODS

Materials

For this study the analytical standards of acesulfame K (ACS-K) (6-methyl-1,2,3-oxathiazin-4(3H)-one 2,2-dioxide potassium salt), aspartame (ASP) (N-(L-α-aspartyl)-L-phenylalanine methyl ester) and sodium saccharin (SAC) (2,3-dihydro-3-oxobenzisosulfonazole sodium salt) were purchased from Supelco. Tetrabutylammonium hydrogen sulphate (97%) and anhydrous sodium acetate were supplied by Sigma-Aldrich, as well as the HPLC grade methanol.

Bottled wine samples were purchased from local markets. Red, rosé and white wines classified as dry, medium dry, medium sweet and sweet, were analysed by the presented method. The samples were stored at 4-6°C until analysis.

Standard solutions and samples preparation

Stock standard solutions were prepared in 10% methanol with the following concentrations: 2500 mg/L for ACS K, 4000 mg/L for ASP and 1000 mg/L for SAC. These stock solutions were used in different volumes for each of them to prepare a mixed working standard solution containing 10 mg/L ACS K, 40 mg/L ASP and 10 mg/L SAC. For obtaining the calibration curve, five standard levels were prepared by diluting the mixed working standard solution with ultrapure water. Stock standard solutions and mixed working standard solution were stored at 4-6°C prior to use.

Wine samples were sonicated in order to remove the possible dissolved gases, then they were properly diluted with ultrapure water. After dilution, the samples were filtered using Millex-

HV syringe filter with 0.45 µm pore size PVDF membrane and placed in an UPLC vial for instrumental analysis.
The fortified test samples were prepared by spiking at the level of 40 mg/L ACS K, 60 mg/L ASP and 40 mg/L SAC.

Chromatographic method
In order to identify and quantify the three synthetic sweeteners in wines by liquid chromatography method, we used Waters Acquity UPLC equipment (with binary solvent manager, heater/cooler sample organizer, thermostatic column compartment) with UV detector. The separation was performed with a Luna Omega C18 column (1.6 µm, 100Å, 2.1 mm x 100 mm), at 22°C, by isocratic elution with 0.2 mL/min flow rate. The mobile phase consisted in a mixture of two solvents: 400 mL of 5 mM tetrabutylammonium hydrogen sulphate and 6.1 mM anhydrous sodium acetate solution prepared in ultrapure water mixed with 171 mL 100% methanol. The solvents were degassed before using. The injection volume was 2.0 µL and the run time was 15 minutes. Artificial sweeteners detection was performed at 220 nm. Data were collected and processed using Empower 2 software.

RESULTS AND DISCUSSIONS

The described chromatographic method was applied for quantification of the three synthetic sweeteners. After diluting the stock solutions, each analyte was injected in turn, in order to determine their sequence. All the three sweeteners were identified in the chromatogram obtained for the mixed standard solution, in the following order: ASP (5.98 min retention time), ACS K (6.95 min retention time) and SAC (10.01 min retention time). The analysis revealed a good separation of the three compounds, indicated by resolution and peaks shape and symmetry (Figure 2). Taking into account the retention times and peak width, the calculated values for resolution were 28.7 for ASP, 2.3 for ACS K and 6.5 for SAC.
The method was validated to demonstrate that its performance characteristics are adequate to using for intended purpose (Barwick et al., 2014). There have been established and confirmed specific validation parameters, like selectivity, limit of detection and limit of quantification, working range, analytical sensitivity, trueness, precision, measurement uncertainty, ruggedness (Barwick et al., 2014).
Selectivity is ability of a method to measure the differences of analysis in the presence of other compounds that behave similarly (Rusea, 2016). Absence of interferences and clear identification of each compound were registered. The peaks are completely resolved, with no overlapping (Figure 2).
Limit of detection (*LOD)* of an individual analytical procedure is the smallest amount of analyte in test sample that can be detected, but it doesn't mean that can be quantified as an exact value (Rusea, 2016). A signal-to-noise ratio of 3 was evaluated for LOD.

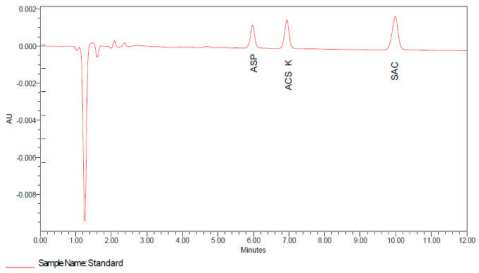

Figure 2. Chromatogram of mixed standard solution

The determined values of LOD were 1.4 mg/L for ASP, 0.3 mg/L for ACS K and 0.2 mg/L for SAC.
Limit of quantification (LOQ) is the smallest amount of analyte in test sample that can be quantitatively determined with acceptable repeatability and accuracy (Rusea, 2016). Evaluation was made considering three times LOD values. The results obtained for LOQ were 4.2 mg/L for ASP, 0.9 mg/L for ACS K and 0.6 mg/L for SAC.
Working range is the interval between lower and upper concentration of analyte in a sample for which the analytical procedure has been adequate (Rusea, 2016). Generally, it is much wider than the linear domain. Our working range was established between 4.0 mg/L and 40.0 mg/L for ASP, 1.0 mg/L and 40.0 mg/L for ACE K and 0.5 mg/L and 40 mg/L for SAC.
Linearity is the ability of a method to provide results directly proportional to the analyte concentration on an established domain (Rusea, 2016). Quantitative analysis were performed

using external calibration method. Calibration curve was obtained with standard solutions with five levels of concentration, with three injections per each level. The correlation coefficient was higher than 0.99 for all the three artificial sweeteners. The obtained values of r^2 were 0.9974 for ASP, 0.9959 for ACS K and 0.9935 for SAC.

Analytical sensitivity is the modification in response of a measuring instrument divided to the corresponding change of the stimulus (Rusea, 2016). It expresses the ability of a method to record small variations in concentration of a certain analyte and we confirmed that this method has analytical sensitivity.

Trueness or accuracy of an analytical procedure express how close the experimental value is to the true value. It indicates the concordance between average value of a set of results and an accepted reference value (Rusea, 2016). Measure of trust is expressed in terms of bias which represents a total systematic error. Our bias is 6.9% for ASP, 4.5% for ACS K and 6.8% for SAC.

Repeatability or precision means the approaching results from a series of measurements obtained from different aliquots of the same homogeneous samples, under the same conditions (Rusea, 2016). It is expressed as relative standard deviation (RSD%) and it is a component of measurement uncertainty. Repeatability was assessed by injecting 6 times in a row three levels of the mixed standard solution. The average values obtained for RSD were 1.6% for ASP, 2.1% for ACS K and 1.9% mg/L for SAC.

Reproducibility is when repeatability is made by another analyst, or using another equipment (with the same configuration), or in another laboratory, but following the same analytical procedure for analysing the same sample. For this study, the reproducibility was assessed by analysing the same three levels of mixed standard solution, 6 times in a row by two analysts. The registered average values for RSD were 8.4% for ASP, 7.3% for ACS K and 4.4% mg/L for SAC.

Recovery is the percentage of the real concentration of a substance recovered during the analytical procedure. It is a measure of method efficiency for detecting the all analyte (Rusea D., 2016). It is expressed as a ratio between response obtained for the samples extracted at three concentrations of analyte and the response measured without the extraction step. Recovery test was performed analysing wine samples fortified with 40 mg/L ACS K, 60 mg/L ASP and 40 mg/L SAC. The determined recovery values were 92% for ASP, 90% for ACS K and 101% SAC.

Measurement uncertainty is a parameter associated with the measurement result that characterizes dispersion of attributed values to the measurement. Uncertainty means evaluation of sources of errors at each stage and estimation of associated uncertainty (Rusea D., 2016) and it is an essential component of the validation process. The global uncertainty it is based on available data from validation method, internal quality control and comparison tests. For this method we established a measurement uncertainty up to 8%.

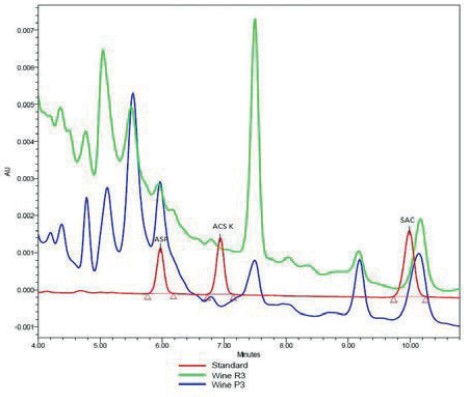

Figure 3. Overlay chromatogram of two positive wine samples for aspartame (R3 and P3) and mixed standard solution

The described method was used for assessing the presence and the content of the three NNSs in 20 wine samples from Romanian market. According to the obtained data, two samples were positive for aspartame, one red dry wine (R3) with 4.701 mg/L and one rosé sweet wine (P3) with 22.858 mg/L. Overlaying the chromatograms of the two samples and the one of the standard, it is obvious the presence of aspartame and the absence of the other two sweeteners (Figure 3). Acesulfame K and saccharin were not detected in any sample.

CONCLUSIONS

The presented UPLC-UV method is suitable for determination of the concentration of illegally added acesulfame K, aspartame and saccharin in wine. The reliability of this method is assured by the obtained values for the validation parameters. The method is rather simple, with no need of complicated sample preparation.

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REFERENCES

Barwick V., Morillas Bravo P.P., Ellison S.L.R., Engman J., Gjengedal E.L.F., Oxenbøll Lund U., Magnusson B., Müller H.T., Patriarca M., Pohl B., Robouch P., Sibbesen L.P., Vanstapel F., Vercruysse I., Yilmaz A., Ömeroglu, P.Y., Örnemark U. (2014). *Eurachem Guide, The Fitness for Purpose of Analytical Methods, A Laboratory Guide to Method Validation and Related Topics*, Second European Edition.

Brown R.J., Walter M., Rother K.I. (2009). Ingestion of diet soda before a glucose load augments glucagon-like peptide-1 secretion. *Diabetes Care*, 32(12), 2184–6.

Carocho M., Morales P. and Ferreira I. C.F.R (2017), Sweeteners as food additives in the XXI century: A review of what is known, and what is to come, *Food and Chemical Toxicology*, Vol. 107, Part A, 302–317.

Chakraborty R. and Das A. (2019). *Artificial sweeteners*, Encyclopaedia of Food Chemistry, pp.30–34

Corkey B.E. (2012). Banting lecture 2011: hyperinsulinemia: cause or consequence? *Diabetes*, 61(1), 4–13.

Jang H.J., Kokrashvili Z., Theodorakis M.J., Carlson O.D., Kim B.J., Zhou J., Kim H.H., Xu X., Chan S.L., Juhaszova M., et al. (2007). *Gut-expressed gustducin and taste receptors regulate secretion of glucagon-like peptide-1,* Proceedings of National Academy of Science of USA, 104(38), p.15069–15074.

Lim, H.S., Park, S.K., Kwak, K.I.S., Kim, H., Sung, J.H., Jang, S.J., Byun, M.Y., Kim, S.H. (2013). HPLC-MS/MS analysis of 9 artificial sweeteners in imported foods. *Food Sci. Biotechnol.*, 22(1), 233–240

Martyn D., Darch M., Roberts A., et al. (2018). Low-/No-Calorie Sweeteners: A Review of Global Intakes. *Nutrients*, 10(3):357. Published 2018 Mar 15. doi:10.3390/nu10030357

Moran A.W., Al-Rammahi M.A., Arora D.K., Batchelor D.J., Coulter E.A., Daly K., Ionescu C., Bravo D., Shirazi-Beechey S.P. (2010). Expression of Na+/glucose co-transporter 1 (SGLT1) is enhanced by supplementation of the diet of weaning piglets with artificial sweeteners. *British Journal of Nutrition*,104, 637–46.

Mitsutomi K., Masaki T., Shimasaki T., Gotoh K., Chiba S., Kakuma T., Shibata H. (2014). Effects of a nonnutritive sweetener on body adiposity and energy metabolism in mice with diet-induced obesity. *Metabolism*, 63(1), 69–78.

Palmnas M.S., Cowan T.E., Bomhof M.R., Su J., Reimer R.A., Vogel H.J., Hittel D.S., Shearer J. (2014). Low-dose aspartame consumption differentially affects gut microbiota-host metabolic interactions in the diet-induced obese rat. *PloS one*, 9(10):e109841.

Pepino M.Y. (2015). Metabolic effects of non-nutritive sweeteners. *Physiol Behav*, 152 (Part B), 450–455. doi:10.1016/j.physbeh.2015.06.024

Rogers P.J., Hogenkamp P.S., De Graaf C., Higgs S., Lluch A., Ness A.R., Penfold C., Perry R., Putz P., Yeomans M.R., et al. (2016). Does low-energy sweetener consumption affect energy intake and body weight? A systematic review, including meta-analyses, of the evidence from human and animal studies. *Int. J. Obes*, 40:381–394. doi: 10.1038/ijo.2015.177

Rusea D. (2016). *Validation of analyzes methods*, Course support, FiaTest, Bucuresti

Shah R., de Jager L.S. (2017). *Recent Analytical Methods for the Analysis of Sweeteners in Food: A Regulatory Perspective*, Food Safety: Innovative Analytical Tools for Safety Assessment, Scrivener Publishing LLC, pp.13–32.

Suez J, Korem T, Zeevi D, Zilberman-Schapira G, Thaiss C.A., Maza O., Israeli D., Zmora N., Gilad S., Weinberger A., et al. (2014). Artificial sweeteners induce glucose intolerance by altering the gut microbiota. *Nature*, 514(7521), 181–6.

World Health Organization (WHO) (2015). *Guideline: Sugars Intake for Adults and Children,* Geneva, Switzerland

Zygler, A., Wasik, A., Kot-Wasik, A., Namieśnik, J. (2011). Determination of nine high-intensity sweeteners in various foods by high-performance liquid chromatography with mass spectrometric detection. *Anal. Bioanal. Chem*., 400(7), 2159–2172.

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INFLUENCE OF CORN SYRUP AND INVERTED SUGAR ADULTERATION ON PHYSICOCHEMICAL PROPERTIES OF ROMANIAN ACACIA HONEY

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Abstract

The aim of this study was to analyze the influence of corn and inverted sugar syrups adulteration on physicochemical parameters of acacia honey. The adulteration of the acacia honey samples analyzed was done by the addition of concentrated corn syrup. For this purpose, acacia honey was adulterated with different percentages (5%, 10%, 20% and 50%) of corn syrup and inverted sugar. The color, pH, free acidity, electrical conductivity, moisture content, hydroxymethylfurfural content, as well as the sugar content (fructose, glucose, sucrose, turanose, maltose, trehalose, melesitose, and raffinose) were chosen as parameters for evaluating the influence of adulteration agents in honey. The moisture content of the honey samples were below the maximum level established by Codex Alimentarius (≤ 20%). The hidroxymethylfurfural content increased considerably from 18.97 in authentic honey to 337.31 mg/kg honey adulterated with 50% inverted sugar syrup, while a decrease occurred from 18.97 to 10.89 mg/kg honey adulterated with 50% corn syrup. Electrical conductivity decreased from 122.26 µS/cm to 107.08 µS/cm and from 122.26 µS/cm to 69.34 µS/cm with the addition of inverted sugar syrup and of corn syrup respectively. The fructose/glucose ratio decrease from 1.42 in authentic honey to 1.25 in honey adulterated with 50% inverted sugar syrup and from 1.42 to 1.34 in honey adulterated with 50% corn syrup. The results indicated that most of the values of the physicochemical parameters showed considerable changes on the adulterated honey with both types of syrups.

Key words*: adulteration, honey, physicochemical parameters, syrups.*

INTRODUCTION

Honey is a natural product used since ancient times due to its nutritional and therapeutic properties (antibacterial, antiviral, anti-inflammatory, antioxidant activity) (Sakač et al., 2019). According to international standards, authentic honey should not contain additives or other substances intentionally added (Amiry et. al., 2016). Furthermore, honey should not present uncharacteristic flavor, aroma and foreign matter that could affect its processing or storage (Frew et al., 2013).

Increasing consumption options for bee products stimulates the development of food products that use honey as a raw material, thus requiring a higher production of honey (Geană et al., 2020). This high market demand has led to illegal practices among producers, where the direct or indirect adulteration is widely used to increase both production and profit, while significantly affecting the quality of honey.

Adulteration of honey reduces its quality and safety. Honey adulterated with chemicals loses its medicinal value and may also harm the consumers (Naila et al., 2018). Adulterated honey may present changes in some chemical and/or biochemical parameters such as enzyme activity, electrical conductivity, content of specific honey compounds (HMF, glucose, fructose, sucrose, maltose, isomaltose, proline, and ash) when compared to pure honey. However, some parameters are ambiguous with respect to the accuracy of the results (Soares et al., 2017). For example, when honey is subjected to heat treatment or stored for a long time, pentoses and hexoses are broken down by slow enolization and rapid β-elimination of three water molecules, resulting in undesirable compounds such as furans. The main products of sugar degradation are: furfural derived from pentoses and 5-hydroxymethylfurfural (5-HMF) derived from hexoses such as glucose and fructose. Their presence in products is often

associated with Maillard reactions, degradation of sugars in acidic environment and caramelization reactions (Da Silva et al., 2016).
Sugars are the main components of honey, as this product mainly contains glucose, fructose, and also other oligosaccharides in lower concentrations. Therefore, adulteration through the addition of carbohydrates is a type of fraudulent practice that requires special control due to the fact that the variations of these compounds in honey and the similarities with the sugar syrup composition makes difficult the detection of these adulteration agents (Morales et al., 2008). The presence of sugars in a high content following the adulteration of honey can be linked to a direct addition of different types of sugar syrups in certain concentrations after production in order to enhance the sweetness of honey or by stimulating the bees with sugar syrups during the main nectar period to produce a larger quantity of honey. For this purpose, the producers use low-cost industrial sugar syrups such as corn syrup, high fructose corn syrup, glucose syrup, sucrose syrup, invert sugar syrup, with high fructose inulin syrup (Soares et al., 2017).
In this study is presented the influence of corn and inverted sugar syrups adulteration on the physicochemical parameters of acacia honey, because this type of honey is widely used by the Romanian population due to its characteristic properties and benefits on human health. Analysis of variance (ANOVA) was used in this study as a means to emphasize the differences between authentic and adulterated acacia honey samples.

MATERIALS AND METHODS

Acacia honey was purchased from a local beekeeper in Suceava Country, Romania. Honey was adulterated with corn syrup and inverted sugar, each adulteration agent was added in the authentic honey in 5%, 10%, 20% and 50% (w/w), respectively. The inverted sugar syrup was obtained using sucrose. Citric acid was used for the hydrolysis process to correct the sugar solution. The corn syrup was purchased from DAESANG EUROPE B.V. Importing company (product of South Korea). All samples were liquefied (50°C) and homogenized prior to any analysis.
The following physicochemical characteristics of honey samples were analysed: color (analysed with a portable chromameter and a photometer Pfund), pH, free acidity, electrical conductivity, moisture content, hydroxymethylfurfural content and sugar content. All the analysis were made in triplicate.

Moisture content

The moisture content was analyzed using the refractometric method (Abbé refractometer, Leica Mark II Plus), which is a method that determines the refractive index of honey and uses a Chataway table to determine the water content (%) (Bogdanov et al., 1999). Prior to analysis the samples were liquefied at 50ºC.

Hydroxymethylfurfural content (HMF)

In order to determine HMF presence in honey samples, 5 g of honey were dissolved in 25 g of distilled water. Then Carrez I and Carrez II solutions were added and the volume was made up with distilled water in a 50 ml volumetric flask. The solution was filtered and then divided into two clarified solutions, one containing 0.2% sodium bisulphite solution as the reference sample and the other containing distilled water as the sample. The absorbance was read at 284 nm and 336 nm using a UV-VIS-NIR 3600 spectrophotometer (Schimadzu Corporation, Japan). Calculations were made according to the formula below and the results were expressed in mg/kg honey:

$$HMF = (A_{284}\text{-}A_{336}) \times 149.7 \times 5 \times D/W,$$

where: D is the dilution factor and W is the weight of honey sample (g) (White, 1979).

Color

Two instruments were used to measure the color of the samples. The first one was a portable chromameter CR-400 (Konica Minolta, Japan) which uses the CIE L*a*b* color space for color analysis, namely Cartesian coordinates to determine a value in color space. The color is described by chromatic qualities, which are found in three components: brightness, hue and chroma (saturation) (Tuberoso et al., 2014).
The second instrument that was used for honey samples color analysis was a photometer Pfund HI 96785 (Hanna Instrumets, USA). The Pfund colorimeter is a simple tool by which allow the comparison of the samples color with a reference sample. The reference unit is the Pfund scale, whose variation is between 0 and

140 mm, from very light honey colors to the darkest hue (Dominguez, 2015).

pH

To determine the pH, a 10% honey aqueous solution was prepared and the measurements were made with a METTLER TOLEDO Five Go pH-meter (Mettler Toledo, USA).

Free acidity

The free acidity was determinated by following the steps below: 10 g of honey were dissolved in 75 mL of carbon dioxide-free water, it was measured the pH of the solution, and then it was titrated with 0.1 M sodium hydroxide to pH = 8.3 with a TITROLINE easy device (Schott Instruments, Germany).

The calculation was performed according to the formula presented below and the results were expressed in milliequivalents/kg of honey:

Free acidity = mL of 0.1 M NaOH × 10

Electrical conductivity

The electrical conductivity of 20 g of honey sample dissolved in 100 mL of distilled water was measured with a portable conductometer HQ14d (HACH, USA). The results were expressed in microSiemens per centimeter ($\mu S \cdot cm^{-1}$).

Sugar content

The sugar content was determined by high performance liquid chromatography (HPLC) with RI (refractive index) detection according to the method published by Bogdanov et al. (1988). Fructose, glucose, sucrose, turanose, maltose, trehalose, melesitose and raffinose were used as standard substances. Sample preparation was made as follows: 5 g of honey were dissolved in 40 mL of distilled water. 25 mL of methanol were added and the volume was made up with distilled water in a 100 mL volumetric flask. Then resulting solution was filtered and afterwards injected into the instrument. The mobile phase was a acetonitrile: water (80:20) mixture.

RESULTS AND DISCUSSIONS

Moisture content

A very important constituent of honey composition is water. Its content may vary depending on different factors such as botanical origin and maturity level, storage conditions and also processing technique (Da Silva et al., 2016). According honey standard of the Codex Alimentarius and EU Honey Directive, the moisture content cannot be more than 20% (Bogdanov and Martin, 2002).

Some parameters of honey (viscosity, degree of crystallization, color, flavor, taste, specific weight, solubility, preservation degree) can be influenced by the moisture content (Da Silva et al., 2016). In this study the moisture content did not exceed the admitted limit of 20%. Its variation with the degree of adulteration was between 15.96% and 16.85% for honey adulterated with inverted sugar syrup and between 15.96% and 18.99% for honey adulterated with corn syrup (Table 1.b.).

Hydroxymethylfurfural content (HMF)

The HMF content is a quality indicator of the freshness and purity of honey (Naila et al., 2018). Depending on the degree of adulteration, the HMF content increased considerably from 18.97 mg/kg to 337.31 mg/kg in honey adulterated with inverted sugar syrup. This increase was due to inverted sugar syrup, which has a high HMF content, thus determining values that exceed the maximum allowed limit of 40 mg/kg honey, as shown in Table 1.b. Zábrodská (2015) reported a value of 200 mg/kg that was determined for honey adulterated with invert sugar syrup obtained by acid hydrolysis.

In the case of honey adulterated with corn syrup, the HMF content decreased from 18.97 mg/kg in authentic honey to 10.89% in honey adulterated with 50% syrup.

High HMF content may also be due to other conditions, such as heat treatment and long storage. For example, honey samples stored for more than 1-2 years contained 128-1131 mg/kg of HMF (Naila et al., 2018).

Color

Color is the most important feature from a commercial point of view. Honey color varies depending on different factors such as the geographical origin of the honey, the content of pigments (carotene and xanthophylls), and the content of polyphenols. Proper color measurement allows exporters to choose the most profitable commercial market for their products, this parameter being the only sensory

examination with precise coding within the regulations in force (Dominguez, 2015).

Depending on the degree of adulteration, the values of L* ranged between 44.07 and 45.68 ($p > 0.05$) for honey adulterated with inverted sugar syrup and between 44.07 and 43.94 ($p > 0.05$) for honey adulterated with corn syrup. The values of a* and b* varied between -3.34 and -2.62 ($p < 0.001$) and 24.76 and 31.13 ($p < 0.001$) for honey adulterated with inverted sugar syrup and between -3.34 and -3.91 ($p < 0.001$) and 24.76 and 21.17 ($p < 0.01$) for honey adulterated with corn syrup, respectively. ΔE* ranged from 3.60 to 8.06 for honey adulterated with inverted sugar syrup and from 1.91 to 3.64 for honey adulterated with corn syrup. Negative and positive values of a* and b* coordinates indicate that all the samples had nuance of colour between green and yellow (fourth quadrant of CIE L*a*b* color space). On the Pfund scale, the color of adulterated honey with inverted sugar syrup ranged from 12.87 mm Pfund to 26.40 mm Pfund (the color changed from extra white to white according to the Pfund scale) and of honey adulterated with corn syrup ranged from 12.87 to 6.93 (the color changed from extra white to water white according to the Pfund scale). Depending on the adulteration agent, the differences were significant ($p < 0.001$) for the both measurement methods. All these results are presented in Table 1.a. and Table 1.b.

pH

The results presented in Table 1.b. show that the pH values decreased with the increase of the degree of adulteration from 4.30 to 3.98 ($p > 0.05$) for honey adulterated with inverted sugar syrup. For honey adulterated with corn syrup the pH values increased from 4.30 to 4.40 ($p > 0.05$). Oroian et al. (2018) argued that the invert sugar syrup added in authentic honey leads to a decrease of pH due to the addition of citric acid in the syrup solution to prevent crystallization or hydrolysis. It can be assumed that the addition of citric acid to invert sugar syrup in a higher content would greater impact the pH of honey.

A pH level between 3.2 and 4.5 and the natural acidity of honey inhibits the growth of microorganisms, since the optimum pH for most organisms is between 7.2 and 7.4. The addition of high-fructose corn syrup to honey from Brazil has led to a significant increase in pH value compared to pure honey (Ribeiro et al., 2014).

Free acidity

The free acidity of honey is an important characteristic that can indicate microbial spoilage. When the values of the free acidity exceed the maximum allowed limit a fermentation of the sugar with formation of acetic acid resulting by the alcoholic hydrolysis should be expected (Geană et al., 2020).

The free acidity significantly increased with the degree of adulteration from 3.86 meq/kg to 5.10 meq/kg ($p < 0.001$) for honey adulterated with inverted sugar syrup, as it can be observed in Table 1.b. For honey adulterated with corn syrup the values decreased from 3.86 meq/kg to 3.61 meq/kg ($p < 0.05$). Oroian et al. (2018) found that the adulteration of honey with inverted sugar syrup in addition to the decrease of the pH produces an increase of free acidity. During honey deterioration, fermentation of sugars with the formation of organic acids leads to increased acidity. The maximum acidity level established by the Codex Alimentarius Commission (2001) is 50.00 meq/kg (Sakač et al., 2019). In this study the free acidity did not exceed the maximum allowed limit for any analyzed sample.

Electrical conductivity

The electrical conductivity of honey is related to the ash content and acidity. This parameter increases with the increase of the organic acid ions and proteins content. According to Codex Alimentarius, the maximum admitted value of electrical conductivity is 800 $\mu S \cdot cm^{-1}$ (Da Silva et al., 2016). In this study, the electrical conductivity showed a partially significant decrease ($p < 0.01$) from 122.26 $\mu S \cdot cm^{-1}$ to 107.08 $\mu S \cdot cm^{-1}$ for honey adulterated with inverted sugar syrup and a significant decrease ($p < 0.001$) from 122.26 $\mu S \cdot cm^{-1}$ to 69.34 $\mu S \cdot cm^{-1}$ for honey adulterated with corn syrup (Table 1.b.).

Sugar content

The content of sucrose, turanose, melesitose and also raffinose changed significantly ($p < 0.001$) depending on the degree of adulteration for the both agents with values that ranged from 0.44% to 0.23% (sucrose), 0.17% to 0.70% (turanose), 1.32% to 0.68% (melesitose) and from 0.51% to

0.28% (raffinose) for honey adulterated with inverted sugar syrup and values that ranged from 0.44% to 0.22% (sucrose), 0.17% to 0.08% (turanose), 1.32% to 18.17% (melesitose) and from 0.51% to 0.31% (raffinose) for honey adulerated with corn syrup, respectively, as shown in Table 1.b.

Depending on the degree adulteration the content of maltose and trehalose showed the same ranges for honey adulterated with both types of syrup, from 2.18% to 1.09% (maltose) and 1.15% to 0.58% (trehalose).

The decrease of the content of fructose was not significant ($p > 0.05$) for honey adulterated with inverted sugar syrup (the range was from 37.18% to 35.90%), while for honey adulterated with corn syrup was significant ($p < 0.001$) with values from 37.18% to 18.62%.

The content of glucose and F/G ratio changed partially significant ($p < 0.01$) for honey adulterated with inverted sugar syrup with values from 25.93% to 28.43% and from 1.42 to 1.25, respectively. For honey adulterated with corn syrup the content for glucose decrease significantly from 25.93% to 13.74%, and for F/G ratio the decrease was less significant with values from 1.42 to 1.34.

Different types of honey have different fructose/glucose ratios. This ratio indicates the ability of honey to crystallize, a ratio greater than 1 indicating a liquid honey (Geană et al., 2020). Floral honey has a fructose/glucose ratio of about 1. The crystallization of glucose has a stronger effect on honey with higher glucose content (Gleiter et al., 2006).

Regardless of the adulteration agent (inverted sugar syrup or corn syrup) the changes for maltose and trehalose content were not significant ($p > 0.05$), as shown in Table 1.a.

Detecting inverted sugar syrup adulteration honey can be difficult by common methods, because it can be adapted to imitate the sucrose-fructose-glucose profile of authentic honey. By adding a small amount of invert sugar the changes in glucose and fructose levels are not significant when compared to those of authentic honey (Geană et al., 2020). In Figure 1 is presented the chromatographic profil of acacia honey, inverted sugar syrup and corn syrup, it can be observed that corn syrup has a high peak for melesitose, while fructose peak is similar for acacia honey and inverted sugar syrup.

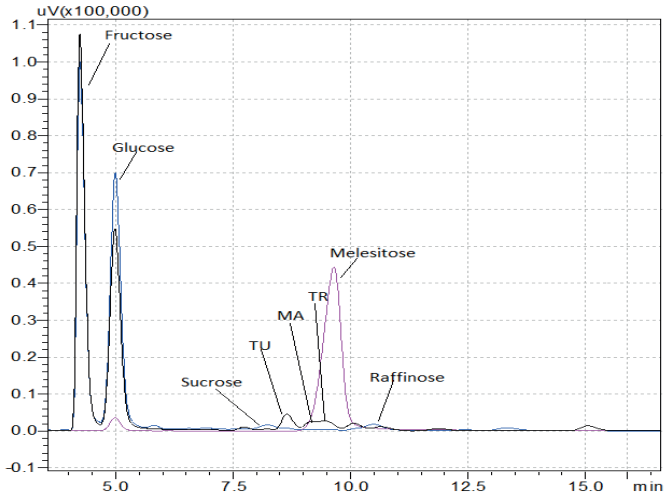

Figure 1. Chromatografic profile of acacia honey (black line), inverted sugar (blue line) and corn syrup (red line)
TU - turanose, MA - maltose, TR - trehalose

Table 1.a. Physicochemical parameters of adulteration agent and interaction agent-degree of adulteration

Parameter	Adulteration agent		F - ratio	Interaction
	Corn syrup	Inverted sugar syrup		Agent - Degree of adulteration
L*	43.01(1.01)b	44.84(0.88)a	42***	3.95*
a*	-3.34(0.32)b	-2.94(0.26)a	380***	143.79***
b*	23.76(1.51)b	28.73(2.32)a	868***	97.20***
ΔE*	1.90(1.21)b	4.72(2.93)a	11262***	1482.33***
Color (mm Pfund)	11.74(2.86)b	18.74(4.78)a	4647***	1168.68***
pH	4.33(0.05)a	4.19(0.13)b	27***	7.47**
Free acidity (meq/kg)	3.77(0.10)b	4.28(0.46)a	375***	102.81***
Electrical conductivity (μS/cm)	103.14(19.86)b	115.97(5.82)a	323***	88.29***
Moisture (%)	16.99(1.14)a	16.26(0.37)b	47***	12.92***
HMF (mg/kg)	16.22(3.03)b	127.20(119.31)a	20633***	5640.43***
Fructose (%)	30.87(6.96)b	36.75(0.62)a	719***	196.78***
Glucose (%)	21.78(4.57)b	26.77(0.97)a	1007***	275.41***
Sucrose (%)	0.32(0.08)a	0.27(0.08)b	1183***	231.80***
Turanose (%)	0.13(0.03)b	0.35(0.20)a	13595***	3742.20***
Maltose (%)	1.81(0.41)a	1.81(0.41)a	0ns	0ns
Trehalose (%)	0.95(0.21)a	0.95(0.21)a	0ns	0ns
Melesitose (%)	7.05(6.31)a	1.10(0.24)b	19731***	5391***
Raffinose (%)	0.44(0.07)a	0.43(0.08)b	13**	3*
F/G ratio	1.39(0.03)a	1.36(0.06)b	21***	4*

ns - not significant ($p > 0.05$), *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, a-e - different letters in same row indicate significant differences between sample

Table 1.b. Physicochemical parameters of acacia honey adulterated with inverted sugar syrup and corn syrup

Acacia honey analysis results												
Parameter	**Degree of adulteration for inverted sugar syrup**					**F - ratio**	**Degree of adulteration for corn syrup**					**F - ratio**
	0.00%	**5.00%**	**10.00%**	**20.00%**	**50.00%**		**0.00%**	**5.00%**	**10.00%**	**20.00%**	**50.00%**	
L*	44.07(0.63)a	45.68(0.65)a	44.16(0.63)a	44.6(0.64)a	45.68(0.65)a	3ns	44.07(0.62)a	42.18(0.60)b	42.04(0.60)b	42.82(0.62)ab	43.94(0.63)a	4ns
a*	-3.34(0.05)e	-3.06(0.04)d	-2.9(0.04)c	-2.79(0.04)b	-2.62(0.04)a	82***	-3.34(0.05)b	-3.01(0.04)a	-3.12(0.04)a	-3.32(0.05)b	-3.91(0.06)c	103***
b*	24.76(0.35)	29.39(0.42)	28.33(0.40)	30.06(0.43)	31.13(0.45)	70***	24.76(0.35)a	24.68(0.35)a	24.92(0.35)a	23.28(0.33)b	21.17(0.30)c	43**
ΔE*	0e	8.06(0.12)a	3.60(0.05)d	5.36(0.07)c	6.62(0.09)b	3198***	0d	1.91(0.03)c	2.04(0.03)b	1.93(0.03)c	3.64(0.05)a	3230***
Color (mm Pfund)	12.87(0.18)e	15.84(0.23)d	18.81(0.27)c	19.8(0.28)b	26.40(0.37)a	680***	12.87(0.18)b	14.85(0.21)a	13.19(0.19)b	10.89(0.16)c	6.93(0.10)d	618.50***
pH	4.30(0.06)	4.27(0.06)	4.24(0.06)	4.17(0.06)	3.98(0.06)	9*	4.30(0.06)a	4.31(0.06)a	4.32(0.06)a	4.34(0.06)a	4.40(0.06)a	0.77ns
Free acidity (meq/kg)	3.86(0.06)d	3.98(0.06)cd	4.11(0.06)c	4.35(0.06)b	5.1(0.07)a	127***	3.86(0.06)a	3.84(0.05)a	3.81(0.06)a	3.76(0.06)a	3.61(0.05)b	6.42*
Electrical conductivity (μS/cm)	122.26(1.75)a	120.75(17.72)a	119.23(1.71)ab	116.19(1.66)b	107.08(1.53)c	25**	122.26(1.75)a	116.97(1.67)b	111.68(1.60)c	101.09(1.45)d	69.34(0.99)e	385***
Moisture (%)	15.96(0.23)b	16.05(0.23)b	16.13(0.23)b	16.31(0.23)ab	16.85(0.24)a	4ns	15.96(0.23)c	16.26(0.23)c	16.57(0.23)bc	17.17(0.24)b	18.99(0.27)a	49***
HMF (mg/kg)	18.97(0.27)e	50.80(0.72)d	82.64(1.18)c	146.31(2.09)b	337.31(4.82)a	5414***	18.97(0.27)a	18.16(0.26)b	17.35(0.25)c	15.73(0.22)d	10.89(0.156)e	377***
Fructose (%)	37.18(0.53)a	37.06(0.53)a	36.93(0.53)a	36.67(0.53a	35.90(0.51)a	1ns	37.18(0.53)a	35.33(0.50)b	33.48(0.48)c	29.76(0.42)d	18.62(0.27)e	536***
Glucose (%)	25.93(0.37)c	26.18(0.38)bc	26.43(0.38)bc	26.93(0.38)b	28.43(0.41)a	13**	25.93(0.37)a	24.71(0.35)b	23.49(0.34)c	21.05(0.30)d	13.74(0.20)e	465***
Sucrose (%)	0.44(0.007)a	0.23(0.005)b	0.23(0.005)b	0.23(0.005)b	0.23(0.005)b	666***	0.44(0.007)a	0.42(0.007)b	0.40(0.007)c	0.35(0.007)d	0.22(0.007)e	311***
Turanose (%)	0.17e	0.22(0.003)d	0.27(0.006)c	0.38(0.005)b	0.70(0.008)a	3686***	0.17a	0.16(0.001)b	0.15(0.002)c	0.13(0.004)d	0.08(0.004)e	294***
Maltose (%)	2.18(0.03)a	2.08(0.03)b	1.97(0.03)c	1.75(0.03)d	1.09(0.02)e	535***	2.18(0.03)a	2.08(0.03)b	1.97(0.03)c	1.75(0.03)d	1.09(0.02)e	535***
Trehalose (%)	1.15(0.01)a	1.09(0.02)b	1.03(0.02)c	0.92(0.01)d	0.58(0.008)e	485***	1.15(0.01)a	1.09(0.02)b	1.03(0.02)c	0.92(0.01)d	0.58(0.008)e	485***
Melesitose (%)	1.32(0.01)a	1.25(0.02)b	1.19(0.02)c	1.06(0.02)d	0.68(0.01)e	513***	1.32(0.01)e	3.01(0.04)d	4.69(0.07)c	8.06(0.12)b	18.17(0.26)a	5075***
Raffinose (%)	0.51(0.007)a	0.49(0.004)b	0.47(0.009)c	0.42(0.004)d	0.28(0.005)e	438***	0.51(0.007)a	0.49(0.006)b	0.47(0.006)c	0.43(0.005)d	0.31(0.004)e	402***
F/G ratio	1.42(0.01)	1.40(0.02)	1.38(0.02)	1.35(0.02)	1.25(0.02)	26**	1.42(0.01)a	1.41(0.02)a	1.41(0.02)a	1.40(0.02)a	1.34(0.02)b	6*

ns - not significant ($p > 0.05$), *$p<0.05$, **$p<0.01$, ***$p<0.001$, a-e - different letters in same row indicate significant differences between samples

CONCLUSIONS

In this study it was analyzed the influence of honey adulteration through the addition of corn syrup and inverted sugar syrup on the physicochemical properties of the product.

The statistical analysis of the results highlighted the significant differences between authentic and adulterated honey. Depending on the degree of adulteration, for honey adulterated with inverted sugar syrup, the differences were significant ($p < 0.001$) and partially significant ($p < 0.01$) for physicochemical parameters such as color parameters on both color CIE L*a*b* (a*, b*, ΔE* color parameters) and Pfund scales respectively, free acidity, electrical conductivity, HMF content, and sugar content (glucose, sucrose, turanose, maltose, trehalose, melesitose, raffinose and F/G ratio).

For honey adulterated with corn syrup, the significant and partially significant differences were for the same physicochemical parameters of honey adulterated with inverted sugar syrup with some exceptions: moisture content and fructose content presented significant changes and free acidity content and F/G ratio were the parameters with the less significant changes.

It is important to note that with the increase of the degree of adulteration, the changes of physicochemical parameters were more significant (for example, the HMF content increased 17.78 times in honey adulterated with inverted sugar syrup compared to the content of authentic honey, exceeding the maximum allowed level and the electrical conductivity ranged from 122.26 $\mu S \cdot cm^{-1}$ in authentic honey to 101.09 $\mu S \cdot cm^{-1}$ in adulterated honey with 20% corn syrup and 69.34 $\mu S \cdot cm^{-1}$ in adulterated honey with 50% corn syrup).

The less significant differences for honey adulterated with inverted sugar were obtained for pH ($p < 0.05$) and for honey adulterated with corn sugar were obtained for free acidity and F/G ratio. In the case of L* color parameter on color CIE L*a*b* color space the results obtained for honey adulterated with both types of syrup showed that the changes were not significant ($p > 0.05$).

Depending on the adulteration agent (corn syrup and inverted sugar syrup) the differences were significant or partially significant for almost all parameters presented in this article (physicochemical parameters and the sugar content), except maltose and trehalose ($p > 0.05$).

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REFERENCES

Amiry, S., Esmaiili, M., & Alizadeh, M. (2017). Classification of adulterated honeys by multivariate analysis. *Food chemistry*, *224*, 390-397.

Bogdanov, S., & Baumann, E. (1988). Bestimmung von Honigzuckermit HPLC. *Mitt GebLebensmitteluntersHyg*, *79*, 198-206.

Bogdanov, S., Lüllmann, C., Martin, P., von der Ohe, W., Russmann, H., Vorwohl, G., ... & Flamini, C. (1999). Honey quality and international regulatory standards: review by the International Honey Commission. *Bee world*, *80*(2), 61-69.

Bogdanov, S., & Martin, P. (2002). Honey authenticity. *MitteilungenausLebensmitteluntersuchung und Hygiene*, *93*(3), 232-254.

DaSilva, P. M., Gauche, C., Gonzaga, L. V., Costa, A. C. O., &Fett, R. (2016). Honey: Chemical composition, stability and authenticity. *Food Chemistry*, *196*, 309-323.

Dominguez, M. A., & Centurión, M. E. (2015). Application of digital images to determine color in honey samples from Argentina. *Microchemical Journal*, *118*, 110-114.

Frew, R., McComb, K., Croudis, L., Clark, D., & Van Hale, R. (2013). Modified sugar adulteration test applied to New Zealand honey. *Food chemistry*, *141*(4), 4127-4131.

Geană, E. I., Ciucure, C. T., Costinel, D., &Ionete, R. E. (2020). Evaluation of honey in terms of quality and authenticity based on the general physicochemical pattern, major sugar composition and δ13C signature. *Food Control*, *109*, 106919.

Gleiter, R. A., Horn, H., & Isengard, H. D. (2006). Influence of type and state of crystallisation on the water activity of honey. *Food chemistry*, *96*(3), 441-445.

Morales, V., Corzo, N., & Sanz, M. L. (2008). HPAEC-PAD oligosaccharide analysis to detect adulterations of honey with sugar syrups. *Food Chemistry*, *107*(2), 922-928.

Naila, A., Flint, S. H., Sulaiman, A. Z., Ajit, A., & Weeds, Z. (2018). Classical and novel approaches to the

analysis of honey and detection of adulterants. *Food Control*, *90*, 152-165.

Oroian, M., Paduret, S., & Ropciuc, S. (2018). Honey adulteration detection: voltammetric e-tongue versus official methods for physicochemical parameter determination. *Journal of the Science of Food and Agriculture*, *98*(11), 4304-4311.

Sakač, M. B., Jovanov, P. T., Marić, A. Z., Pezo, L. L., Kevrešan, Ž. S., Novaković, A. R., & Nedeljković, N. M. (2019). Physicochemical properties and mineral content of honey samples from Vojvodina (Republic of Serbia). *Food chemistry*, *276*, 15-21.

Soares, S., Amaral, J. S., Oliveira, M. B. P., & Mafra, I. (2017). A comprehensive review on the main honey authentication issues: Production and origin. *Comprehensive Reviews in Food Science and Food Safety*, *16*(5), 1072-1100.

Tuberoso, C. I. G., Jerković, I., Sarais, G., Congiu, F., Marijanović, Z., & Kuś, P. M. (2014). Color evaluation of seventeen European unifloral honey types by means of spectrophotometrically determined CIE L∗Cab∗hab∘ chromaticity coordinates. *Food chemistry*, *145*, 284-291.

White Jr, J. W. (1979). Spectrophotometric method for hydroxymethylfurfural in honey. *Journal of the Association of Official Analytical Chemists*, *62*(3), 509-514.

Zábrodská, B., & Vorlová, L. (2015). Adulteration of honey and available methods for detection - a review. *Acta Veterinaria Brno*, *83*(10), 85-102.

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PACKAGING AND TECHNOLOGICAL SOLUTIONS FOR BREAD SHELF LIFE IMPROVING

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Abstract

Bread shelf life in our society is a problem if we look in food waste zone. In the studies of bread shelf-life, an important role is carried out by the various packaging materials and technological solutions that exist. Traditionally, packaging materials had to be as inert as possible (this method is so-called passive packaging), and because of this bread was protected against the main causes of spoilage, namely the presence of oxygen and mold. Most recent, films made of synthetic polymers that have low gas permeability, coupled with the modification of the packaging headspace through decreasing oxygen levels below 0.1% and also the use of a new concept called active packaging that allows the packaging material to interact with the food products, thus improving the bread shelf-life drastically. The new concepts of active packaging and intelligent packaging, in which the new developed functional materials deliberately interact with bread in order to prolong or monitor the shelf life and the use of nanomaterials represent the top of innovation in this field. Technological aids such as natural antimicrobial compounds or sourdough utilization can be very useful tools used to improve bread quality and shelf life. Finding adequate bread packaging systems, in addition with technological interventions on formulation and breadmaking technologies, can increase significantly bread shelf life. This paper will review the literature for various state of the art packaging and technological solutions for bread shelf life and quality improving.

Key words*: bread packaging, shelf life, natural antimicrobials, sourdough.*

INTRODUCTION

The shelf life of cereal products and their derivates, can be in general influenced by the packaging materials and used technologies, with special regards in our case, bread it is mainly dependent on the staling rate (Cencic et al., 1996; Del Nobile et al., 2003; Fava et al., 2000; Lanza et al., 2000; Latou et al., 2010; Licciardello et al., 2013; Pagani et al., 2006; Piergiovanni & Fava, 1997; Rodríguez et al., 2000). Shelf life testing represents a tool for selecting the most suitable packaging systems. The bread staling is a complex phenomenon, which cannot be described by only one parameter (Karim et al., 2000, Sidhu et al., 1997). For the reason mentioned above, various tests are usually performed simultaneously and supplying complementary information which can be associated with bread staling. Karim et al. (2000) reviewed the methods for the study of starch retrogradation, which include based on the changes in physical and chemical properties. In the last years, both producers and consumers have become more sensitive towards the sustainability of food production, with some special regards for the role of packaging. Estimations of the impact of packaging are in the range of 5–10% of the total environmental impact of a food item (Hanssen, 1998). Sometimes it is necessary to increase the packaging environmental impact in order to reduce food losses (Wikström & Wilsson, 2010). This is not always true and new packaging solutions that have a lower environmental impact can be able to guarantee certain shelf life standards.

Bread is considered to be a fundamental food product and it is generally viewed as a perishable commodity, due to its fast decrease of freshness features and due to its rapid staling (Minervini et al., 2014). Bread is always present

in the human daily diet, being one of the main produced products of Romanian food industry. The annual average consumption of bread per capita, is estimated at 97 kg/capita, exceeding the European average consumption levels. About 55% of the Romanian households consume unpacked bread, acquired mainly from small supermarkets and bakeries (Tamba-Berehoiu et al., 2014). Bakery products made by using highly refined white flour contain lower amounts of vitamin B1 and vitamin E compared with those containing whole-wheat flour or rye flour. Addition of supplementary ingredients (olive oil, garlic, onion, mixed seeds) to some types of bakery products resulted in obtaining B1 and E vitamins enriched food products. These supplementary vegetal ingredients were used to improve the taste and to diversify the range of bakery products, aiming to be an encouragement and an orientation for healthier food consumption. (Gherghina et al., 2015). Lactic acid bacteria (LAB) and yeasts in the form of sourdough have been reported to have positive effects on wheat bread quality and staling (Clarke et al., 2002; Corsetti et al., 2000; Crowley et al., 2002) as they are responsible for the capacity of dough to leaven, while acidifying it (De Vuyst & Neysen, 2005). Traditional sourdough obtained with selected microorganisms is able to increase bread shelf life by delaying staling (Chavan & Chavan, 2011) and improve bread properties through enhancing its nutritional value, taste, and aroma profile (Arendt et al., 2007; Hansen & Schieberle, 2005; Poutanen et al., 2009). The use of LAB may affect the rheology of leavened bakery products through a strain-dependent proteolytic activity (Gobbetti et al., 1996).

Modified atmosphere packaging (MAP) is one of the methods used to extend product shelf life. This can be done by using different gases to replace air around non-respiring foods regardless of whether or not the atmosphere changes over time of storage or packing. The most used MAP technique used for bakery products is the addition of CO_2 inside the food packaging in order to decrease the O_2 levels. In this way the shelf-life of the MAP packaged products will be prolongedIn high water content food like bakery products, CO_2 can dissolve in water to form carbonic acid, thus lowering the pH. This acidification of the cell contents causes the death of bacteria. It is concluded that mold growth could not be prevented but could be delayed by N_2 and/or CO_2 up to 5-10 days (Smith et al., 1986). The only possibility to prevent mold growth was to maintain the level of O_2 below 0.4%. Results of the dependency of O_2 content on fungi growth on other types of products under MAP were confirmed. A study carried out by Marta Taniwaki about the use of modified atmospheres to prevent fungal growth and mycotoxin production in cheese was evaluated. Eight fungal species: *Mucor plumbeus*, *Fusarium oxysporum*, *Byssochlamys fulva*, *Byssochlamys nivea*, *Penicillium commune*, *Penicillium roqueforti*, *Aspergillus flavus* and *Eurotium chevalieri* were inoculated onto cheese samples and incubated under conditions of decreasing concentrations of O_2 (0.5% to 5%) and increasing concentrations of CO_2 (20-40%). Fungal growth was measured by colony diameter and ergosterol content. All fungi examined grew in atmospheres containing 20% and 40% CO_2 with 1% or 5% O_2, and growth was reduced by 20-80%, depending on species, compared with growth in air (Stamatiset al., 2007; Halouat et al., 1997; Sanguinetti et al., 2016; Taniwaki et al., 2001). However, the high concentration of CO_2 may lead to increased perceived acidity in the organoleptic proprieties (Fik et al., 2012; Suppakul et al., 2016). Furthermore, the conclusive effect of CO_2 in the MAP on the bread nutritional quality currently cannot be made due to conflicting results from various researches. Another packaging technique that has been studied is active packaging (AP), in which the packages not only act as a protective barriers but also interact with the packaged product in order to protect it from adulteration, for example, moisture, oxygen, ethylene, and microorganisms.

Two types of AP, i.e. sachet-based and plastic film-based packaging, have been developed for food industry. Sachets, containing active ingredients such as oxygen absorbers, ethylene scavengers, and moisture absorbers, were developed in the late 1970s in Japan. Although this technique is considerably practical for packaging industries, some disadvantages are inevitable. They cannot be used in liquid products or in the tight-fitting film as their

functionalities would be restrained. Furthermore, risks of accidental ingestion of these sachets are a serious concern. These drawbacks can be alleviated if those functionality components are incorporated in the packaging material matrix itself. In addition to that, recent studies have revealed that combinations of various packaging techniques to prolong shelf life are more effective. The study of Berenzon and Saguy (1998) suggested that even though using oxygen absorber sachets was very effective in terms of controlling the headspace oxygen, they could not slow down lipid oxidation of crackers when stored at high temperatures, attaining comparable sensory evaluation of crackers kept in absence of oxygen absorber sachets. An oxygen absorber sachet was found to extend the shelf life of pita bread and bakery products by impeding mold and yeast growth even in CO_2/N_2 MAP due to the fact that it absorbed oxygen trapped in food and in the air that permeated through the package (Smith et al., 1986).

MATERIALS AND METHODS

Web of Science database was electronically searched for articles published in the last decades. The literature search included as documents research articles and reviews. Keywords used were: “sourdough”, “packaging in bread industry”, “bread making”, “bread shelf life” while the articles title contained the word “flour”.

RESULTS AND DISCUSSIONS

Baked products are perishable foods that undergo severe physical, physiochemical, organoleptic and microbial changes during storage (Robertson, 1993). The time-dependent loss in quality of flavor and texture is generally described as bread staling. Crumb firmness significantly increases, crispness of the bread crust decreases, and the bread loaf loses its fragrance, assuming a stale flavor. These complex physical and chemical phenomena are a consequence of a retrogradation of the starch granules gelatinized during baking, an interchange of moisture between the starch and protein constituents of bread, an increase in interaction between the protein fraction and starch, a redistribution of water in bread and a removal of aromatic molecules (Parker & Ring, 2001; Piazza & Masi, 1995; Schiraldi & Fessas, 2001). The use of sourdough has a long tradition and still plays an important role in the bread-making process. Sourdough is obtained by spontaneous fermentation of a mixture of flour, water and salt; recent years have seen the use of specific cultures and control of the fermentation process. Sourdough is used in baking and its ability to improve the quality and extend the shelf life of bread has been widely studied (Arendt et al., 2007; Gocmen et al., 2007; Katina et al., 2006; Martinez-Anaya, 2003). The impact of processing conditions on the microbial quality of par-baked wheat and sourdough bread was investigated by Debonne et al. (2017). Processing conditions included par-baking time (8 and 13 min), temperature (150 and 200 °C), amount of steam (200 and 600 mL), and the use of MAP. Total anaerobic mesophilic plate counts, moulds, yeasts and spore-forming bacteria, together with pH (power of hydrogen) and a_w (water activity) of the par-baked breads were analysed. The obtained data was used to make predictive models showing the impact of the main effects and their interactions. Sourdough addition could extend the time of acceptable bread quality based on the anaerobic counts from 8 to more than 13 days. Visual growth of moulds and yeasts (presence/absence of single spots) was most efficiently obtained by the combination of MAP and the use of highest baking temperature and time. Microbiological analysis of moulds and yeasts however, showed that sourdough had the best preservation potential, followed by MAP. This study showed that adjusting the par-baking conditions, bread composition and packaging can increase the shelf-life of par-baked bread in a natural way. (Els Debonne et al., 2018).

Lactic acid bacteria (LAB) constitute a heterogeneous group of industrially important bacteria that are used to produce fermented foods and beverages, using various substrates, such as milk, vegetables, cereals, meat, cocoa beans etc. The most important advantage of LAB, making them suitable for the use in food biotechnology, is that they are generally recognized as safe (GRAS - Generally

Recognized as Safe). LAB have been shown to contribute to the improvement of the shelf life of fermented foods, due to the production of a wide variety of compounds, acting in a synergistic way to prevent or eliminate microbial contamination.

In fermented foods, LAB also contributes to the nutritional and organoleptic characteristics of the final products and they are traditionally used as starter cultures for the industrial production of many types of foods and beverages.

The so-called "functional foods" concept was recently proposed and has shown a remarkable growth over the last few years. Such foods should promote well-being and health improvements, while at the same time should reduce the risk of some major chronic and degenerative diseases, such as cancer, cardiovascular diseases, obesity and gastrointestinal tract disorders (Zamfir M. et al., 2014).

Lactic acid bacteria (LAB) produce several metabolites which have been shown to have a positive effect on the texture and staling of bread, e.g. organic acids, exopolysaccharides (EPS) and/or enzymes. EPS can improve the viscoelastic properties of dough, increase loaf volume, reduce crumb hardness and prolong shelf life (Poutanen et al., 2009; Tieking & Gänzle, 2005). Moreover, the transformation of amino acids or peptides to aroma compounds contributes substantially to food flavor. In particular, the conversion of glutamate by LAB enables the targeted optimization of food flavour (Gänzle, 2009; Plessas et al., 2011). *In situ* production of EPS has the advantage of avoiding the use of bread improvers such as expensive hydrocolloids (Arendt et al., 2007; Palomba et al., 2012; Pepe et al., 2013; Tieking et al., 2003). However, *in situ* production of exopolysaccharides during sourdough fermentation is challenged by simultaneous acidification due to metabolic activities of the bacteria, which may significantly diminish the positive technological impact of EPS (Katina et al., 2009). Formation of alternative products from sucrose like organic acids are of special importance for application of *in situ* produced EPS. Lactate and acetate have previously been identified to significantly affect dough rheology, bread volume and crumb hardness, and may counterbalance the positive effect of EPS (Kaditzky & Vogel, 2008). Lacaze et al. (2007) have developed a new process used to obtain a dextran-rich sourdough by using a specific LAB strain (*Leuconostoc mesenteroides* LMGP-16878) able to produce a enough high molecular weight (HMW) dextran, ensuring a significant impact on bread volume.

The sourdough obtained allows improvements in freshness, crumb structure, mouth feel and softness of all kinds of baked goods from wheat-rich dough products to rye sourdough breads. Katina et al. (2009) showed the potential of *Weissella confusa* to produce significant amounts of polymeric dextran and isomaltooligo-saccharides in wheat sourdough without strong acidification. Dextran-enriched *W. confusa* sourdoughs showed increased viscosity and improved bread quality. Di Cagno et al. (2006) reported that the synthesis of EPS was found from sucrose only as shown by carbohydrate consumption, Moreover, compared with EPS-negative strain (*Lactobacillus sanfranciscensis* SF17), sourdough started with EPS positive strains (*Weissella cibaria* WC4, *Lactobacillus plantarum* PL9), fermented at 30°C for 24 h, increased its viscosity, and the resulting bread had higher specific volume and lower firmness. The performance of *L. sanfranciscensis* TMW 1.392 and its levansucrase deletion mutant in wheat dough and their impact on bread quality was studied by Kaditzky, et al. (2008). The authors reported that *in situ* production of EPS was not enough to achieve the same positive effects of EPS, as they partially overlapped with effects resulting from enhanced acidification. LAB strains and/or fermentation conditions must be found to maximize *in situ* EPS production while at the same time optimizing acid production to a certain quotient which allows acceptable volume, crumb structure and flavor of breads. Thus, when EPS-producing strains are screened for dough applications, their metabolite pattern, the pH at the end of fermentation and fermentation quotient (the molar ratio of lactate/acetate) should be considered.

CONCLUSIONS

Advanced packaging technologies have been playing an important role in food industries for more than a decade and becoming more and more common in recent years especially in a health-concerned society. As these types of packaging technologies successfully extend the shelf life for several foodstuffs including bakery products, uses of chemical preservatives which cause health hazard problems can be reduced significantly.

Food packages are required to have multi-functions in terms of chemical, physical, and biological alterations of the food to prolong its shelf life. Chemically, the packages should be able to control and/or prevent oxidation and some other chemical reactions, such as hydrolytic rancidity and Maillard reaction, in food products. Packages should maintain the moisture level in the products which is a critical parameter in controlling bread staling and changes in texture and physical appearance. Most importantly, microbial growth in food during storage is a serious problem and a major cause to shorten food shelf life. Packages that can inhibit microbial proliferation are currently in demand. The use of sourdough in bread-making influences all aspects of bread quality. Technological effects of the sourdough on flavor, texture, shelf life, and nutritional quality of products depends on the bioconversion of flour components at dough stage (Gänzle M. G., 2014). Apart from generating a unique flavor, the products of sourdough fermentation have been linked to various health benefits (Poutanen et al., 2009).

REFERENCES

Arendt, E. K., Ryan, L. A., Dal Bello, F. (2007). Impact of sourdough on the texture of bread. *Food Microbiology*, 24(2), 167-174.

Berenzon, S., & Saguy, I. S. (1998). Oxygen absorbers for extension of crackers shelf-life. *LWT Food Sci. Technol.*, 31 (1), 1-5.

Cencic, L., Bressa, F., Dalla Rosa, M. (1996). Influence of modified atmosphere on bread staling during storage. *Italian Food & Beverage Technology*, 71, 20-24.

Chavan, R. S., & Chavan, S. R. (2011). Sourdough technology-a traditional way for wholesome foods: A review. *Comprehensive Reviews in Food Science and Food Safety*, 10(3), 169-182.

Clarke, C. I., Schober, T. J., Arendt, E. K. (2002). Effect of single strain and traditional mixed strain starter cultures on rheological properties of wheat dough and on bread quality. *Cereal Chemistry Journal*, 79(5), 640-647.

Corsetti, A., Gobetti, M., De Marco, B., Balestrieri, F., Paoletti, F., Russi, L. (2000). Combined effect of sourdough lactic acid bacteria and additives on bread firmness and staling. *Journal of Agricultural and Food Chemistry*, 48(7), 3044-3051.

Crowley, P., Schober, T. J., Clarke, I. C., Arendt, E. K. (2002). The effect of storage time on textural and crumb grain characteristics of sourdough wheat bread. *European Food Research and Technology*, 214, 489-496.

De Vuyst, L., & Neysen, P. (2005). The sourdough microflora: Biodiversity and metabolic interactions. *Trends in Food Science & Technology*, 16, 43-56.

Debonne, E., Van Bockstaele, F., Philips, E., De Leyn, I., & Eeckhout, M. (2017). Impact of par-baking and storage conditions on the quality of par-baked and fully baked bread. LWT-Food *Science and Technology*, 78, 16-22.

Debonne, E., Bockstaele, F. V., Driessche, M. V., De Leyn, I., Eeckhout, M., Devlieghere, F. (2018). Impact of par-baking and packaging on the microbial quality of par-baked wheat and sourdough bread, *Food Control*, 91, 12-19.

Del Nobile, M.A., Martoriello, T., Cavella, S., Giudici, P., Masi, P. (2003). Shelf life extension of durum wheat bread. *Italian Journal of Food Science,* 15(3), 383-393.

Di Cagno, R., De Angelis, M., Limitone, A., Minervini, F., Carnevali, P., Corsetti, A., Gaenzle M, Ciati R, Gobbetti M. (2006). Glucan and fructan production by sourdough Weissella cibaria and Lactobacillus plantarum. *Journal of Agricultural and Food Chemistry*, 54, 9873-9881.

Di Cagno, R., Gobbetti, M. (2014). Ecological parameters influencing microbial diversity and stability of traditional sourdough. *International Journal of Food Microbiology*, 3(171), 136-146.

Fava, P., Limbo, S., Piergiovanni, L. (2000). La previsione della shelf life di prodotti alimentari sensibili agli scambi di umidità. *Industrie Alimentari*, 39, 121-128.

Fik, M., Surówka, K., Maciejaszek, I., Macura, M. Michalczyk, M. (2012). Quality and shelf life of calcium-enriched wholemeal bread stored in a modified atmosphere. *Journal of Cereal Science*, 56(2), 418-424.

Gänzel, M. G. (2009). From gene to function: metabolic traits of starter cultures for improved quality of cereal foods. *International Journal of Food Microbiology*, 134, 29-36.

Gänzle M. G. (2014). Enzymatic and bacterial conversions during sourdough fermentation, *[J] Food Microbiology*, 37, 2-10.

Gherghina E., Israel-Roming F., Balan D., Luta G., Simion V., Zachia M. (2015). Assessment of some nutrients in bakery products. *Scientific Bulletin. Series F. Biotechnologies*, Vol. XIX, 140-144.

Gobbetti, M., Smacchi, E., & Corsetti, A. (1996). The proteolytic system of Lactobacillus sanfrancisco CB1: Purification and characterization of a proteinase, a dipeptidase, and an aminopeptidase. *Applied and Environmental Microbiology*, 62(9), 3220-3226.

Gocmen, D., Gurbuz, O., Kumral, A. Y., Dagdelen, A. F., & Sahin, I. (2007). The effects of wheat sourdough on glutenin patterns, dough rheology and bread properties. *European Food Research and Technology*, 225, 821-830.

Halouat, A. E., & Debevere, J. M. (1997). Effect of water activity, modified atmosphere packaging and storage temperature on spore germination of moulds isolated from prunes. *International Journal Food Microbiology*, 35(1), 41-48.

Hanssen, O. J. (1998). Environmental impacts of product systems in a life cycle perspective: A survey of five product types based on life cycle assessments studies. *Journal of Cleaner Production*, 6(3-4), 299-311.

Hansen, A., & Schieberle, P. (2005). Generation of aroma compounds during sourdough fermentation: Applied and fundamental aspects. *Trends in Food Science & Technology*, 16, 85-94.

Kaditzky, S., & Vogel, R. F. (2008). Optimization of exopolysaccharide yield in sourdoughs fermented by lactobacilli. European *Food Research and Technology*, 228, 291-299.

Kaditzky, S., Seitter, M., Hertel, C., & Vogel, R. F. (2008). Performance of *Lactobacillus sanfranciscensis* TMW 1.392 and its levansucrase deletion mutant in wheat dough and comparison of their impact on bread quality. *European Food Research and Technology*, 227, 433-442.

Karim, A., Norziah, M., Seow C. (2000). Methods for the study of starch retrogradation. *Food Chemistry*, 71, 9-36.

Katina, K., Heinio, R. L., Autio, K., & Poutanen, K. (2006). Optimization of sourdough process for improved sensory profile and texture of wheat bread. *LWT - Food Science and Technology*, 39, 1189-1202.

Katina, K., Maina, N. H., Juvonen, R., Flander, L., Johansson, L., Virkki, L., et al. (2009). In situ production and analysis of *Weissella confusa* dextran in wheat sourdough. *Food Microbiology*, 26, 734-743.

Lacaze, G., Wick, M., & Cappelle, S. (2007). Emerging fermentation technologies: development of novel sourdoughs. *Food Microbiology*, 24, 155-160.

Lanza, C. M., Tomaselli, F., Muratore, G., Pulvirenti A. (2000). Study on shelf-life of a pastry for ice-cream. *Industrie Alimentari*, 39(392), 565-571.

Latou, E., Mexis, S., Badeka, A., Kontominas M. (2010). Shelf life extension of sliced wheat bread using either an ethanol emitter or an ethanol emitter combined with an oxygen adsorber as alternatives to chemical preservatives. *Journal of Cereal Science*, 52, 457-465.

Licciardello, F., Rizzo, V., Grillo, O., Venora, G., Muratore, G. (2013). Bread-making performances of durum wheat semolina, as affected by ageing. *Journal of Cereal Science*, 57(3), 372-376.

Martinez-Anaya, M. A. (2003). Association and interaction of microorganisms in dough fermentations: effects on dough and bread characteristics. *Handbook of dough fermentations*, New York: Marcel Dekker, 63-95. Minervini, F., De Angelis, M.

Pagani, M., Lucisano, M., Mariotti, M., Limbo S. (2006). Influence of packaging material on bread characteristics during ageing. *Packaging Technology and Science*, 19(5), 295-302.

Palomba, S., Cavella, S., Torrieri, E., Piccolo, A., Mazzei, P., Blaiotta, G., et al. (2012). Wheat sourdough from Leuconostoc lactis and Lactobacillus curvatus exopolysaccharide-producing starter culture: polyphasic screening, homopolysaccharide composition and viscoelastic behavior. *Applied and Environmental Microbiology*, 78(8), 2737-2747.

Parker, R., & Ring, S. G. (2001). Macromolecular aspect of bread staling. *Series in contemporary food science Bread staling,* New York: CRC Press LLC. 61-71.

Piergiovanni, L., Fava, P. (1997). Minimizing the residual oxygen in modified atmosphere packaging of bakery products. *Food Additives and Contaminants*, 14(6-7), 765-773.

Pepe, O., Ventorino, V., Cavella, M., Fagnano, M., & Brugno, R. (2013). Prebiotic content of bread prepared with flour from immature wheat grain and selected dextran-producing lactic acid bacteria. *Applied and Environmental Microbiology*, 79, 3779-3785.

Piazza, L., & Masi, P. (1995). Moisture redistribution throughout the bread loaf during staling and its effect on mechanical properties. *Cereal Chemistry*, 72(3), 320-325.

Plessas, S., Alexopoulos, A., Bekatorou, A., Mantzourani, I., Koutinas, A. A., & Bezirtzoglou, E. (2011). Examination of freshness degradation of sourdough bread made with kefir through monitoring the aroma volatile composition during storage. *Food Chemistry*, 124, 627-633.

Poutanen, K., Flander, L., & Katina, K. (2009). Sourdough and cereal fermentation in a nutritional perspective. *Food Microbiology*, 26, 693-699.

Robertson, G. L. (1993). Food packaging - Principles and practice. New York, USA. Marcel Dekker, Inc.

Rodríguez, M., Medina, L. M., Jordano, R. (2000). Effect of modified atmosphere packaging on the shelf life of sliced wheat flour bread. *Nahrung*, 44(4), 247-252.

Sanguinetti, A. M., Del Caro, A., Scanu, A., Fadda, C., Milella, G., Catzeddu, P., Piga, A. (2016). Extending the shelf life of gluten-free fresh filled pasta by modified atmosphere packaging. *LWT - Food Sci. Technol*., 71, 96-101.

Schiraldi, A., & Fessas, D. (2001). Mechanism of staling: an overview. In P. Chinachoti, & Y. Vodovotz (Eds.), *Series in contemporary food science Bread staling.* CRC Press LLC.

Sidhu, J. S., Al-Saqer, J., Al-Zenki, S. (1997). Comparison of methods for the assessment of the extent of staling in bread. *Food Chemistry*, 1-2, 161-167.

Smith, J. P., Ooraikul, B., Koersen, W. J., Jackson, E. D., Lawrence, R. A. (1986). Novel approach to oxygen control in modified atmosphere packaging of bakery products. *Food Microbiology*, 3(4), 315-320.

Stamatis, N., Arkoudelos, J. (2007). Quality assessment of Scomber colias japonicus under modified atmosphere

and vacuum packaging. *Food Control*, 18 (4), 292-300.

Suppakul, P., Thanathammathorn, T., Samerasut, O., Khankaew S. (2016). Shelf life extension of "fios de ovos", an intermediate-moisture egg-based dessert, by active and modified atmosphere packaging. *Food Control*, 70, 58-63.

Tamba-Berehoiu, R., Popa, C., Vișan, L., Popescu, S. (2014). Correlations between the quality parameters and the technological parameters of bread processing, important for product marketing. *Romanian Biotechnological Letters*, 19(3).

Taniwaki, M. H., Hocking, A. D., Pitt, J. I., Fleet, G. H. (2001). Growth of fungi and mycotoxin production on cheese under modified atmospheres. *International Journal Food Microbiology*, 68(1-2), 125-133.

Tieking, M., Korakli, M., Ehrmann, M. A., Gänzle, M. G., & Vogel, R. F. (2003). *In situ* production of exopolysaccharides during sourdough fermentation by cereal and intestinal isolates of lactic acid bacteria. *Applied and Environmental Microbiology*, 69, 945-952.

Tieking, M., & Gänzle, M. (2005). Exopolysaccharides from cereal - associated lactobacilli. *Trends in Food Science and Technology*, 79-84.

Wikström, F., & Wilsson, H. (2010). Potential environmental gains from reducing food losses through development of new packaging - A Life-Cycle model. *Packaging Technology and Science*, 23, 403-411.

Zamfir M., Cornea C. P., De Vuyst L., Grosu T. S. S. (2014). Biodiversity and biotechnological potential of lactic acid bacteria. *AgroLife Scientific Journal*, Volume 3, Number 1, ISSN 2285-5718, 169-176.

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UP-TO-DATE KNOWLEDGE ON YEASTS FOR FOOD INDUSTRY

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Abstract

Yeasts have been used for food processing since ancient times due to their special fermentative characteristics. In present, they represent a major group of microorganisms used in food industry as well in related domains of biotechnology. Yeasts are a rich source of proteins, vitamins (especially those from B complex) and are main producers of biocatalysts (lipases, esterases, phospholipases) used to improve aromas and flavours and of different natural additives such as food colorants. Yeasts isolated from traditional dairy products produce inulinases and β- galactosidases important for production of foods intended for weight loss or for people with lactose intolerance. Inulinases exhibit also prebiotic potential, catalysing the growth of probiotic microorganisms. Certain yeast species are used as probiotics since they not participate in horizontal antibiotic resistance genes exchanges. Recently, the yeasts with antioxidant activity are considered as attractive for development of biopreservatives, an economic alternative for classical food preservatives of chemical origin. With a great history developed in the food field, the yeasts continue to surprise the scientific world even in the 21st century through their special metabolic abilities.

Key words*: yeasts, natural food additives, prebiotics/ probiotics, biocatalysts, biopreservatives.*

INTRODUCTION

Yeasts have a rich history in food industry but their potential exceeds their use in baking, beer and wine production. Although they play an important role in obtaining fermented foods and beverages, during past decades, yeasts proved to have huge potential in functional foods development. Functional food is a generic term referring to different products which, in addition to the nutritional value of the ingredients from which they are prepared, offers also great support for maintaining or improving human health. Yeasts are an interesting research model, some of the species are considered GRAS (Generally Regarded as Safe), they do not participate in horizontal antibiotic genes exchanges and they have special metabolic properties that make them suitable for a variety of industrial processes.

Food industry is a constantly changing field. Developing products with improved quality or properties is a hard challenge. Due to their metabolic versatility, yeasts represent a potential solution for many of these challenges. For example, various yeast species have been used as probiotics, while different yeasts cell components proved to be useful for nutraceuticals development. Also, yeasts are able to secret different bioactive metabolites, such as antioxidants, vitamins or enzymes that are both ecological and highly valuable from nutritional point of view (Rai et al., 2018). Also, yeasts showed great potential for producing new flavours and aroma food ingredients. Using yeasts as bioflavouring agents represent a step forward meant to replace the synthetic compounds used in present (Willaert et al., 2005).

The aim of this review is to highlight some important discoveries regarding the biotechnological potential of yeasts in food industry.

YEASTS AS NATURAL ANTIOXIDANTS PRODUCERS

Recently, the interest for developing natural antioxidants has increased significantly since their synthetic alternatives proved to be sometimes harmful for human health. Synthetic antioxidants that are still frequently used as food additives, such as butylated hydroxyanisole (BHA), butylated hydroxytoluen (BHT) and tertbutylhydroquinone (TBHO), have been an intensely debated topic, since their use in high

concentrations can cause cancer in some animal species (Shahidi & Zhong, 2010). In food industry, antioxidants are used as food preservatives that protect fat-based foods (meat, dairy products) against oxidative rancidity (Shahidi, 2015). Oxidation may occur during different stages of food processing and determine the development of different off-flavours, loss of essential nutrient such as fatty acids and fat-soluble vitamins, and last, but not least, the appearance of toxic compounds (Shahidi &. Zhong, 2005). Based on their mode of action antioxidants are grouped into 2 main classes: (1) primary antioxidants or chain-breaking antioxidants, that donate protons or electrons to a free radical thus transforming them to their more stable form and (2) secondary antioxidants or hydroperoxide decomposers, that convert hydroperoxides into nonradical/nonreactive products (Hermund, 2018).

Many natural antioxidants have already been identified and introduced into the food industry among which: tocopherols, ascorbic and erythrobic acids and their salts, different plants extracts and short chain peptides. Natural antioxidants of microbial origin are of great economic importance since the microorganisms require rather simple and economic growth substrates and are able to produce large quantities of products in a short period of time. Yeasts are already known as a great source of antioxidants, being able to synthesize citric acid, carotenoids (torulene and torularhodine), glutathione, ubiquinone, riboflavin and hydroxymethyl/hydroxyethyl furanone (Abass, 2006).

Glutathione (GSH) is a non-proteic thiol compound with great potential both in biomedical field (being used for pancreatic inflammations and liver cirrhosis treatment) and food industry. Glutathione has great importance in oenology being involved in the control of oxidative spoilage of wine. White wine, in particular, is very sensitive to oxygen exposure which determines the development of atypical aging aroma and colour changing (Kritzinger et al., 2012; De Vero et al., 2017). In present, glutathione is produced via two main methods. The first method is based on using glutathione-generating enzymes and the amino acids precursors in a highly controlled environment with ATP consumption (Forman et al., 2009). This method is expensive since using ATP increases the production cost. The second method is a direct fermentative method based on using different microorganisms with natural ability to accumulate glutathione in their cells (Tahmasebi et al., 2016). *Saccharomyces cerevisiae* and *Candida utilis* are recognized as having high ability to produce large amounts of glutathione in low-cost growth media with sugar cane molasses and glycerol as carbon sources (Anschau et al., 2013; Rollini et al., 2010). The biosynthesis of glutathione in yeasts dependents on sulphate and nitrogen uptake. Sulphate intake is mediated through specific membrane permeases (Sul1p and Sul2p). After internalisation, the sulphate enters the metabolic pathway for homocysteine synthesis followed by interconversion of homocysteine to cysteine. Cytoplasmic biosynthesis of glutathione is catalysed by γ-glutamylcysteine synthetase that forms γ-glutamylcysteine as intermediate and GSH synthetase which adds glycine- the third amino-acid from glutathione chemical structure (Suzuki et al., 2011).

Apart from cytoplasmic biosynthesis of glutathione, yeasts are able to assimilate this compound by direct internalisation via Opt1p/Hgt1p transporter. Different studies showed that nitrogen deprivation decreases the amount of intracellular glutathione because it induces the gene expression of an enzyme (γ-glutamyltranspeptidase) that hydrolyses glutathione into L-glutamate and cysteinylglycine (De Vero et al., 2017). Although many yeasts were characterised as being able to produce large amounts of glutathione, winemakers are using different strategies in order to enhance glutathione production by yeasts. Random mutagenesis is one of the main methods used and is based on exposing yeast cells to chemical (ethyl methanesulphonate - EMS, nitrous acid, intercalating agents) or physical mutagens (UV radiation, γ-raddiation) (Li et al., 2004). Another technique frequently used is sexual hybridization, the most efficient way to increase yeast diversity and to improve industrially relevant traits such as flavour profile, stress tolerance and fermentative performance

(Steensels et al., 2014). The most advanced method is evolutionary engineering, in which the yeast strains are exposed to different mutagens, then exposed to specific selective pressure followed by the final selection of best glutathione producing strains (Perez-Torrado et al., 2015).

Ubiquinone (coenzyme Q) is a redox active lipid involved in the electron transfer system. This compound is synthesized *via* a complex metabolic pathway that uses chorismate and polyprenyl diphosphate as precursors. Ubiquinone is highly studied due to its high biomedical potential. This compound proved to be of particular importance in the prevention of cardiovascular disease, neurodegenerative and mitochondrial conditions, diabetes and periodontal disease. Since 1999 when functional foods term was briefly defined in European Union as food products fortified with different active compounds that can improve health and well-being, ubiquinone has gained notoriety among researchers. Ubiquinone can be produced by chemical synthesis, obtained from plants/ animals by tissue extraction or by microbial fermentation. Since chemical synthesis involves using solvents or chemicals during the process, microbial fermentation becomes more attractive both from economic and ecological point of view (Berekova et al., 2008). Different yeast species belonging to *Candida*, *Sporidiobolus* and *Rhodotorula* genera proved to be highly valuable for ubiquinone production. Moreover, this alternative implies low production costs which rends it very attractive for industrial production (Tokdar et al., 2014; Dixson et al., 2011).

Citric acid is the most common organic acid commercialised in large quantities around the world since 1930. In 2007 global production of citric acid was estimated around 1.7 million tons with an annual increase of almost 4% (Tong et al., 2019). This compound is widely used in food industry as an acidifier/antioxidant to preserve and to improve the flavour of different foods and beverages (fruit juices, ice cream, marmelades etc.). Also, it is frequently used for detergents (as phosphate substitute), pharmaceuticals (for vitamins preservation, as pH corrector, blood preservative) and cosmetic products (Soccol et al., 2006). More than 90% of the required citric acid is obtained from microbial surfaces or submerged cultures. Many microorganisms are already known as being able to produce citric acid among which yeasts occupy a special position. *Candida tropicalis*, *Candida oleophila*, *Pichia guilliermondii*, *Candida citroformans*, *Pichia anomala* and *Yarrowia lipolytica* are known as being able to synthetize citric acid when cultivated under special conditions. Since the citric acid is part of energy metabolism, some of the microbial strains are not suitable for industrial purposes. Therefore, there is a growing interest for improvement of citric acid producing strains by mutagenesis of characterised strains or by isolation and selection of new yeasts with natural producing potential (Ridrigues et al., 2006). Many studies reported high citric acid production using as substrate different raw materials such as: starch, molasses, coffee husk, wheat bran, pineapple waste, citrus waste etc. (Max et al., 2010).

Carotenoids are a group of compounds ubiquitous in nature with yellow, orange or red colour. In general, carotenoids have a polyene backbone form by conjugated C=C bonds that is involved in pigmentation and also in their antioxidant activity assuring the interaction of these compounds with free radicals (Young and Lowe, 2018). Carotenoids are a great source of vitamin A and are recognized as being able to reinforce immune system. Also, these compounds proved to be very efficient for the treatment of eye diseases such as cataract and macular degeneration and for skin protection against ultraviolet radiation (Stahl & Sies, 2007). Since the human body cannot produce carotenoids, it is recommended their use as food additives. Apart from lutein, astaxanthin, zeaxanthin which are already used at industrial scale as natural antioxidants, more natural carotenoids were described lately. Among them, torulene and torularhodin have gained a growing attention. These two compounds are a group of carotenoids mainly produced by yeasts including *Rhodotorula*, *Rhodosporidium* and *Sporobolomyces* species and by filamentous fungi. Torulene and torularhodine present a β-ionone ring connected to a polyene chain (Herz et al., 2007) and their colour varies directly in proportion to their concentration from pale pink to red. The carotenoids act mainly in microbial

cell protection against the reactive form of oxygen and radiation. Different studies proved that torularhodin is even more efficient than β-carotene and α-tocopherol in terms of antioxidant activity (Sakaki et al., 2002; Sakaki et al., 2001). As a consequence, they have huge biotechnological potential, a special attention being granted to the genetic background involved. Therefore, a number of genes were characterised as being involved in carotenoid synthesis in yeasts, coding different enzymes such as: phytoene synthase, phytoene dehydrogenase, lycopene cyclase, pytoene desaturase etc. Many of these genes were overexpressed in order to enhance carotenoids production in yeast cells (Wang et al., 2008). Torulene and torularhodine biosynthesis is strongly influenced by cultivation conditions, the carbon and nitrogen source being extremely important. Some of the most frequently used substrates for enhancing carotenoids production in yeasts are the grape must as carbon source (Buzzini and Martini, 2007) and ammonium sulphate as nitrogen source (El Banna et al., 2012) in adequate aeration conditions (Simova et al., 2003).

Hydroxymethyl/hydroxyethyl furanones are chemical compounds that present a five-membered heteroaromatic ring containing an oxygen atom. The members of these group are highly valuable for the biomedical field as it has been shown that they can be used as analgesics, antimicrobials, anti-inflammatories, etc. (Husain et al., 2019). Probably the best known furanones is vitamin C (5-(1,2-dihydroxethyl)-3,4-dihydroxy-2(5*H)*-furanone) but there are also other naturally occurring furanones with great biotechnological potential, such as EMHF (4-hydroxy-2 (or 5)-ethyl-5 (or 2)-methyl-3(*2H*)-furanone), a furanone derivate produced by different yeast species. This compound was first identified in soy sauce and miso and it was proven to be responsible for the characteristic flavour. First, the Maillard reactions were thought to be responsible for the particular flavour, but lately it was shown that EMHF occurrence in soy sauce was due to fermentative action of different yeasts such as: *Zygosaccharomyces rouxii*, *S. cerevisiae* and *Y. lipolytica* (Slaughter, 1999). The EMHF was also found in roasted coffee, melons and beer (Uehara et al., 2015). The 3(*2H*)furanones exhibit both anti-oxidative and pro-oxidative activity depending on the availability of oxygen species from the environment (Schwab et al., 2013).

Riboflavin, also known as vitamin B2, is a water-soluble vitamin with great importance for human health. Riboflavin is a precursor of flavin mononucleotide (FMN) and flavin dinucleotide (FAD) coenzymes that act as electron acceptors for various oxidoreductases. Humans are not able to synthetize riboflavin being forced obtain it from their diet. Nevertheless, riboflavin occurs naturally in liver or egg yolk but it is added in many other types of foods such as breakfast cereals or bread. Also, riboflavin is used as food colorant due to its yellowish colour. Chemical production of riboflavin proved to be very expensive since lot of waste is produced and also it requires many organic solvents. Microbial fermentation is much cheaper and has better yield (Kato & Park, 2012). Many yeasts including *Candida famata, P. guilliermondii*, *Candida membraniefaciens* subsp. *flavinogenie*, *Debaryomyces hansenii, Schwanniomyces occidentalis* are already used for industrial production of riboflavin through microbial fermentation (Wang et al., 2008). It seems that overproduction of riboflavin in yeasts is a metabolic response to iron deficiency and is induced by the presence of cobalt ions (Boretsky et al., 2007).

Apart from riboflavin, yeasts are known as an important source of other vitamins such as thiamine, nicotinic acid, pyridoxine, pantothenic acid, cyancobalamin, biotin and folic acid. *Kloeckera apiculata*, *S. cerevisiae* and *Saccharomyces uvarum* accumulate or release during ethanol fermentation very large quantities of thiamine (Abbas, 2005). Ergosterol, a precursor of vitamin D, is an important constituent of cell membrane lipids, many studies reporting *Candida tropicalis* as an important ergosterol producing yeast species (Liu et al., 2019; Abbas, 2005). Also, *Candida guilliermondii*, *C. utilis* and *Saccharomyces fragilis* grown on media containing lard or waste fats as carbon sources and ammonium salts or uree as nitrogen sources, produced high amount of nicotinic acid, pantothenic acid, riboflavin, pyridoxine and cobalamine (Abbas, 2005).

AROMA FORMATION IN YEASTS

The importance of yeasts in obtaining fermented foods (mainly alcoholic and non-alcoholic beverages such as wine, spirits beverages, bear) is also due to the fact that they produce many flavours and aromas. Among them, most known compounds synthetized by yeasts are fusel alcohols, fatty acids and their derived esters.

Fusel alcohols are a group of aroma compounds characteristic mainly to alcoholic beverages such as rums, brandies and whiskeys. Isoamyl alcohol and isoamyl acetate are the main fusel alcohols produced by yeasts (Abe & Horikoshi, 2005). *Torulaspora delbrueckii*, *Pichia fermentas* and *Kluyveromyces maxianus* are known as yeast able to produce satisfactory quantities of fusel alcohols or derived esters (Hernández-Carbajal et al., 2013).

Fatty acids and their esters represent another major group of aroma compounds synthetized by yeasts. Short chain volatile fatty acids (propionic acid, butyric and isobutyric acid, caproic acid, capric acid, caprylic acid, etc.) are probably the best known fatty acids highly valuable for food industry. The presence of the fatty acids with 6 to 10 carbon atoms in alcoholic beverages gives the final product musty and rancid specific aromas. Many yeast species are characterised as aroma producers. *S. cerevisiae* is used for ethyl caproate synthesis in culture conditions characterised by small concentration of inositol. This compound is important for Scotch whisky production conferring its specific aroma (Chen et al., 2014).

Carbonyl, sulphur and phenolic compounds. The carbonyl compounds of great interest for food industry are the aldehydes, such as diacetyl and 2,3-pentanedione that offers beer a specific buttery flavour. These compounds have an undesirable effect on beverages quality since its specific aroma is not well tolerated by individuals (Zhang et al., 2005). Sulphur compounds (hydrogen sulphide, diethyl sulphide), derived mainly from sulphur containing amino-acids (cysteine and methionine), have also undesirable effect on beverage due to their offensive smell. Despite that, these compounds acts as antioxidants preventing thus oxidation that can definitely affect the final product. In this group, the phenolic compounds occupy a central position. Compounds such as 4-ethylphenol,4-ethylguaiacol, 4-methylguaiacol, in small concentration are desirable. Commonly known yeast involved in aroma development based on carbonyl, sulphur and phenolic compounds are *S. cerevisiae*, *Dekkera bruxellensis* and *Dekkera anomala* (Dzialo et al., 2017).

The **lactones** are also an important group among the aromas produced by yeasts. They confer specific aroma of peach, apricot or coconut to various types of foods. Chemically, lactones have a carbon ring with an oxygen atom and are produced by many yeasts especially *Sporobolomyces odorus, Y. lipolytica, Sporidiobolus ruinenii* (Abbas, 2005).

THE YEASTS - SOURCE OF ENZYMES

Yeasts are able to synthesize various enzymes such as lipases, esterases, amylases and glycosidases more stable than plant or animal enzymes, which act as biocatalysists improving food flavour, appearance or processing. For example, yeast proteases, lipases, β-glucosidase and invertase have high impact on the organoleptic characteristics of the bread and, also, influence the dough structure, the crust colour, crumb texture and firmness of the bread. The ***α-amylases*** (1,4-α-D-glucan-glucanohydrolases, E.C. 3.2.1.1) are used in the backing industry not only as flavour enhancers, but also for starch conversion in dextrins, maltose and glucose, representing the carbon substrates for yeast metabolism. The main yeast species producing α-amylases belong to *Schwanniomyces* - *S.* (*Debaryomyces*) *occidentalis*, *S. aluvius*, *Cryptococcus* (*C. flavus*), *Saccharomycopsis* (*S. fibuligera*) and *Candida* - *C. utilis*, *C.* (*Pichia*) *guilliermondii*, *C. famata* (*Debaryomyces hansenii*), *C. antarctica*. The characteristics of yeast α-amylases depend on the yeast species, presenting variable molecular weight (38-75 kDa) and optimal activity at temperatures from 30 to 70°C and pH values from 4 to 6 (Djekrif et al., 2016).

Glycosidases have important role in wine industry, hydrolyzing the sugar-conjugated precursors existent in the grapes, releasing thus the terpens (aglycons) responsible for the

flavour and odor of the wine. Yeast species belonging to *Candida*, *Kluyveromyces*, *Debaryomyces*, *Hanseniaspora*, *Hansenula (Wickerhamomyces)*, *Pichia*, *Metschnikowia*, *Rhodotorula* and *Trichosporon* genera synthesize β-glucosidases (EC 3.3.1.21). These enzymes cleave the non-reducing terminal β-D-glucosyl residues from cellulose and remove the β-D-glucose. For some yeast species, the genes coding the β-glucosidases are regulated by the substrate or the growth conditions. For example, the gene *WaExg2* from *Wickerhamomyces anomalus* is active at low pH (3.5-4.0), high sugar (20% w/v) and ethanol (10-15% v/v) concentrations and presence of sulphites or cations, and inhibited by glucose. In *D. hansenii* the highest activity was observed under aerobic conditions, at pH 4.0-5.0, during the exponential growth phase, the enzyme production being inhibited by high glucose concentrations (Maicas and Mateo, 2015; Claus & Mojsov, 2018). Some species from *Candida*, *Kluyveromyces*, *Debaryomyces* and *Pichia* genera synthesize extracellular glucose-tolerant β-glucosidases, but only a third of them showed high glucose tolerance (Rosi et al., 1994). Yeasts β-glucosidases are also of great interest in table olive processing contributing to oleuropein hydrolysis which allows removing the natural bitterness without using large amounts of water (Anagnostopoulos et al., 2017). On the other hand, *Candida*, *Hanseniaspora* and *Pichia* cells have been used as hosts for cloning β-glucosidase genes from *Aspergillus oryzae* for obtaining terpenols from monoterpenyl glycosides from wort and must (Verstrepen et al., 2006).

Xilanases are a group of enzymes extensively used in food industry due to their ability of cleaving the xylan, a major component of hemicellulose. In backing, the xylanase break down the hemicellulose from the wheat flour and increase the binding of water in the dough, improving thus the bread quality and volume. In beverages, the xylanases hydrolyze the cell wall of barley in beer production and also assure a better quality improving the organoleptic properties of the juices (Raveendran et al., 2018). Yeast β-D-xylosidase (EC 3.2.1.37) is important in wine making. Few yeast species, such as *Pichia anomala*, present extracellular, cell-wall-bound and intracellular β-D-xylosidase acivity, while other yeast species present only one form of activity, *i.e*. *Hanseniaspora uvarum* - cell-wall-bound. The thermostability, pH variations and resistance to stress conditions (glucose and ethanol concentrations) of yeast β-D-xylosidases, represent an important asset for their application in enhancing wine aroma and flavour (Romano et al., 2006; Burlacu et al., 2016).

Lipases (triacylglycerol acylhydrolyses) catalyze the hydrolysis of long-chain triacylglycerides and have wide applications in food industry for processing of meat and dairy products or in baked foods. For example, *Y. lipolytica* synthesize intracellular and extracellular lipases, assuring approximately 60% of the flavours associated with ripening in meat products based on pork fat. The enzymes are able to reduce mainly the content of free fatty acids after a short period of incubation, lowering thus the probability of *rancid* odor and consistency in the products (Romano et al., 2006). The expression of several genes coding for lipases in *Y. lipolytica* seems to be substrate-driven and is induced by the presence of the oleic acid (*LIP2*) or glucose (*LIP11* and *LIP13*). However, lipases were synthesized also in the presence of olive oil, oleic acid, sunflower oil, tributyrin and Tween 80 (Csutak & Sarbu, 2018).

Candida rugosa lipases (CRL) have GRAS status and are used in food industry. The coding genes and lipase structure are well studied, many of the lipases being synthesized as isoforms with different thermal stabilities and substrate specificities. In order to increase the thermostability and activity and to improve the industrial processes, various technologies of enzyme immobilization in celite, nylon fiber, polymethyl methacrylate, glass beads, silicage a.s.o, were developed: covalent methods, the use of photo-cross-linkable resins or adsorption. Free or immobilized *C. rugosa* lipases are successfully used in production of ice-cream, of fermented foods used for storage and as food supplies, or for obtaining specific fragrances in dairy products (Benjamin & Pandey, 1998).

Candida (*Pseudozyma*) *antarctica* synthesize cold-active lipases A and B (CALA and CALB) with optimal activity at 20ºC. The lipase CALB

is used in Novozym®435 (used for the production of human milk fat substitute) where it promotes the replacement of the palmitic acid from tripalmitin with unsaturated free fatty acids allowing thus to obtain of triacylglycerols with similar structure from human milk (Szczęsna-Antczak et al., 2013; Guerrand, 2017).

Another yeast species, *Pichia pastoris* is recognized as QPS (Qualified Presumption of Safety) by the European Food Safety Authority and can be used for production of enzymes for food industry. *P. pastoris* cells are used as hosts for production of 34.6% of total recombinant lipases mentioned in research studies, due to the presence of the powerful promotor *AOX1*/*MOX1* and to a reduced level of hyperglycosylation. These lipases are mainly used in the synthesis of short-chain flavour esters. Also, the CALB lipase from *C. antarctica* was expressed on the surface of *P. pastoris* cells, acting as a biocatalysis for sugar monoester production (Borrelli & Trono, 2015). Other *Pichia* lipases are also commercialized (*P. roqueforti* - Lipase R, Amano; Lipomod™, Biocatalysts; *P. camemberti* - Lipase G, Amano) and used in the dairy industry for hydrolysis of milk-fat triacylglycerols.

PROBIOTIC/PREBIOTIC YEASTS

Due to their presence in many fermented foods, the yeasts are an important part of human daily diet by providing vitamins of the B group. Therefore, the GRAS yeasts represent a growing interest for obtaining probiotics. In order to become a true candidate as a probiotic, a microorganism must present several attributes including: growth at low pH, a specific degree of cell surface hydrophobicity and the ability to tolerate bile (Rai et al., 2019). Yeasts are generally resistant to antibiotics and proved to be able to resist in low pH environments (for the passage through the gastrointestinal tract). Also, there are no reports regarding yeast implication in the transfer of antibiotic resistance genes. Therefore, some yeast species can be used successfully for development of new probiotics (Rima et al., 2012; Fakruddin et al., 2017). *S. cerevisiae* and *Saccharomyces boulardii* are able to produce biotherapeutic agents very efficient in treatment of different types diarrhea, such as antibiotic associated diarrhea (Duman et al., 2005), *Clostridium difficile* associated diarrhea (McFarland et al., 2006) or traveler`s diarrhea (McFarland, 2007). It seems that these species are able to produce polyamines that increase short fatty acids and disaccharide enzymes activity in order to stimulate the well function of intestinal cells (Ali et al., 2012). Also, some probiotic yeast exhibit hypocholesterolemic activity (Saikia et al., 2017) and present the ability to reduce oxidative stress (Romanin et al., 2015) being thus an emerging tool for improvement of human health. Many yeast species are able to produce lipases that hydrolase tributyrin to glycerol and butyric acid, an important phenomenon since the butyric acid is useful for colonocytes health (Glueck et al., 2018). The butyric acid acts as a fuel source for the colonocytes, regulates the water and electrolyte absorption and provide protection against mucosal inflammation and oxidative stress (Canani et al., 2012). Also, some studies reported butyric acid as a valuable anticarcinogen drug being able to limit the evolution of several types of cancers (Kuefer et al., 2004).

Although the mechanism is not fully understood, *Saccharomyces* probiotic species inhibit growth of pathogens belonging to the *Enterobacteriaceae* (*Escherichia coli, Salmonella typhi, Shigella dysenteriae, Salmonella enteritidis* and *C. difficile*) family and increase the population of *Bifidobacteria,* a beneficial microorganism from the gut microbiota (Ali et al., 2012). Also, *S. boulardii* is able to inactivate the bacterial toxins, to stimulate host immune defenses and to enhance nutrient absorption (Fakruddin et al., 2017). Therefore, yeasts can also be used for prebiotics synthesis (Nascimento et al., 2012).

The *Kluyveromyces* yeasts (*K. lactis*, *K. marxianus, K. fragilis*) are seldom isolated from dairy products and are able to assimilate lactose and to degrade inulin. The synthesis of β-galactosidase (lactase) is an important step in the intracellular metabolism of lactose to glucose and galactose which is subsequently degraded through the Leloir pathway (Csutak, 2014). The yeast β-galactosidase are used in the dairy industry, to improve the sweetness of products

and to degrade the whey, major waste from the dairy industry.

The *K. lactis* enzyme shows optimal activity as high pH values (6.0-7.0) and 30-35ºC, making it suitable for milk and sweet whey hydrolysis (Saquib et al., 2017). In present, the *K. lactis* β-galactosidase is produced and commercialized at industrial scale as Maxilact (DSM Food Specialties, The Netherlands) and is used for the production of lactose-free dairy products for the benefit of lactose intolerant individuals. The stability of the enzyme produced by *K. lactis* can be increased by immobilization on polyacrylamide beads, using freeze-dried liposomes (Rubio-Texeira, 2005), or by entrapement in cellulose triacetate fibers – the product commercialized by Centrale del Latte of Milan, Italy (Xavier et al., 2017). On the other hand, the enzyme from *K. fragilis* has optimal activity at pH at 4.8 and 50ºC, which recommends it for treatment of acid whey (Raveendran et al., 2018).

An important process that appear during lactose hydrolysis is transgalactosylation wich is associated with β-galactosidase and formation of galactooligosachharides (GOS), important prebiotic compounds. The *K. lactis* enzyme is sucessfully used in this process using skimmed milk permeate fortified with lactose (Xavier et al., 2017), while the company Amano Enzyme, Inc. (Amano, Japan) obtained the GRAS notification for an enzyme derived from *Papiliotrema* (*Cryptococcus*) *terrestris* for use as a processing aid in the production of GOS (Keller & Heckman, 2017).

According to the RDC Resolution 205/2006, besides the β-galactosidases synthesized by *Kluyveromyces* sp., in the dairy industry are also accepted the enzymes produced by *Saccharomyces* sp. or *Candida tropicalis*. Thus, Morioka et al. (2019) obtained a β-galactosidase from permeabilized *S. fragilis* IZ 275 cells, at 44ºC and pH 7.0.

The *Kluyveromyces* yeasts are also able to produce inulinases (β-2,1-D-fructan fructanohydrolase; EC 3.2.1.7), enzymes that degrade the inulin, a polysaccharide with important role of energy storage in plants. The inulin is formed from β-(2,1)-linked glucose and fructose units with a polymerisation degree of 2 to 60. Fructose is considered as GRAS and is extensively used in food industry as glucose replacement and also for improving food flavour and product stability. The inulin is not naturaly decomposed in the human body in the gastrointestinal tract where it acts as a dietary fiber for controling the body weight and as a probiotic compound assuring an enhanced absorbtion of calcium and magnesium. *K. marxianus* produces extracellular and cell wall-bound inulinases at pH = 4.5 and 52-55ºC, allowing the production of fructooligosaccharides (FOS) that act as a growth factor for many *Bifidobacteria* (Jain et al., 2012; Csutak, 2014) and as noncarnicogenic sweeteners with low caloric value for use by diabetic patients. Singh et al., 2007 described an thermostable exoinulinase (β-D-fructan fructohydrolase, EC 3.2.1.80) with high pH stability and significant kinetic properties that hydrolized the raw inulin from *Asparagus racemosus* producing a high-fructose syrup. Also, Stuyf et al. (2018) presented the strain *K. marxianus* CBS6014 able to synthesize inulinase that can be used for production of whole meal breads with low content of Fermentable Oligo-, Di-, Monosaccharides And Polyols (FODMAPs), molecules that are poorly absobted in the small intestine representing thus the main cause of irritable bowel syndrome. An inulinase was also isolated from the marine strain of *Cryptococcus aureus* G7a able to produce high ammount of mono- and oligisaccharides (Sheng et al., 2007).

CONCLUSIONS

Based on their rich history as fermentative microorganisms, the yeasts remain one of the main players in food industry as well in numerous biotechnology domains. Yeasts have shown great potential for processing and improving the quality of fermented foods. Although, in present, many studies are based on using modern technologies to improve the biotechnological potential of yeasts, the interest for exploring the microbiota of traditional fermented foods gains more interest. These type of products continue to surprise the scientific world as they represent a valuable source of numerous new yeasts strains with exceptional metabolic qualities.

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REFERENCES

Abbas, C. A. (2006). Production of antioxidants, aromas, colours, flavours, and vitamins by yeasts. *In Yeasts in food and beverages* (pp. 285-334). Springer, Berlin.

Abe, F. U., Horikoshi, K. (2005). Enhanced production of isoamyl alcohol and isoamyl acetate by ubiquitination-deficient *Saccharomyces cerevisiae* mutants. *Cellular and Molecular Biology Letters*, 10(3), 383-388.

Ali M. A. E., Abdel-Fatah O. M., Janson J. C., Elshafei A. M. (2012). Antimicrobial potential of *Saccharomyces boulardii* extracts and fractions. *Journal of Applied Sciences Research*, 8(8), 4537-4543.

Anagnostopoulos D., Bozoudi D., Tsaltas D. (2017). Chapter 6: Yeast ecology of fermented table olives: A tool for biotechnological applications. In: *Yeast - Industrial applications* (pp.135-152). Rijeka, Croatia: IntechOpen.

Anschau, A., Santos, L. O. D., Alegre, R. M. (2013). A cost effective fermentative production of glutathione by *Saccharomyces cerevisiae* with cane molasses and glycerol. *Brazilian Archives of Biology and Technology*, 56(5), 849-857.

Benjamin, S., Pandey, S. (1998). *Candida rugosa* lipases: molecular biology and versatility in biotechnology, *Yeast* 14, 1069-1087.

Borekova, M., Hojerova, J., Korpra, V., Bauerova, K. (2008). Nourishing and health benefits of coenzyme Q. *Czech Journal of Food Science,* 26(4), 229-241.

Boretsky, Y. R., Protchenko, O. V., Prokopiv, T. M., Mukalov, I O., Fedorovych, D. V., Sibirny, A. A. (2007). Mutations and environmental factors affecting regulation of riboflavin synthesis and iron assimilation also cause oxidative stress in the yeast *Pichia guilliermondii*. *Journal of Basic Microbiology*, 47(5), 371-377.

Borrelli, G. M., Trono, D. (2015). Recombinant lipases and phospholipases and their use as biocatalysts for industrial applications, *International Journal of Molecular Sciences*, 16, 20774-20840.

Burlacu A., Cornea C. P., Israel-Romıng F. (2016). Mıcrobıal xylanase: a revıew. *Scientific Bulletin. Series F. Biotechnologies,* Vol. XX, 335-342.

Canani, R. B., Di Costanzo, M., Leone, L. (2012). The epigenetic effects of butyrate: potential therapeutic implications for clinical practice. *Clinical epigenetics*, 4(1), 4-14.

Chen, Y., Li, F., Guo, J., Liu, G., Guo, X., Xiao, D. (2014). Enhanced ethyl caproate production of Chinese liquor yeast by overexpressing EHT1 with deleted FAA1. *Journal of Industrial Microbiology & Biotechnology*, 41(3), 563-572.

Claus, H., Mojsov, K. (2018). Enzymes for wine fermentation: *Current and Perspective Applications, Fermentation*, 4. 52-71.

Csutak, O. (2014). *Genetics and biodiversity of yeasts with biotechnological applications*, Ed. Universităţii din Bucureşti.

Csutak, O., Sarbu, I. (2018). Chapter 6: Genetically modified microorganisms: Harmful or Helpful, In: *Genetically engineered foods. Handbook of Food Bioengineering.* (pp. 143-175) Volume 6, Eds: A.M. Holban, A.M. Grumezescu, Academic Press, Elsevier,.

De Vero, L., Bonciani, T., Verspohl, A., Mezzetti, F., & Giudici, P. (2017). High-glutathione producing yeasts obtained by genetic improvement strategies: A focus on adaptive evolution approaches for novel wine strains. *AIMS Microbiology*, 3(2), 155-175.

Dixson, D. D., Boddy, C. N., & Doyle, R. P. (2011). Reinvestigation of coenzyme Q10 isolation from *Sporidiobolus johnsonii*. *Chemistry & biodiversity,* 8(6), 1033-1051.

Djekrif, D. S., Gillmann, L., Bennamoun, L., Ait-Kaki, A., Labbani, K., Nouadri, T., Meraihi, Z. (2016). Amylolytic Yeasts: Producers of α-amylase and pullulanase. *International Journal Life-Sciences Scientific Research*, 2(4). 339-354.

Duman, D. G., Bor. S., Özütemiz, Ö., Sahin, T., Oguz, D., Istan, F., Soytürk, M. (2005). Efficacy and safety of *Saccharomyces boulardii* in prevention of antibiotic-associated diarrhoea due to *Helicobacter pylori* eradication. *European Journal of Gastroenterology & Hepatologyc*. 17(12) 1357-1361.

Dzialo, M. C., Park, R., Steensels, J., Lievens, B., & Verstrepen, K. J. (2017). Physiology, ecology and industrial applications of aroma formation in yeast. *FEMS Microbiology Reviews*, 41, 95-128.

El-Banna, A. A., El-Razek, A. A. M., El-Mahdy, A. R. (2012) Some factors afecting the production of carotenoids by *Rhodotorula glutinis* var. *glutinis*. *Journal of Food and Nutrition Science*, 3, 64-71.

Buzzini, P., Innocenti, M., Turchetti, B., Libkind, D., Van Broock, M., Mulinacci, N. (2007) Carotenoid profles of yeasts belonging to the genera *Rhodotorula, Rhodosporidium, Sporobolomyces*, and *Sporidiobolus*. *Canadian Journal of Microbiology*, 53, 1021-1031.

Fakruddin, M. D., Hossain, M. N., Ahmed, M. M. (2017). Antimicrobial and antioxidant activities of *Saccharomyces cerevisiae* IFST062013, a potential probiotic. BMC *Complemenary and Alternative Medicine*, 17(1), 64-74.

Forman, H. J., Zhang, H., Rinna, A. (2009). Glutathione: Overview of its protective roles, measurement, and biosynthesis. *Molecular Aspects of Medicine*, 30(1-2), 1-12.

Glueck, B., Han, Y., Cresci, G. A. M. (2018). Tributyrin supplementation protects immune responses and

vasculature and reduces oxidative stress in the proximal colon of mice exposed to chronic-binge ethanol feeding. *Journal of İmmunology Research*, e-9671919.

Guerrand, D. (2017). Lipases industrial applications: focus on food and agroindustries, *Oilseeds & fats crops and lipids*, 24(4). D403.

Hermund, D. B. (2018). Antioxidant properties of seaweed-derived substances. In *Bioactive seaweeds for food applications* (pp. 201-221). Massachusetts, United Syates Academic Press.

Hernández-Carbajal, G., Rutiaga-Quiñones, O. M., Pérez-Silva, A., Saucedo-Castañeda, G., Medeiros, A., Soccol, C. R., & Soto-Cruz, N. Ó. (2013). Screening of native yeast from Agave duranguensis fermentation for isoamyl acetate production. *Brazilian Archives of Biology and Technology*, 56(3). 357-363.

Herz, S., Weber, R. S., Anke, H., Mucci, A., Davoli, P. (2007). Intermediates in the oxidative pathway from torulene to torularhodin in the read yeasts *Cystofilobasidium infirmominiatum* and *C. capitatum* (Heterobasidiomycetes, Fungi). Phytochemistry, 68, 2503-2511.

Husain, A., Khan, S. A., Iram, F., Iqbal, M. A., & Asif, M. (2019). Insights into the chemistry and therapeutic potential of furanones: A versatile pharmacophore. *European Journal of Medicinal Chemistry,* 171. 66-92

Jain, S. C., Jain, P. C., Kango, N. (2012). Production of inulinase from *Kluyveromyces marxianus* using *Dahlia tuber* extract. *Brazilian Journal Microbiology*, 43(1), 62-69.

Kato, T., Park, E. Y. (2012). Riboflavin production by Ashbya gossypii. *Biotechnology Letters*, 34(4), 611-618.

Keller and Heckman L. L. P. (2017). GRAS Notification for β-Galactosidase Enzyme Preparation Derived from *Papiliotrematerrestris*, GRAS Notice (GRN) No. 743

Kritzinger, E. C., Bauer, F. F., & Du Toit, W. J. (2013). Role of glutathione in winemaking: a review. *Journal of Agricultural And Food Chemistry*, 61(2), 269-277.

Kuefer, R., Hofer, M. D., Altug, V., Zorn, C., Genze, F., Kunzi-Rapp, K., Gschwend, J. E. (2004). Sodium butyrate and tributyrin induce *in vivo* growth inhibition and apoptosis in human prostate cancer. *British Journal Cancer*, 90(2), 535-541.

Liu, J. F., Xia, J. J., Nie, K. L., Wang, F., & Deng, L. (2019). Outline of the biosynthesis and regulation of ergosterol in yeast. *World Journal of Microbiology and Biotechnology*, *35*(7), 98-107.

Maicas, S., Mateo, J. J. (2015), Enzyme contribution of non-saccharomyces yeasts to wine production. *Universal Journal of Microbiology Research*, 3(2). 17-25.

Max, B., Salgado, J. M., Rodríguez, N., Cortés, S., Converti, A., & Domínguez, J. M. (2010). Biotechnological production of citric acid. *Brazilian Journal of Microbiology*, 41(4), 862-875.

McFarland, L. V. (2007). Meta-analysis of probiotics for the prevention of traveler's diarrhea. *Travel Medicine and Infectious Disease*, 5(2), 97-105.

McFarland, L. V., Elmer, G. W., McFarland, M. (2006). Meta-analysis of probiotics for the prevention and treatment of acute pediatric diarrhea. *International Journal Probiotics and Prebiotics*, 1(1), 63-74.

Morioka, L. R. O., dos Santos Viana, C., de PáduaAlaves, É., Gonzales Paiao, F., Takihara, A. M., Kakuno, A. S. S., Suguimoto, H. H. (2019), Concentrated beta-galactosidase and cell permeabilization from *Saccharomyces fragilis* IZ 275 for beta-galactosidase activity in the hydrolysis of lactose, Food and Sciences Technology. Campinas, 39(3). 524-530.

Nascimento, D. S., Valasques Junior, G., Fernandes, P., Ribeiro, G. C., Lima, D. M., Góes-Neto, A., Assis, S. A. D. (2012). Production, characterization and application of inulinase from fungal endophyte CCMB 328. *Anais Academia Brasileira Ciências*, 84(2). 443-454.

Pérez-Torrado, R., Querol, A., Guillamón, J. M. (2015). Genetic improvement of non-GMO wine yeasts: Strategies, advantages and safety. *Trends Food Sciences Technolgyes*, 45, 1-11.

Steensels, J., Snoek, T., Meersman, E., et al. (2014). Improving industrial yeast strains: exploiting natural and artificial diversity. *FEMS Microbiology Review*, 38, 947-995.

Rai, A. K., Pandey, A., Sahoo, D. (2019). Biotechnological potential of yeasts in functional food industry. *Trends Food Sciences. Technology*, 83, 129-137.

Raveendran, S., Parameswaran, B., Ummalyma, S. B., Abraham, A., Mathew, A. K., Madhavan, A., Rebello, S., Pandey, A.(2018). Applications of Microbial enzymes in food industry. *Food Technologtes and Biotechnologyes*, 56(1), 16-30.

Ridrigues, C., Soccol, C. R., Pandey, A., Vandenberghe, L. P. S. (2006). New perspectives for citric acid production and application, *Food Technology and Biotechnology*, 44(2), 141-149.

Rima, H., Steve, L., Ismail, F. (2012). Antimicrobial and probiotic properties of yeasts: from fundamental to novel applications. *Froniers in Microbiology*, 3, 421-431.

Rollini, M., Musatti, A., Manzoni, M. (2010). Production of glutathione in extracellular form by *Saccharomyces cerevisiae*. *Process Biochemistry*, 45(4), 441-445.

Romanin, D. E., Llopis, S., Genoves, S., Martorell, P., Ramon, V. D., Garrote, G. L. (2015). Probiotic yeast *Kluyveromyces marxianus* CIDCA 8154 shows antiinflammatory and anti-oxidative stress properties in vivo models. *Beneficial Microbes*, 7(3), 73-83.

Romano, P., Capece, A., Jespersen, L. (2006). Chapter 2: Taxonomic and ecological diversity of food and beverage yeasts, (pp. 13-54) In: Querol, A., Fleet, G. (Eds.) *The yeast handbook. Yeasts in food and beverages,* vol. 2. Springer-Verlag, Berlin Heidelberg.

Rosi, I., Vinella, M., & Domizio, P. (1994). Characterization of β-glucosidase activity in yeasts of oenological origin. *Journal of Applied Bacteriology*, 77(5), 519-527.

Rubio-Texeira, M. (2006). Endless versatility in the biotechnological applications of *Kluyveromyces* LAC genes. *Biotechnology Advances*, 24, 212-225.

Saikia, D., Manhar, A. K., Deka, B., Roy, R., Gupta, K., Namsa, N. D., Chattopadhyay. P., Doley, R., Mandal,

M. (2017). Hypocholesterolemic activity of indigenous probiotic isolate *Saccharomyces cerevisiae* ARDMC1 in a rat model, *Journal of Food and Drug Analysis*, 26, 154-162.

Sakaki, H., Nakanishi, T., Komemushi S., Namikawa, K., Miki, W. (2001). Torularhodin as a potent scavenger against peroxyl radicals isolated from a soil yeast, *Rhodotorula glutinis*. *Journal of Clinical Biochemistry and Nutrition*, 30, 1-10.

Saqib, S., Akram, A., Halim, S. A., Tassaduq, R. (2017). Sources of β-galactosidase and its applications in food industry, *Biotechology*, 3, 7-79.

Schwab, W. (2013). Natural 4-Hydroxy-2, 5-dimethyl-3 (2H)-furanone (Furaneol®). *Molecules*, 18(6), 6936-6951.

Shahidi, F. (Ed.). (2015). Antioxidants in *Handbook of antioxidants for food preservation*. Woodhead Publishing.

Shahidi, F., & Zhong, Y. (2010). Novel antioxidants in food quality preservation and health promotion. *European Journal of Lipid Science and Technology*, 112(9), 930-940.

Shahidi, F., Zhong, Y., Lipid oxidation: measurement methods, (pp. 357-386) In: *Bailey's Industrial Oil and Fat Products*, Vol. 1, Shahidi, F. (Ed.), John Wiley & Sons Ltd., Hoboken, NJ 2005.

Sheng, J., Chi, Z., Li, J., Gao, L., Gong, F. (2007). Inulinase production by the marine yeast *Cryptococcus aureus* G7a and inulin hydrolysis by the crude inulinase. *Process Biochemistry*, 42(5), 805-811.

Simova, E. D., Frengova, G. I., Beshkova, D. M. (2003). Efect of aeration on the production of carotenoid pigments by *Rhodotorula rubra-Lactobacillus casei* subsp. *casei* co-cultures in whey ultrafltrate. *Zeitscrift fur Naturforsch*, 58, 225-229

Singh, R. S., Dhaliwal, R., Puri, M. (2007). Partial purification and characterization of exoinulinase from *Kluyveromyces marxianus* YS-1 for preparation of high-fructose syrup. *Journal of Microbiology and Biotechnology*, 17(5), 733-738.

Slaughter, J. C. (1999). The naturally occurring furanones: formation and function from pheromone to food. *Biological Reviews*, 74(3), 259-276.

Soccol, C. R., Vandenberghe, L. P., Rodrigues, C., &Pandey, A. (2006). New perspectives for citric acid production and application. *Food Technology & Biotechnology*, 44(2), 141-149

Stahl, W., Sies, H. (2007). Carotenoids and flavonoids contribute to nutritional protection against skin damage from sunlight. *Molecular Biotechnology*, 37, 26-30.

Struyf, N., Verspreet, J., Verstrepen, K. J., Courtin, C. M. (2017). Investigating the impact of α-amylase, β-glucosidase and glucoamylase action on yeast-mediated bread dough fermentation and bread sugar levels. *Journal of Cereal Science*, 75, 35-44.

Suzuki, T., Yokoyama, A., Tsuji, T., Ikeshima, E., Nakashima, K., Ikushima, S., & Yoshida, S. (2011). Identification and characterization of genes involved in glutathione production in yeast. *Journal of bioscience and bioengineering,* 112(2), 107-113.

Szczęsna-Antczak, M., Kamińska, J., Florczak, T., Turkiewicz, M. (2013). Chapter 16: Cold-Active Yeast Lipases: Recent Issues and Future Prospects, (pp 353-375) In: *Cold-adapted Yeasts*, P. Buzzini and R. Margesin (eds.), Springer-Verlag Berlin Heidelberg, Germany.

Tahmasebi, T., Nosrati, R., Zare, H., Saderi, H., Moradi, R., & Owlia, P. (2016). Isolation of indigenous glutathione producing *Saccharomyces cerevisiae* strains. *Iranian Journal of Pathology*, 11(4), 354-365.

Tong, Z., Zheng, X., Tong, Y. (2019). Systems metabolic engineering for citric acid production by *Aspergillus niger* in the post-genomic era. *Microbial Cellular Factory* 18, 28-43.

Tokdar, P., P. Ranadive, R., Kshirsagar, S. S., Khora & Deshmukh S. K. (2014). Influence of substrate feeding and process parameters on production of coenzyme Q10 using *Paracoccus denitrificans* ATCC 19367 mutant strain P-87. *Advances in Bioscience and Biotechnology*, 5, 966-977.

Uehara, K., Watanabe, J., Akao, T., Watanabe, D., Mogi, Y., Shimoi, H. (2015). Screening of high-level 4-hydroxy-2 (or 5)-ethyl-5 (or 2)-methyl-3 (2H)-furanone-producing strains from a collection of gene deletion mutants *of Saccharomyces cerevisiae*. *Applied and Environmental Microbiology*, 81(1), 453-460.

Verstrepen, K. J., Chambers, P. J., Pretorius, I. S. (2006). Chapter 13: The development of superior yeast strains for the food and beverage industries: challenges, opportunities and potential benefits. (pp. 399-444) In: *The Yeast Handbook. Yeasts in Food and Beverages,* vol. 2. Springer-Verlag, Berlin Heidelberg.

Wang, L., Chi, Z., Wang, X., Ju, L., Chi, Z., &Guo, N. (2008). Isolation and characterization of *Candida membranifaciens* subsp. *flavinogenie* W14-3, a novel riboflavin-producing marine yeast. *Microbiological Research*, 163(3), 255-266.

Willaert, R., Verachtert, H., van den Bremt, K., Delvaux, F., & Derdelinckx, G. (2005). Bioflavouring of Foods and Beverages. In: *Applications of Cell Immobilisation Biotechnology* (pp. 355-372). Springer, Dordrecht.

Xavier J. R., Ramana K. V., Sharma R. K. (2017). β-galactosidase: Biotechnological applications in food processing, *Journal of Food Biochemistry*, vol. 42:e12564.

Young, A. J., & Lowe, G. L. (2018). Carotenoids-antioxidant properties, *Antioxidants*, 7, 28, 1-4.

Zhang, J. N., He, X. P., Guo, X. N., Liu, N., Zhang, B. R. (2005). Genetically modified industrial brewing yeast with high-glutathione and low-diacetyl production. *Chinese Journal of Biotechnology*, 21(6), 942-946.

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MIXING AND PASTING CHARACTERISTICS OF THE PUMPKIN SEEDS-WHEAT FLOUR BLENDS

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Abstract

Due to it high nutritional and sensorial characteristics of pumpkin seed flour (PSF) it is suistanable to be used as ingredient in different food products such as cereal products. The objective of this study was to analyze the effect of PSF addition (from 0% up to 20%) in a meal form (PSF) in a refined wheat flour on the mixing dough rheological properties by using Farinograph and Extensograph devices and pasting dough rheological properties by using Amylograph and Falling Number devices. It seems that by PSF addition the water absorption capacity increased, whereas compared to the control sample the dough stability and development time decreased. According to Extensograph device the dough extensibility decreased with the increased level of PSF addition in wheat flour. The dough pasting properties showed that by PSF addition the Falling Number and peak viscosity decreased whereas the gelatinization temperature and temperature at peak viscosity increased.

Key words*: wheat flour, pumpkin seeds, Farinograph, Extensograph, Amylograph, Falling Number.*

INTRODUCTION

The pumpkin belongs to the family *Cucurbitaceae,* genus *Curcubita*. The nutritional value of pumpkin seeds are high (Xanthopoulou *et al.,* 2009) those containing 37-46% proteins, 25-37% fat content, 16-24% dietary fibers and arround 4.5% minerals (Mironeasa and Codină, 2016). These seeds are rich in sources of unsaturated fatty acids (especially linoleic and oleic acids), vitamins (vitamin E and the main isomers alpha-tocopherol and gamma-tocopherol), minerals, phytosterols, pigments, pyrazine derivatives and phenolic compounds (Murković *et al.*, 2000; Xanthopoulou *et al.,* 2009). Its fibers content contains 40% cellulose, arround 4% hemicellulose and 4% lignin. Along its high nutritional value pumpkin seeds presents a highly desired aromas, sweetness taste and greenish color (Mirhosseini *et al.,* 2015).

Due to it high nutritional and pleasant sensorial characteristics pumpkin seed flour (PSF) may be used as ingredient in different food products. In cereal products, PSF may be used as ingredient in order to improve the nutritional, physical and sensory qualities of bakery products. It was reported that it was used as ingredient in different cereal products such as: breads, cakes, instant noodles and spices, as well as a natural coloring agent in flour and flour mixtures (Hosseini, *et al.,* 2018; Minarovičová *et al.,* 2017).

Different studies reported that up to a certain level PSF addition may improve bread quality from the sensory and technological point of view. Mironeasa and Codină (2016) concluded that up to 10% PSF addition was obtained bread of a good quality. El-Soukkary et al. (2001) reported that the addition of pumpkin seeds leads to an acceptable bread quality up to 17% for raw, roasted and autoclaved meal forms, up to 19% for germinated, fermented and pumpkin protein concentrate forms and up to 21% for pumpkin protein isolate form. An increase of bread volume and the scores for organoleptic acceptability has also been reported by Ptitchkina, *et al.* (1998) which added pumpkin powder in wheat flour and by Jeevitha and Bhuvana (2019) which concluded that pumpkin seed meal can be added to the whole wheat bread in order to improve the nutritional value and the physical properties of the bread even at high levels. However, Dabash, *et al.* (2017) did not

reported any significant improvement of bread quality by PSF addition in wheat flour. They concluded that, in general, the increase of the amount of PSF addition in wheat flour conducted to an decrease to the specific volume of bread and it sensory acceptabiliy.
Thus, the addition of PSF influenced bread quality and therefore the technological properties of bread making. With the addition of PSF, the baking loss decreased with 9.18% compared to the control bread (Jeevitha and Bhuvana, 2019). El-Soukkary (2001) reported an increased of the water absorption, dough development time and softening values with the increase level of PSF addition whereas Costa *et al.* (2018) reported an decreased of water absorption, stability and dough development time values for the samples in which pumpkin seeds with high fat content were incorporated in wheat flour. It seems that this different results obtained by different reserchers are due to the type and level of PSF used in wheat flour. Many studies are focused on using pumpkin seeds after drying and grinding the meal or seeds (El-Demery, 2011) others are focused on using PSF in a grounded form (Mironeasa and Codină, 2016) under germinated and fermented form, under concentrate and isolate protein form, e.g (El-Soukkary, 2001).
The objective of this study was to analyze the effect of pumpkin seed addition (from 0% up to 20%) in a meal form (PSF) in a refined wheat flour on the mixing and pasting dough rheological properties.

MATERIALS AND METHODS

Commercial wheat flour (harvest of 2019) from S.C. MOPAN S.A. (Suceava, Romania) and partially deffated pumpkin seed flour (Marbacher Ölmühle GmbH, Germany) was used. The flours were analyzed according to ICC methods as: ash content (ICC 104/1), moisture content (ICC methods 110/1), protein content (ICC 105/2). The wheat flour was also analyzed through the international and Romanian standard methods: falling number (ICC 107/1), wet gluten (SR 90:2007) and gluten deformation index (SR 90:2007).
Dough rheological properties during mixing were analyzed using the devices Farinograph (Brabender OGH, Duisburg, Germany with a 300 g capacity) and Extensograph (Brabender OGH, Duisburg, Germany) according to ICC method 115/1.
Dough rheological properties during pasting were analyzed using the devices Amylograph (Brabender OGH, Duisburg, Germany) according to ICC method 126/1 and Falling Number (Perten Instruments, Sweden) according to ICC method 107/1.
Statistical analysis was performed with XLSTAT (Version 2019.14.1, free trial; Addinsfot's Corporation, USA). Results are presented as means±standard deviation. An ANOVA and Tukey tests were performed with a 95% confidence interval.

RESULTS AND DISCUSSIONS

The wheat flour analytical characteristics are the fallowings: 0.65% ash content, 14.0% moisture content, 12.67% protein content, 30% wet gluten content, 6 mm gluten deformation index, 1.5% fat content and 442 s Falling Number value. According to the results obtained the wheat flour is a very good one for bread making with a low α amylase activity. The PSF presented the fallowing characteristics: 29.7% ash content, 5.68% moisture content, 62.27% protein content and 10.81 fat content.
According to Farinograph measurements shown in the Figure 1 the PSF addition in wheat flour significantly increased ($p < 0.05$) the water absortion (WA) value up to 4.2% to the PSF20 sample compared to the control one. These results are in agreement with those reported by El-Soukkary (2001), Costa *et al.* (2018) for dough samples in which pumpkin seeds flours in a defatted form were incorporated in wheat flour. This may be attributed to the high protein content of pumpkin seeds flour which absorbs water in the dough system limiting the water availability for the gluten network development. The dough development time (DDT) and dough stability (ST) increased from 1.9 to 7.7 and 2.3 to 11.0 respectively and further decreased when high levels of PSF were incorporated in dough samples. Also the degree of softening at 10 min (DS) decreased and at high levels begin to increased due to the gluten dilution by PSF

addition, a non gluten flour in wheat flour as it may be seen in Figure 2.

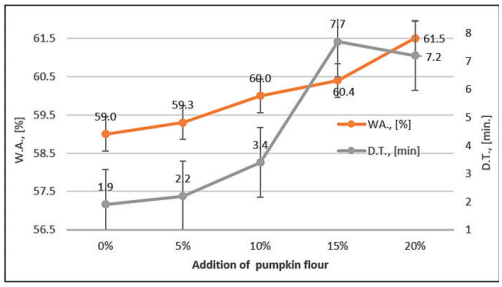

Figure 1. Farinograph water absorption (WA) and dough development time (DT) parameters of pumpkin seeds-wheat flour blends

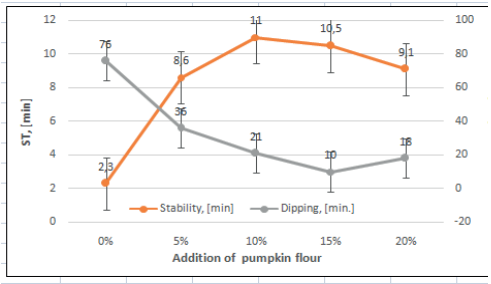

Figure 2. Farinograph stability (ST) and degree of softening (DS) parameters of pumpkin seeds-wheat flour blends

Dough extensibility for the samples with different levels of PSF addition is shown in Figures 3-6.

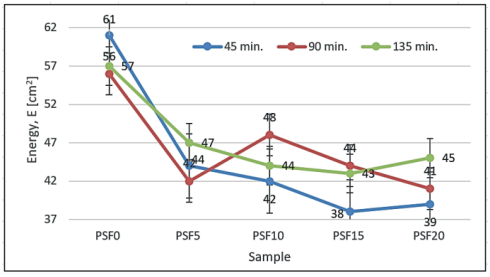

Figure 3. Extensograph energy (E) parameter of pumpkin seeds-wheat flour blends

As it may be seen compared to the control sample the Extensograph parameters decreased probably due to the fact that PSF decreased the gluten availability which affect the development of three-dimensional dough structure. According to El-Soukkary, (2001) and Sundy, (2004) which obtained similar data for dough samples with PSF addition the dough weakening may be due to the following reasonns: gluten dilution, the presence of sulphhydryl groups from PSF, the competition between proteins of wheat flour and PSF for water. The pumpkin seeds-wheat flour blends presented a ratio number higher than the recommended values of 1.2 facts that make the use of these blends feasible in bread making.

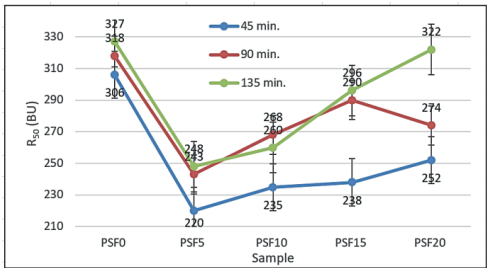

Figure 4. Extensograph resistance to extension (R_{50}) parameter of pumpkin seeds-wheat flour blends

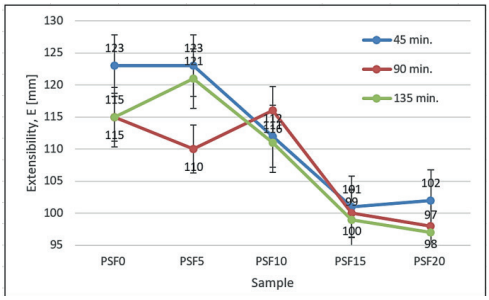

Figure 5. Extensograph extensibility (E) parameter of pumpkin seeds-wheat flour blends

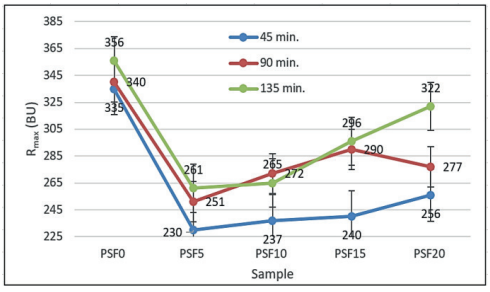

Figure 6. Extensograph maximum resistance to extension (R_{max}) parameter of pumpkin seeds-wheat flour blends

The pasting characteristics of pumpkin seeds-wheat flour blends are shown in Figure 7 and Figure 8.

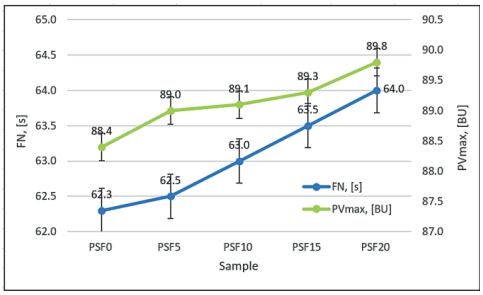

Figure 7. Falling Number value and Amylograph peak viscosity (PV_{max}) parameter of pumpkin seeds-wheat flour blends

The falling number value of wheat flour dough decreased with the increased level of PSF addition, showing a decrease in dough viscosity of the blends. These data are in agreement with those obtained by Khan *et al.* (2019) which also has been reported an decreased of FN with pumpkin seed flour addition.

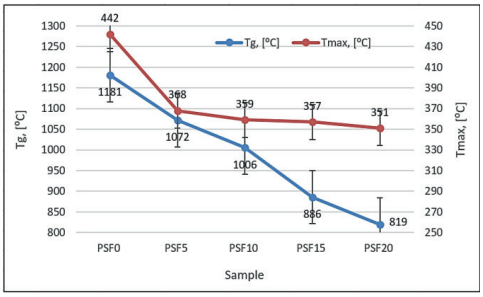

Figure 8. Amyograph gelatinization temperature (Tg,) and temperature at peak viscosity (T_{max}) parameters of pumpkin seeds-wheat flour blends

According to the Amylograph data, the gelatinization temperature increased ($p < 0.05$) with the increased level of PSF addition indicating a delay of starch gelatinization, these data being in agreement with those obtained by Wongsagonsup, *et al.* (2015) regarding final RVA pasting temperature for wheat flour in which PSF was incorporated. Also the temperature at peak viscosity had a similar trend with the gelatinization temperature values which increased when PSF were incorporated in wheat flour dough.

Regarding the peak viscosity, these values were significantly decreased ($p < 0.001$) when PSF were incorporated in wheat flour. This decreased with the increase level of PSF addition may be attributed to lower starch content from the pumpkin seeds-wheat flour blends. As the addition level of PSF in wheat flour increased, the non-starch component from the dough system decreased, leading to a decrease of the PV_{max} when temperature increased above the starch gelatinization. According to Wongsagonsup, *et al.* (2015) the lower peak viscosity for samples with PSF addition are due to higher lipid content of PSF and less nitrogen-free extractives (mainly starch) from it. They explain the undetectable breakdown of pumpkin seeds-wheat flour blends to the swelling restriction of starch by lipids fact that causes a stronger structure of starch granules. When helical complexes develop between starch chains and lipids, the helices hold amylopectin and amylose molecules together, which restrict granule swelling fact that will led to an increase in pasting temperature, and in a decrease in the pumpkin seeds-wheat flour blends viscosity and increase resistance to shear-thinning of paste.

CONCLUSIONS

By using the PSF up to 20% level addition in wheat flour of a low α amylase activity and a very good quality for bread making according to the wheat flour analytical data, the dough rheological properties were improved. This fact showed that PSF addition in wheat flour is feasible from rheological point of view in bread making. Therefore the dough stability and development time increased up to 15% PSF addition and dough extensibility decreased with the increased level of PSF incorporated in wheat flour. With the increase temperature of dough samples the dough viscosity decreased showing a decrease in falling number values and peak viscosity.

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REFERENCES

Costa, L.L., Tomé, P. H. F., Jardim, F. B.B., Silva, V.P., Castilho, E.A., Damasceno, K. A. Campagnol, P. C. B. (2018). Physicochemical and rheological characterization of pan bread made with pumpkin seed flour.

Dabash, V., Burešová, I., Tokár, M., Zacharová, M., Gál, R. (2017).The effect of added pumpkin flour on sensory and textural quality of rice bread. *Journal of Microbiology, Biotechnology and Food Sciences*, 6(6), 1269-1271.

El-Demery, M.E. (2011). Evaluation of physico-chemical properties of toast bread fortifi ed with pumpkin (Cucurbita moschata) flour. *The 6th Arab and 3rd International Annual Scientific Conference on Development of Higher Specific Education Programs in Egypt and the Arab World in the Light of Knowledge Era Requirements, Faculty of Specifi c Education, Mansoura University, Mansoura, Egypt,* 13-14.

El-Soukkary, F.A.H. (2001). Evaluation of pumpkin seed products for bread fortification. *Plant Foods for Human Nutrition*, 56**:** 365-384.

Hosseini G., S.H., Seyedain Ardabili, S.M., Kashaninejad, M. (2018). Physico-chemical, textural and sensory evaluation of sponge cake supplemented with pumpkin flour. *International Food Research Journal*, 25(2): 854-860.

Jeevitha GR and Dr. S Bhuvana (2019). Study on the impact of pumpkin seed flour on whole wheat bread characteristics. *International Journal of Chemical Studies*, ISSN: 2321–4902 IJCS 2019; 7(4): 273-277.

Khan, M.A., Mahesch, C., Vineeta, P., Sharma, G.K., Semwal., A.D. (2019). Effect of pumpkin flour on the rheological characteristics of wheat flour and on biscuit quality. *Journal of Food Processing and Technology*, 10 (9), 812.

Minarovičová, L., Lauková, M., Kohajdová, Z., Karovičová, J., & Kuchtová, V. (2017). Effect of pumpkin powder incorporation on cooking and sensory parameters of pasta. *Potravinarstvo Slovak Journal of Food Sciences*, 11, 373-379.

Mirhosseini, H., Rashid, N.F.A., Amid, B.T., Cheong, K.W., Kazemi, M., Zulkurnain, M. (2015). Effect of partial replacement of corn flour with durian seed flour and pumpkin flour on cooking yield, texture properties, and sensory attributes of gluten free pasta. *LWT - Food Science and Technology*, 63,184-190.

Mironeasa S., Codină G.G. (2016). Evaluation the effect of pumpkin seed flour addition in wheat flour of a strong quality for bread making on bread quality, *16th International Multidisciplinary Scientific Geoconference (SGEM 2016)*, 261-268.

Murković M., Pfannhauser W. (2000). Stability of pumpkin seed oil. *European Journal of Lipid Science and Technology*, 102, 607-611.

Ptitchkina N.M., Novokreschonova L.V., Piskunova G.V. & Morris E.R. (1998). Large enhancements in loaf volume and organoleptic acceptability of wheat bread by small additions of pumpkin powder: possible role of acetylated pectin in stabilising gas-cell structure. *Journal of Food Hydrocolloids,* 12(3), 333-337.

Sundy, Y.G. (2004). Dough rheology and quality of wheat bread supplemented with fluted pumpkin seed protein concentrate. *Global Journal of Agriculture Science*, 4 (1), 7-13.

Wongsagonsup, R., Kittisuban, P., Yaowalak, A., Suphantharika, M. (2015). Physical and sensory qualities of composite wheat-pumpkin flour bread with addition of hydrocolloids. International *Food Research Journal*, 22 (2), 745-752.

Xanthopoulou M.N., Nomikos T., Fragopoulou E. & Antonopoulou S. (2009). Antioxidant and lipoxygenase inhibitory activities of pumpkin seed extracts, *Journal of Food Research International*, 42(5-6), 641-646.

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EXPERIMENTAL RESEARCH ON BIOPOLYMERS OBTAINING BASED ON CHITOSAN, GELATIN AND PECTIN

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Abstract

Biopolymers are polymers obtained from biological origins and used for industrial applications in food packaging. A biopolymer should be non-toxic, non-carcinogenic, biodegradable and also adequately available for their widespread applications.
The aim of this study was to obtain packaging materials for food, as films prepared in the laboratory, based on natural polymers obtained from renewable resources:
- chitosan/gelatine and chitosan/gelatine/n-clay respectively;
- chitosan/pectin and chitosan/pectin/n-clay respectively;
- chitosan/gelatine/pectin and chitosan/gelatine/pectin/n-clay respectively. The nano-clay was added 3% to the mixing mass. In order to obtain films with a good flexibility, 5% glycerol was added.
These polymeric films have been characterized in terms of transmittance/transmission by UV/VIS spectroscopy and chemical structure by ATR-FTIR technique.
It was found that the optical properties of these films were slightly affected by the nano-clay concentration in their composition. As for the films based on chitosan - gelatine, chitosan-pectin, which contain nano-clay, this has increased the thickness of the films, giving them mechanical strength, but also opacity.
Analysis of the film microstructure obtained by FTIR exhibited a good compatibility among the gelatine-chitosan blend and showed that this matrix allowed a uniform distribution of the actives throughout the network.
The results obtained from the ATR-FTIR spectra confirmed the existence of specific chemical groups in the analysed polymers (-OH, -NH2, -NH, CH2, CH- groups), according to the data reported in the literature.

Key words**:** *chitosan, gelatine, pectin, polymeric films, nano-clay.*

INTRODUCTION

In recent decades, there has been a sharp increase in the use of films based on natural polymers in packaging for the food industry as a replacement for synthetic plastic films, driven by a societal desire for sustainable food packaging, which protects food products from external contamination and delays their deterioration by extending the shelf life and maintaining quality (Bonnaillie et al., 2014).
In addition to consumer requirements and to replace plastic packaging, a large variety of low-cost natural resource biopolymers have emerged (Ahvenainen et al., 2003). The biopolymers used mainly for edible films in food packaging are proteins, polysaccharides and lipids (Yao et al., 2017).
Films made from chitosan and gelatine, according to studies are increasingly provided for biomedical and food applications, as well as for packaging (Cordoba et al., 2016).
Chitosan is a polysaccharide derived from chitin, being its N-deacetyl form. This polymer has attracted the attention of researchers, due to its antimicrobial properties, being considered a natural preservative (Miteluț et al., 2015; Popescu et. al., 2018;). Chitosan acts on the cell of pathogenic and degrading microorganisms, interacting with the cell membrane, thus causing functional and structural changes, changing the permeability of the cell wall (Bonilla & Sobral, 2016).The intrinsic properties of chitosan make it ideal for fabricating films, and chitosan based films promise great potential as active packaging materials due to its antimicrobial

activity, non-toxicity and low permeability to oxygen (Kanatt et al., 2012).

Gelatine is a protein produced by partial acid/base hydrolysis of collagen. Gelatine can be extracted from various sources: pig skin, fish skin and bovine bone (Alexandre et al., 2016). Gelatine is a water-soluble protein substance, prepared by processes that involve the destruction of the tertiary, secondary and to some extent the primary structure of native collagen (Fernandez-Diaz et al., 2001), specifically by partial hydrolysis of collagen derived from the skin, white connective tissue, and animal bones (Morrison et al., 1999). Gelatine is a digestible protein that contains all the essential amino acids except tryptophan (Benbettaïeb et al., 2014). The composition of amino acids, especially with regard to proline and hydroxyproline, may vary from species to species, as a result of exposure to a wide range of environmental conditions (Ladislaus et al., 2007).

Pectins are a class of complex polysaccharides found in the cell walls of higher plants, where they act as a moisturizing agent and a cementing material for the cellulose network. They are typically produced during the initial stages of primary cell wall growth and account for about one third of the cell wall of the dry matter of dicotyledonous plants and of monocotyledonous plants. The main exceptions are the cell walls of the *Graminae* family, which may contain pectin with normal structure, but in very small quantities.

Nano-clay is one of the most commonly used nanomaterials in biopolymer film technology Nano-clay is characterized by a moderate negative surface charge, which has a perfect crystalline structure, consisting of a two-dimensional layer having a central octahedral sheet of aluminium oxide and magnesium oxide bonded with two external silica tetrahedra (Majdzadeh-Ardakani et al., 2010; Slavutsky et al., 2014).

Nano-clay can be used to control the release of antimicrobial compounds from the film into the packaged product and also to control the interior atmosphere of the package, depending on the improvement of gas and water vapor permeability (Fabra et al., 2009).

MATERIALS AND METHODS

MATERIALS USED IN THE EXPERIMENTS TO OBTAINING FILMS

Materials used in this experimental work were:
- chitosan with average molecular weight, nano-clay and glycerol provided from Center for Research and Eco-Metallurgical Expertise, University Politehnica of Bucharest, Romania;
- gelatine and pectin purchased from the local market (Bucharest, Romania).

METHODS USED TO OBTAIN FILMS

In order to obtain various films, experiments were realized in Center for Research and Eco-Metallurgical Expertise, University Politehnica of Bucharest, Romania.

The following films have been developed: chitosan-gelatine (C/G), chitosan-gelatine-nano-clay(C/G/N-clay), chitosan-pectin (C/P), chitosan-pectin-nano-clay (C/P/N-clay), chitosan-gelatine-pectin (C/G/P), chitosan-gelatine-pectin-nano-clay (C/G/P/N-clay).

Different experimental variants of the three individual polymers were tried, as well as of the mixture between them to obtain a polymer film with a smooth and homogeneous structure.

To prepare film forming solutions, gelatine was dissolved in distilled water at room temperature, while 1.5% chitosan (Average Molecular Weight) was dissolved in 1% acetic acid solution, by stirring on the magnetic plate, at temperature of 120 °C and 400 rpm.

Pectin was used both dissolved in 2.5% distilled water and in powder form. The nano-clay was dispersed in glycerol with distilled water.

After obtaining the individual solutions, they were homogenized in different proportions to obtain the films, according to Table 1. Ultrasonic bath was used, then the homogeneous solutions were placed in square plastic plates (25 ml/plate) (Figure 1) and allowed to dry at room temperature for 48 hours.

Figure 1. Homogeneous solutions placed in square plastic plates

Table 1. Different concentrations of films

Sample	Chitosan 1.5% (g/sol)	Gelatine 10% (g/sol)	Pectin 2.5% (g/sol)	Glycerol (g)	Nano-Clay (g)
1	47.5	47.5	-	5	
2	43.5	43.5	-	5	8
3	47.5	-	47.5 (g/sol)	5	-
4	43.5	-	47.5 (g/sol)	5	8
5	46.25	46.25	2.5 (g)	5	-
6	45.25	45.25	2.5 (g)	5	8

RESULTS AND DISCUSSIONS

ANALYSIS OF FILM TRANSMISSION BY UV-VIS SPECTROMETRY

The obtained films (Figures 2-7) were analysed in the UV-VIS field using the Helyos spectrophotometer to determine their transparency.

Portions of each film obtained were placed in the Thermo Electron Corporation Helyos Spectrophotometer Testing Cell. Their transparency was determined by measuring the absorbance at a wavelength of 800 nm and was calculated by the equation:

$$\text{Transparency} = \frac{A800}{x} = -logT800/x$$

Where: The T800 is transmitting at 800 nm, A is the absorption at 800 nm and x is the film thickness (mm).

To indicate transparency, films must be homogeneous, there should be no phase separation between different components. If the compatibility between the different constituents of the films is not good, then the light transmission is low or the opacity is high due to the reflection or light scattering at the two-phase interface. Films have excellent barrier properties in the UV region, regardless of the cross-section content.

As it can be seen in Figure 8 the most transparent film is the one based on CS-G, having the lowest transparency value, A800/x = 0.39, followed by Cs-G-P with 0.45. The opaquest films are the ones made of Cs-G-P-N/ clay and Cs-G-N/clay with the transparency value A800/x = 1.79, respectively A800/x = 1.70.

FOURIER TRANSFORMED INFRARED SPECTROSCOPY

The structure of the films (Figures 9-14) was determined using, characterized by the Infrared (IR) spectroscopy by ATR spectra, that was carried out using a Perkin Elmer One spectrometer (Spectrum 2000 FTIR-ATR).

FTIR spectroscopy was used to characterize the interactions between chitosan, gelatine, pectin and nano clay. FT-IR spectra were recorded in the wavelength range 700 cm^{-1}-4000 cm^{-1}. For each film, two repetitions were performed. FTIR spectra obtained for each sample showed for all films, carbonyl (C-O) stretches at 1799 cm^{-1} and 2514 cm^{-1}, N-H bending at 1549 cm^{-1}, C-N stretching at 1239 cm^{-1} and C-H, amide II, amide III, and O-H stretching between 2931 cm^{-1} - 3300 cm^{-1} (Pereda et al., 2011). According to researchers Chen et al. (2016) the observed peaks at wavenumbers of 2854 cm^{-1} and 2924 cm^{-1} can be assigned to antisymmetric and symmetric -CH2 stretching vibrations, while peaks in the range 3200 cm^{-1} - 3600 cm^{-1} can be assigned to different -OH stretching vibrations corresponding to inter- and intra-molecular H-bonds (Chen et al., 2016).

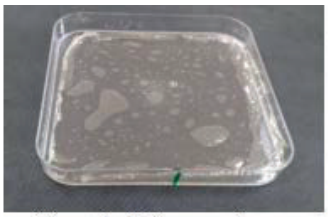

Figure 2. Chitosan and gelatin film (CS/G)

Figure 3.Chitosan and gelatin nano-clay film (CS/G/N-clay)

Figure 4.Chitosan and pectin film (CS/P)

Figure 5. Chitosan, pectin and nano-clay film (CS/P/N-clay)

Figure 6.Chitosan, gelatin and pectin film (CS/G/P/)

Figure 7.Chitosan, gelatin, pectin and nano-clay (CS/G/P/ N-clay)

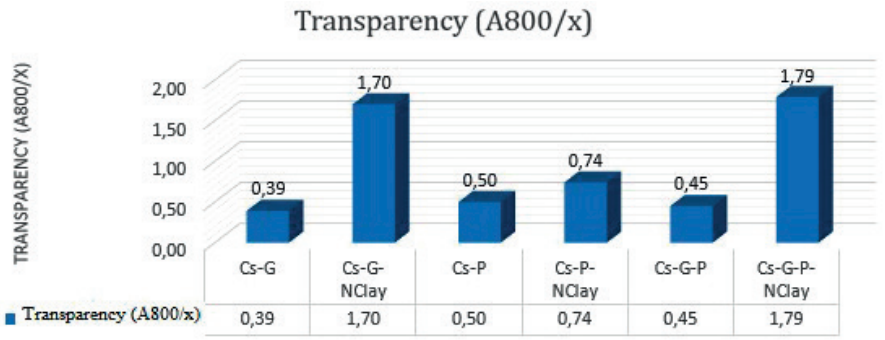

Figure 8. Transparency of the films

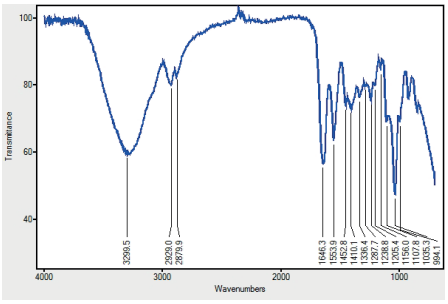

Figure 9. FTIR Spectra of films based on CS/G

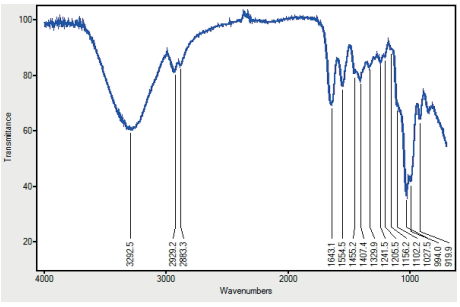

Figure 10. FTIR Spectra of films based on CS/G/N-Clay

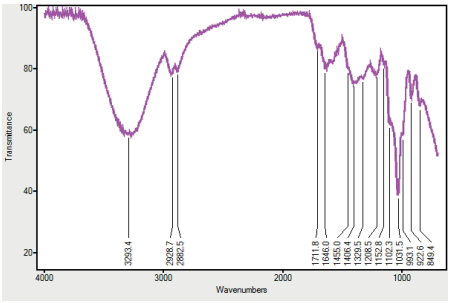

Figure 11. FTIR Spectra of films based on CS/P

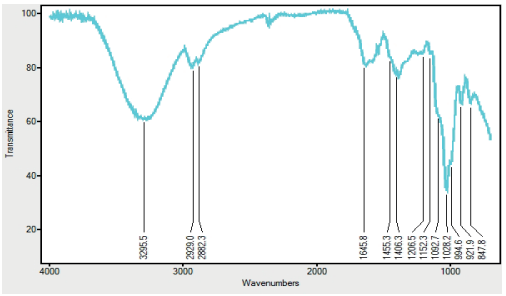

Figure 12. FTIR Spectra of films based on CS/P/N-Clay

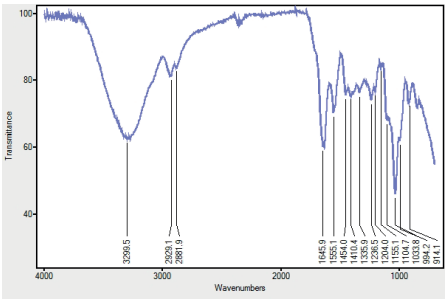

Figure 13. FTIR Spectra of films based on CS/G/P

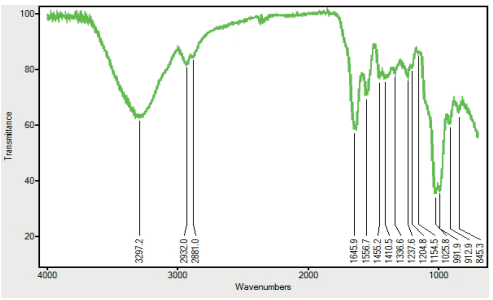

Figure 14. FTIR Spectra of films based on CS/G/P/N-Clay

The attribution of the absorption bands is in accordance with the literature study (Marcos-Fernández et al., 2006). Chitosan shows the absorption band of the C-C valence bond at 850-853 cm^{-1}, C-H at 2790 cm^{-1} -2881 cm^{-1} and the C-H (CH2) bond at 2931 cm^{-1} -2933 cm^{-1}. The absorption band specific for the N-H bond is found at 1316-1410 cm^{-1} and is due to the small amount of chitin present. Also, the valence vibration absorption band of the C-O bond at 1043 cm^{-1} and 1021 cm^{-1} is highlighted (Marcos-Fernández et al., 2006).

According to the literature, the chitosan-specific absorption vibrations are related to the following frequencies: 923 cm^{-1} -1033 cm^{-1} due to the valence vibration of the C–O bond in the secondary OH groups in the pyranose ring, 1152 cm^{-1} due to the valence vibration of the C bond. –O of the glycosidic bond, 1264 cm^{-1} due to the bending vibration of the N – H amide group, 1650-1559 cm^{-1} due to the valence vibration of the C = O bond of the amide I and the bending vibration of the N – H bond amide II, 2870 cm^{-1} attributed to the valence vibration of the CH bond in the pyranose ring, 2928 cm^{-1} attributed to the valence vibration of the CH2 group in the CH_2-OH bond and 3323 cm^{-1} attributed to the valence vibration of the NH2 group in the primary amines and the valence vibration of the OH group in the pyranose ring (Pasela et al., 2019).

Due to its unique amino acid sequence and numerous functional groups (groups), gelatine is a polymer containing free unprotected ε-amino groups (-NH2) derived from lysine or hydroxylisine (Dolete et al., 2019).

According to the data from the specialized literature (Hossana et al., 2014), the gelatine has three specific maxima, respectively at 1640 cm^{-1} due to the tensile vibration of the C = O bond specific to the amide I, at 1535 cm^{-1} the vibrations of the tension of the CN and deformation bond are highlighted of the NH bond of amide II, and at 1243 cm^{-1} -1260 cm^{-1} the vibrations of stretching of the CN bond and of bending of the NH bond specific to the amide III of gelatine appear. Also, in the specialized literature there are also mentioned the tensile vibrations of the bands - OH and NH located around 3200 cm^{-1}, the symmetrical stretching vibrations of the specific carboxylated salt bonds at about 1402 cm^{-1} and of the ester bond at 1090 cm^{-1}.

In the case of pectin, the FTIR spectrum shows two intense absorption bands, at 1748 cm^{-1} and 1637 cm^{-1} attributed to the valence vibration of the C = O bond in the ester and the bending vibration of the H_2O group which overlaps with the asymmetric stretch vibration of the carboxylate anion (COO–) (Bonilla & Sobral, 2016.).

The nano-clay shows the following specific absorption bands: at 1424 cm^{-1}, 876 and 712 cm^{-1} attributed to the valence vibration of the carbonate CO bond and weak absorption peaks at 1799 cm^{-1} and 2514 cm^{-1}, which are bands overlapping.

The absorption spectrum characteristic of nano-clay consists in highlighting the absorption band in the range 3300 cm^{-1}-3500 cm^{-1}, attributed to the valence vibration of the OH bond in the water molecule, the bands from 1685 cm^{-1} and 1622 cm^{-1} (the bending vibration of the O bond –H –O bending) (Kiros e al., 2013).

CONCLUSIONS

Films from natural polymers such as chitosan, gelatine and pectin were developed, in which nano-clay and glycerol were added, to obtain food packaging materials (Arvanitoyannis, 2002).

The combination of chitosan-gelatine, chitosan-pectin, chitosan-gelatine-pectin allows the obtaining of well shaped, homogeneous and semi-transparent films. In order to obtain flexible films, 5% glycerol and 3% nano-clay was added to the mixing mass.

The obtained films were characterized using UV/VIS spectroscopy to determine their degree of opacity and by ATR-FT-IR technique to characterize the interaction between the used polymers.

It was found that the optical properties of these films were slightly affected by the nano-clay concentration in their composition. As for the films based on chitosan-gelatine, chitosan-pectin, which contain nano-clay, the increased thickness of the films gave them mechanical strength, but also opacity.

Based on the results of this study, the developed materials could be suitable to be used as biodegradable based packaging materials in food industry.

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REFERENCES

Ahvenainen, R. (2003). Active and intelligent packaging: an introduction. In: Ahvenainen, R. (Ed.), Novel Food Packaging Techniques. Woodhead Publishing, Cambridge, (2), 5-21.

Alemayehu, Kiros, A. V. Gholap and Giovanni E. Gigante (2013). Fourier transform infrared spectroscopic characterization of clay minerals from rocks of Lalibela churches, Ethiopia, *International journal of physical sciences*, 8(3), 109-119.

Alexandre, E.M.C., Lourenço, R.V., Bittante, A.M.Q.B., Moraes, I.C.F., Sobral, P.J.A. (2016). Gelatin-based films reinforced with montmorillonite and activated with nanoemulsion of ginger essential oil for food packaging applications. *Food Packag. Shelf Life* (10), 87-96.

Arvanitoyannis, I. (2002). Formation and properties of collagen and gelatin films and coatings. In: Gennadios, A. (Ed.), *Protein-based Films and Coatings.* CRC Press LLC, (11), 275-300.

Benbettaïeb, N., Kurek, M., Bornaz, S., Debeaufort, F. (2014). Barrier, structural and mechanical properties of bovine gelatin-chitosan blend films related to biopolymer interactions. *J. Sci. Food Agric.* (94), 2409-2419.

Bonilla, J., Sobral, P.J.A. (2016). Investigation of the physicochemical, antimicrobial and antioxidant properties of gelatin-chitosan edible film mixed with plant ethanolic extracts. *Food Bioscience*, (16), 17-25.

Bonnaillie, Laetitia M., Han, Zhang, Serife Akkurt, Kit L. Yam and Peggy M. Tomasula (2014). Casein Films: The Effects of Formulation, Environmental Conditions and the Addition of Citric Pectin on the Structure and Mechanical Properties. *Book Polymers*, (6), 2018-2036.

Chen, H., Hu, X., Chen, E., Wu, S., Mcclements, D.J., Liu, S., Li, B., Li, Y. (2016). Preparation, characterization, and properties of chitosan films with cinnamaldehyde nanoemulsions. *Food Hydrocoll*, (61), 662-671.

Cordoba L., Perez J., Sobral, P.J.A. (2016). Physical and antioxidant properties of films based on gelatine, gelatine chitosan or gelatine-sodium caseinate blends loaded with nanoemulsified active compounds, *Food Hydrocolloids*, (79), 3-15.

Dolete, G., Bianca, M. Tihăuan, C. Kamerzan, M. , Tutunaru, O., Mocanu, I.-C., Balaş, C., Ardelean, I. L, Sergiu M. (2019). Development and sequential analysis of a collagen-chitosan wound management biomaterial, *Romanian Biotechnological Letters*, 108-117.

Fabra, J., Atarés, Talens & Chiralt (2009). Effect of Fatty Acids and Beeswax Addition on Properties of Sodium Caseinate Dispersions and Films. *Journal of the Science of Food and Agriculture,* 10(6), 2808-2817.

Kanatt, S.R., Rao, M.S., Chawla, S.P., Sharma, A. (2012). Active chitosan-polyvinyl alcohol films with natural extracts. *Food Hydrocolloids*, 29(2), 290-297.

Ladislaus M., Kasankala, Yan Xue Yao, Weilong Sun D., Hong Qian He (2007). Optimization of gelatin extraction from grass carp (Catenopharyngodon idella) fish skin by response surface methodology. School of Food Science, Research Centre of Functional Food Engineering, Ministry of Education, *Bioresource Technology*, 98(17), 3338-3343.

Fernández-Díaz M. D., Montero P., Gomez-Guillen M.C. (2001). Gel properties of collagens from skins of cod (Gadus morhua) and hake (Merluccius merluccius) and their modification by the coenhancers magnesium sulphate, glycerol and transglutaminase, *Food Chemistry* (74), 161-167.

Hossana Jakir , Md., Gafur M.A., Karim M.M., Rana A. (2014). Mechanical properties of Gelatin-Hydroxyapatite composite for bone tissue engineering, *Journal of Scientific and Industrial Research*, 50(1), 15-20.

Pereda M., Ponce A.G., Marcovich N.E, Ruseckaite, R. A., Martucci J.F. (2011). Chitosan-gelatin composites and bi-layer films with potential antimicrobial activity, *Food Hydrocolloids*, 25(5), 1372-1381.

Majdzadeh-Ardakani, Navarchian and Sadeghi, Slavutsky, Bertuzzi, Armada, García and Ochoa (2012). Preparation and characterization of montmorillonite modified by phosphorus–nitrogen containing quaternary ammonium salts, *Food Hydrocolloids*, 258(8), 4054-4062.

Marcos-Fernández A., Abraham G.A., Valentín J.L., San Román J. (2006). Synthesis and characterization of biodegradable non-toxic poly (ester-urethane-urea)s based on poly(ε-caprolactone) and amino acid derivatives. *Polymer*, 47(3), 785-798.

Miteluț A.C., Tănase E.E., Popa V.I., Popa M.E. (2015). Sustainable alternative for food packaging: chitosan biopolymer - a review, *AgroLife Scientific Journal,* 4(2), 52-61.

Morrison N. A., Clark R. C., Chen Y. L., Talashek T., Sworn G. (1999). Gelatin alternatives for the food industry, Part of the Progress in Colloid and *Polymer Science book series* (PROGCOLLOID, (114), 127-131.

Pasela Bianca Rae, Acelle Pearl Castillo, Rhenish Simon, Maria Teresa Pulido, Haidee Mana-ay, Ma. Roxan Abiquibil, Rhys Montecillo, Kanjana Thumanu, Doebner von Tumacder, and Kathrina Lois Taaca (2019). Synthesis and Characterization of Acetic Acid-Doped Polyaniline and Polyaniline–Chitosan Composite. *MDPI Biomimetics Journals*, 4(1), 4-15.

Popescu, P.A. ,Mitelut, A.C., Popa, E., Popa, M.E. (2018). State of the art on new processing techniques used for preservation of agricultural products - a critical review, *Scientific Bulletin. Series F. Biotechnologies*, XXII, 113-118.

Slavutsky, Bertuzzi, Armada, García and Ochoa (2014). Preparation and characterization of montmorillonite/brea gum nanocomposites films, *Biopolymers*, (35), 270-278.

Yao Yunzhen, Ding Ding, Hongyuan Shao, Qifan Peng, and Yaqin Huang (2017). Antibacterial Activity and Physical Properties of Fish Gelatin-Chitosan Edible Films Supplemented with D-Limonene, *International Journal of Polymer Science*, 1-9.

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DEVELOPMENT OF A MOLECULAR METHOD FOR QUANTIFYING *Hanseniospora* POPULATIONS DURING THE WINEMAKING PROCESS

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Abstract

In the wine and in the grape must, there is a complex microbial ecosystem that harbours a great diversity of yeast species, amongst other, non-Saccharomyces yeasts (Issatchenkia orientalis, Metschnikowia pulcherrima, Torulaspora delbrueckii, Candida zemplinina and Hanseniaspora spp.). The paper aimed to develop a molecular method for quantifying Hanseniospora populations during the winemaking process. Specific primers and SYBR Green probe have been used for real-time qPCR method and dilutions from a reference strain of Hanseniospara uvarum, it also hs been used for the calibration curve. After the method was developed, it was used to study different samples of grape must and wine, following a natural or controlled fermentation with commercial yeast strains. This study is necessary, useful and shows the relevance of qPCR for studying non-Saccharomyces yeasts in the complex ecosystem of grape must and wine.

Key words*: qPCR, Hanseniospora, SYBR Green, grape must, wine.*

INTRODUCTION

For the grape must to turn into wine, it is necessary the coexistence and the succession of different yeast species. On the surface of grape grains are present mainly non-*Saccharomyces* (NS) yeasts, being predominant in the early stages of alcoholic fermentation. In the intermediate to the final stages of fermentation, non-*Saccharomyces* yeasts are outgrown by the growth of *Saccharomyces cerevisiae* (Fleet et al., 1993; Fleet, 2003).

To prevent the risk of non-*Saccharomyces* yeasts growing in the beginning of the wine making process, addition of sulphites is a usual industrial method, being considered unattractive in traditional wine making. Nowadays, increased knowledge about yeast diversity has shown that there are several non-*Saccharomyces* yeasts with their own benefits, contributing to the sensory growth and complexity of the wines (Jolly et al, 2014; Carrau et al., 2015; Padilla et al., 2016).

The mechanism involved in the cell death of two *Hanseniospora* species (*Hanseniospora guillermondii* and *Hanseniospora uvarum*) during mixed fermentation, under oenological growth conditions with *Saccharomyces cerevisiae*, was studied by Perez-Nevado et al. (2006). When *S. cerevisiae* reached a cell concentration of close to 10^7 CFU/mL, a reduction in the population of *Hanseniospora* was observed regardless of ethanol concentration. The authors hypothesized that certain toxic compounds produced by *S. cerevisiae* trigger early death of *Hanseniospora* cells.

Mills et al. (2002), using direct molecular methods, detected an active population of *Hanseniospora* strains during fermentation processes, which could not be observed by classical cultivation methods. Hierro et al. (2006a) detected a permanent population of *H. uvarum* and *H. osmophila*, during fermentation processes, not knowing to what extent these species contribute to the total population of yeasts during wine fermentation. The use of molecular methods independent of the classical methods of cultivation on the plate, can lead us as close as possible to the true diversity of yeast populations throughout the fermentative process of wine (Vrajmasu et al., 2018).

In recent studies, microorganisms as *Saccharomyces* and non-*Saccharomyces* yeasts involved in wine making process, have been studied using real-time quantitative PCR

(qPCR) to detect and quantify yeasts strains without the need for plating (Hierro et al., 2007; Zott et al., 2010; Andorra et al., 2011; Portillo et al., 2016).

The aim of this study was to use and develop a molecular method based on DNA (qPCR), to detect and quantify the population levels of different *Saccharomyces* and non-*Saccharomyces* (*Hanseniospora* spp.) yeasts strains during the various stages of alcoholic fermentation process.

MATERIALS AND METHODS

Grape must and microorganisms

In this study, one type of white wine (Feteasca Regală 2018) and two musts (with and without sulphite addition) were tested. Samples were harvested on September 2018 and 50 mg/L of sulphate was added, debourbage of samples was realized next day. The must having 23.2% Brix and 3.94 g/L total acidity, was inoculated with 20g/hL of selected commercial yeasts: Y1, Y2, Y3, Y4, and Y5 (Y1 and Y2 are non-killer *Saccharromyces* strain; Y3, Y4 and Y5 are killer Saccharomyces strain). Grape must samples were coded as M (simple must) and MS (sulphite must). The temperature of fermentation was 16±1.5°C and was conducted during almost 3 weeks. Sampling was done from the first fermentation day till the end of the fermentation; *Hanseniospora* detection was performed in all fermentative phases (lag, exponential and stationary phase).

For the calibration curve was used a strain with a known concentration of *Hanseniospora uvarum* (CSIII2) with a microbial load of 1.4 x 10^{10} cells/mL, from the UASMVB-Faculty of Biotechnology collection.

DNA extraction

Extraction of DNA was performed with Fast ID Genomic DNA Extraction Kit (Genetic ID NA, Fairfield, IA, USA). For each extraction, 1000 µl of Genomic Lyse buffer premixed with 10 µl of Proteinase K solution was mixed with 1000 µl of sample. After a vortex, samples were incubated at 65ºC for 30 minutes, followed by a spin at 10.000 rpm for 5 minutes in a microcentrifuge. Supernatant was passed through the DNA Binding Column. The columns were washed one time with 1000 µl Genomic Wash and three times with 1000 µl of 75% ethanol, after each wash columns were spin for one minute at 10.000 rpm. At the final 100 µl of 1xTE were added and incubated for 10 minutes at 65ºC, followed by a spin at 13.000 rpm for one minute and eluted DNA collected.

The DNA quality was checked using a spectrophotometer (NABI UV/Vis Nano Spectrophotometer, MicroDigital Co. Ltd., Korea).

Real Time PCR method

The DNA samples were treated with Maxima SYBR Green qPCR Master Mix (Thermo Fisher Scientific, Hampton, NH, USA), in the presence of *Hanseniospora* specific primers Hauf2L (5'-CCCTTTGCCTAAGGTACG-3') and Hauf2R reverse primer (5'-CGCTGTTCTCGCTGTGATG-3') recommended by (Zott et al., 2010) The coupling protocol for one sample is: 12.5 µl SYBR Green qPCR Master Mix, Hauf2L forward primer 0.1 µl (final concentration of 0.3 µM), Hauf2R reverse primer 0.1 µl (final concentration of 0.3 µM), DNA template 5 µl (The DNA concentration in the extracted samples had values between 2.3 and 7.8 ng/µl) and nuclease free water 7.3 µl, total reaction volume 25 µl.

The reaction was performed in a Real-time PCR System (7500 Real-time PCR System, Applied Biosystems, Thermo Fisher Scientific, Foster City, CA, USA) following the program: initial denaturation 10 min at 95ºC; 40 cycles of denaturation 15 s at 95ºC followed by an extension of 60 s at 62ºC. Each sample was amplified in duplicate in every experiment. To generate the standard curve, a 10-fold dilution series of DNA from *Hanseniospora uvarum* strain was subjected to qPCR under the same conditions as described above. All reagents used were molecular biology grade reagents.

RESULTS AND DISCUSSIONS

The purpose of this work was to develop a method for quantifying *Hanseniospora* populations during fermentation in winemaking process. In order to validate the qPCR quantification method, a calibration curve using a *Hanseniospora* strain with a known microbial

loading of 1.4 x 10^{10} cells/mL was required. For the standard curve a very good correlation coefficient was obtained, respectively R^2 = 0.9927 (Figure 1). The limit of detection showed the necessity of the presence of a maximum of Ct equal to 32 for a positive reaction with SYBR-Green. The population level of *Hanseniospora* was obtained by interpolation and expressed in $\log_{10}$ no. cell / mL. The value of efficiency of amplification was 90% for this qPCR reaction, fitting in the validation criteria with an R^2 greater than 0.98 and a slope with a value between -3.1 and -3.6 (Uțoiu et al., 2018).

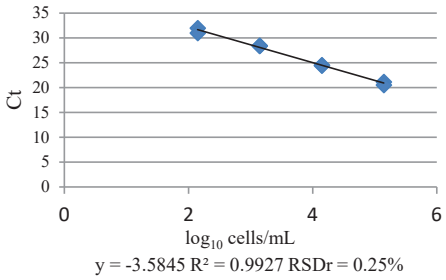

Figure 1. Standard curve obtained from serially diluted *H. uvarum*

Dilutions were performed up to a concentration of 1.4 x 10^2 cells/mL, this value being the limit of detection of the method (Figure 1). The evolution of the *Hanseniospora* population in five types of wine and two musts, were analysed during the alcoholic fermentation, obtaining values for all the analysed samples, the *Hanseniospora* population coming from the grape berries, not being added as a fermentation supplement. Level of *Hanseniospora* population were almost similar for wine samples and significantly higher for wine must samples.

For the Y1 wine samples, the dynamics of the concentration was more constant than in the case of Y2 wine samples, the initial concentration value was 2.91 x 10^4 cells/mL, in the following days of the fermentation period there was a decreasing trend with slight variations, reaching a minimum concentration value on the thirteenth day. 4.15 x 10^3 cells/ mL, so that at the end of the monitoring period, it reaches a maximum recorded value of 3.55 x 10^4 cells/mL, slightly higher than the initial one (Figure 2).

In Y4 wine samples, the initial concentration was 8.4 x 10^4 cells/mL, on the sixth day of fermentation to reach a maximum concentration of 1.18 x 10^5 cells/mL, on the thirteenth day reaching a minimum concentration of 2.84 x 10^4 cells/mL, at the end of the period a concentration of 3.53 x 10^4 cells / mL was recorded (Figure 3). The same trend was registered for Y3 wine samples as for Y5 samples, the initial value recorded was 2.43 x 10^4 cells/mL, on the second day of fermentation the maximum concentration value of 4.22 x 10^4 cells/mL was recorded, the following day recorded a minimum concentration of 8.42 x 10^3 cells/ mL, at the end of the period there was registered a value of 1.94 x 10^4 cells/mL, slightly lower than the initial one (Figure 3). In the Y5 wine samples, an initial concentration value of 5.75 x 10^4 cells/mL was recorded, on the sixth day it reached a maximum value of 8.5 x 10^4 cells/mL, on the fifteenth day of monitoring a minimum value of 7.8 x 10^3 cells/ mL, in the end of the periods a value of 1.9 x 10^4 cells/mL was recorded, a value that is lower than the value of the initial recorded concentration (Figure 3). In Y4 wine samples, the concentration was generally constant, starting from an initial value of 3.62 x 10^4 cells/mL, with slight variations, reaching a minimum value on day 13 of 3.53 x 10^4 cells/ mL, at the end of the period to reach a maximum value of 2.84 x 10^5 cells/mL (Figure 3).

In the case of the must sample, as general remark, the *Hanseniospora* population was higher than in the natural or inoculated wines.

For the simple must samples, an initial concentration value of 4.14 x 10^5 cells/mL was recorded, a slight variation was recorded until the tenth day, when the concentration had a maximum of 4.32 x 10^5 cells/mL and reaching a minimum value on the last day of 2.9 x 10^4 cells/mL (Figure 4). In the case of must samples with sulphites, the highest values of the *Hanseniospora* populations level was recorded in the beginning of the fermentation (6.42 x 10^5 cells/mL): the minimum population level was recorded as 4.36 x 10^5 cells/mL on the eleventh day of fermentation; an increased level of 1.32 x 10^6 cells/mL was recorded on the sixth day. The maximum value of the population level for the sulphite must samples was 2.35 x 10^6 cells/ mL, decreasing slightly on the last day of monitoring at 1.77 x 10^6 cells/mL (Figure 4).

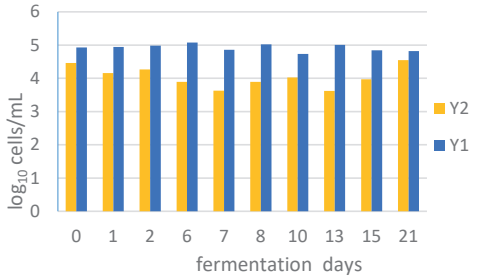

Figure 2. Evolution of *Hanseniospora* population during fermentation with Y1and Y2 non-killer *Saccharomyces* strain

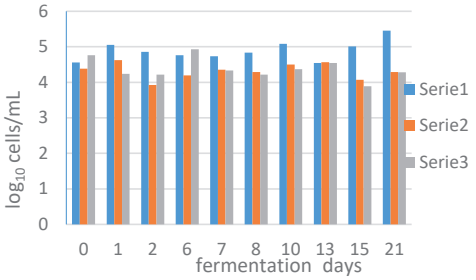

Figure 3. Evolution of *Hanseniospora* population during fermentation with Y3, Y4 and Y5 killer *Saccharomyces* strain

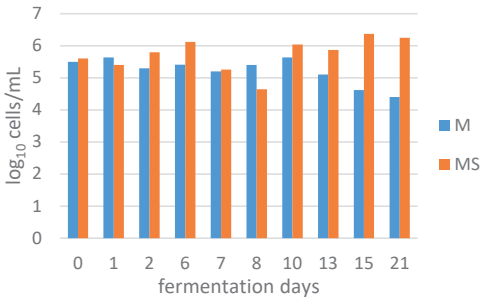

Figure 4. *Hanseniospora* population evolution during fermentation in grape must (M) and grape must with sulphites (MS)

A general image of cardinal *Hanseniospora* level during wine fermentation (beginning of fermentation, end of fermentation and maximum level are presented in Table 1).

In all analysed wine samples, similar values were obtained, with an increasing trend for Y4 wine samples and a decreasing trend for Y3, Y5. Wine samples, Y4 and Y2, registered the minimum and maximum value on the same days (minimum on the thirteenth day and maximum on the last day of monitoring), Y3 recorded the maximum concentration on the second day and Y5 on the sixth day. A difference can be observed between the two types of wine, the concentration of *Hanseniospora* population is slightly higher in the case of killer factor samples than in the case of non-killer ones. Grape must samples had a higher initial concentration than wine, and must samples with sulphites recorded the highest concentrations up to 2.35 x 10^6 cells/mL, with similar results being obtained by Hierro et al. (2007) and Lopez et al. (2015).

Table 1. *Hanseniospora* levels on different wines fermentation stages (cells/mL)

Sample	Beginning of fermentation	End of fermentation	Maximum level
M	$4.1x10^5$	$2.9x10^4$	$4.3x10^5$
MS	$6.42x10^5$	$1.8x10^6$	$2.3x10^6$
Y1	$8.4x10^4$	$6.6x10^4$	$1.2x10^5$
Y2	$2.9x10^4$	$3.5x10^4$	$3.5x10^4$
Y3	$2.4x10^4$	$1.9x10^4$	$4.2x10^4$
Y4	$3.6x10^4$	$2.8x10^5$	$2.8x10^5$
Y5	$5.7 x10^4$	$1.9x10^4$	$8.5x10^4$

Rapid and sensitive methods are needed for yeast detection and enumeration to allow winemakers to control and avoid damaging wines. QuantitativePCR is a fast and accurate technique for quantifying microorganisms associated with food. This technique was used to detect and enumerate the total number of yeasts in wine samples (Martorell et al., 2005; Hierro et al., 2006a; Hierro et al., 2006b; Andorra et al., 2012). In all analysed wine samples, almost similar values were obtained, grape must samples had a higher initial concentration than wine. Must samples with sulphites recorded the highest concentrations up to 2.35 x 10^6 cells/mL, it was assumed that non-*Saccharomyces* yeasts were only present at the beginning of fermentation and were eliminated by the main fermentation yeast *Saccharomyces cerevisiae* (Zott et al., 2010). This method has been applied so far for the enumeration of yeasts from wine by Hierro et al., 2007; Andorra et al., 2012; Rizzotti et al., 2015 and our results are similar to those found by Zott et al., 2010 and Hierro et al., 2007.

CONCLUSIONS

In our effort to set-up a non-culture method to quantify *Hanseniospora* population during wine fermentation, we took a molecular approach by the use of Real Time PCR. For the standard curve a very good correlation coefficient was obtained, respectively $R^2 = 0.9927$ and a value of RSDr = 0.25%. The limit of detection showed the necessity of the presence of a maximum of Ct equal to 32 for a positive reaction with SYBR-Green. The value of efficiency of amplification was 90% for this qPCR reaction, fitting in the validation criteria with an R^2 greater than 0.98 and a slope with a value between -3.1 and -3.6.

In all the analyzed wine samples, almost similar values were obtained for *Hanseniospora* population; grape must samples coefficient was had a higher initial concentration than the wines, and must samples with sulphites recorded the highest concentrations. Our results are in line with other reports and support the idea that qPCR is a fast, direct (non-culture), sensitive and reliable technique for quantifying different yeast species.

REFERENCES

Andorra, I., Monteiro, M., Esteve-Zarzoso, B., Albergaria, H., Mas, A. (2011). Analysis and direct quantification of *Saccharomyces cerevisiae* and *Hanseniospora guilliermondii* populations during alcoholic fermentation by fluorescence *in situ* hybridization, flow cytometry and quantitative PCR.

Food Microbiology (28), 1483-1491.

Andorra, I., Berradre, M., Rozès, N., Mas, A., Guillamón, J.M., Esteve-Zarzoso, B. (2012). Effect of mixed culture fermentations on yeast populations and aroma profile. *LWT-Food Science and technology*, (491), 8-13.

Carrau, F., Gaggero, C., Aguilar, P.S. (2015). Yeast diversity and native vigour for flavour phenotypes. *Trends in Biotechnology*, (33), 148-154.

Esteve-Zarzoso, B., Belloch, C., Uruburu, F., Querol, A. (1999) Identification of yeasts by RFLP analysis of the 5.8S rRNA gene and the two ribosomal internal transcribed spacers. *International Journal of Systematic and Evolutionary Microbiology*, (49), 329-337.

Fleet, G.H., Heard, G.M. (1993). Yeasts: growth during fermentation. *Wine Microbiology and Biotechnology* ed. Harwood Publishing, 27-54.

Fleet, G.H. (2003). Yeast interactions and wine flavor. *International Journal of Food Microbiology*, (86), 11-22.

Hierro, N., Gonzales, A., Mas, A., Guillamon, J.M. (2006a). Diversity and evolution of non-*Saccharomyces* yeast population during wine fermentation: effect of grape ripenes and cold maceration. *FEMS Yeast Research*, (6), 102-111.

Hierro, N., Esteve-Zarzoso, B., Gonzales, A., Mas, A., Guilamon, J.M. (2006b). Real-time quantitative PCR (QPCR) and reverse-transcription-QPCR for detection and enumeration of total yeasts in wine. *Applied and Environmental Microbiology Journal*, (72), 7148-7155.

Hierro, N., Esteve-Zarzoso, B., Mas, A., Guillamon, J.M. (2007). Monitoring of *Saccharomyces* and *Hanseniospora* population during alcoholic fermentation by real-time quantitative PCR. *FEMS Yeast Research*, (7), 1340-1349.

Jolly, N.P., Varela, C., Pretorius, I.S. (2014). Not your ordinary yeast: Non-*Saccharomyces* yeasts in wine production uncovered. *FEMS Yeast Research*, (14), 215-237.

Lopez, S., Mateo, J., Maicas, S. (2015). Screening of *Hanseniospora* Strains for the Production of Enzymes with Potential Interest for Winemaking. *Fermentation*, 2(1), 1.

Martorell, P., Querol, A., Fernandez-Espinar, M.T. (2005). Rapid Identification and Enumeration of *Saccharomyces cerevisiae* Cells in Wine by Real-Time PCR. *Applied and Environmental Microbiology*, 71(11), 6823-6830.

Mills, D.A., Johannsen, E.A., Cocolin, L. (2002). Yeast diversity and persistence in *botrytis*-affected wine

fermentations. *Applied and Environmental Microbiology Journal*, (68), 4884-4893.

Padilla, B., Gil, G.V., Manzanares, P. (2016). Past and future of non-*Saccharomyces* yeasts: from spoilage microorganisms to biotechnological tools for improving wine aroma complexity. *Frontiers in Microbiology*, 7(411).

Perez-Nevado, F., Albergaria, H., Hogg, T., Girio, F. (2006). Cellular death of two non-*Saccharomyces* wine – related yeasts during mixed fermentation with *Saccharomyces cerevisiae*. *International Journal of Food Microbiology*, (108), 336-345.

Portillo, M.C., Mas, A. (2016). Analysis of microbial diversity and dynamics during wine fermentation of Grenache grape variety by high-throughput barcoding sequencing. *LWT-Food Science and Technology*, (72), 317-321.

Rizzotti, L., Levav, N., Fracchetti, F., Felis, G., E., Torianni, S. (2015). Effect of UV-C treatment on the microbial population of white and red wines, as revealed by conventional plating and PMA-qPCR methods, *Food Control*, (42), 407-412.

Uțoiu, E., Matei, F., Toma, A., Diguță, C.F., Ștefan, L.M., Mănoiu, S., Vrăjmașu, V., Moraru, I., Oancea, A., Israel-Roming, F., Cornea, C.P., Constantinescu-Aruxandei, D., Moraru, A., Oancea, F. (2018). Bee Collected Pollen with Enhanced Health Benefits, Produced by Fermentation with a Kombucha Consortium. *Nutrients*, (10), 1365.

Vrajmasu V., Diguta C. F., Matei F. (2018). Classic versus modern tools to study microbial population dynamics during food fermentation processes. *Scientific Bulletin. Series F. Biotechnologies*, Vol. XXII, 89-98.

Zott, K., Claisse, O., Lucas, P., Coulon, J., Lonvaud-Funel, A., Masneuf-Pomarede, I. (2010). Characterization of the yeast ecosystem in grape must und wine using real-time PCR. *International Journal of Food Microbiology*, (27), 559-567.

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SCREENING AMONG LACTIC ACID BACTERIA ISOLATED FROM NATURAL SOURCES FOR THEIR ANTI-*CANDIDA* IHIBITORY ACTIVITY

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Abstract

Candida, known as an "opportunistic" human pathogen, has coevolved with humans by persisting in mucosa and on the skin. The mechanism and factors determining the transformation of the "conolization" status into disease status is still under research. However, new preventive solutions for such events are under investigation. In our study we have tried to prove that some lactic acid bacteria (LAB), isolated from natural fermented food sources (Kombucha and fermented cocoa beans) have inhibitory effect on different species of Candida with different pathogenic potential (C. albicans, C. parapsilosis, C. krusei and C. guilliermondii). The inhibitory tests employed two different methods (transversal and radial strike). Among nine LAB strains isolated from cocoa beans fermentation, two have significant inhibitory activity against Candida parapsilosis. In the case of Kombucha source, two out of seven LAB strains have significant inhibitory activity against Candida albicans, Candida krusei and Candida parapsilosis; low level inhibition has been noticed against Candida guilliermondii. When mixing different combination of LAB from both natural sources, the anti-Candida effect was neither augmented, nor decreased

Key words*: lactic acid bacteria, Kombucha, cocoa fermentation, anti-Candida.*

INTRODUCTION

Human microbiota is complex, within which includes both commensal microorganisms, and pathogenic or facultative pathogenic microorganisms. *Candida* species strains is an example of microorganisms present in human microbiota which, in terms of balance of microflora of the human individual, it does not pose any health problems (Ionescu et. al., 2013). However, in cases of mass propagation, the genus *Candida* can produce local infections such as vaginal infectionsor even infections throughout the body, called candidaemia. The main the main species causing candidiasis is *C. albicans* (Silva et al., 2016), followed by *Candida parapsilosis Candida glabrata*, *Candida tropicalis* and *Candida krusei*, representing over 90% of cases of invasive infection caused by the genus *Candida* (Sardi et al., 2013; Pappas et al., 2015). In an article published in 2014 by Yapar N., citing CDC and the National Healthcare Safety Network, he states that the genus *Candida* is in the fifth place in the above hospital-acquired and in fourth place in the case of bloodstream. The use of various antifungal drugs such as azoles (fluconazole or ketoconazole), echinocandins (micafungin, caspofungin), amphotericin B or nucleoside analogues (Spampinato & Leonardi, 2013) have increased, in recent years, Candida's resistance to these treatments. difficult to treat certain types of candidiasis (Sanguinetti et al., 2015). Therefore, research and development of alternative cures for the treatment of candidiasis is a new trend in modern medicine (Vamanu & Voica, 2017). A good research path is represented by probiotics worth mentioning being genus *Lactobacillus*, the genus *Bifidobacterium*, the genus *Bacillus*, *Saccharomyces cerevisiae* (Silva et al., 2016).

All of the above lead researchers to conclude that the use of probiotics in fighting candidiasis may be the best solution in current medical conditions.

The context the aim of of the study was to characterize functional strains of microorganisms with anti-microbial activity and their recovery in the form of products with mixed anti-microbial and probiotic activity.

In the present work, anti-*Candida* activity of different lactic acid bacteria originated from different natural sources were studied: strains of lactic bacteria isolated from fermented cocoa

beans and strains of lactic bacteria isolated from Kombucha SCOBY.
Strains of lactic bacteria isolated from cocoa beans and isolated ones from kombucha are presented in Table 2.

MATERIALS AND METHODS

The *Candida* species used to carry out the experiments and on which the anti-*Candida* activity was carried out are part of the collection of the Faculty of Biotechnologies of the University of Agronomic Sciences and Veterinary Medicine of Bucharest and are presented in Table 1.

Table 1. Strains of *Candida* used

No.	**Strain**
1.	*Candida albicans* ATCC 10231
2.	*Candida guillermondii* MI 40
3.	*Candida krusei* 2016 MI 41
4.	*Candida parapsilosis* ATCC 20019

Table 2. Strains of lactic bacteria used

No.	Code	Species
Isolated from cocoa beans		
1.	Ped-3	*Enterococcus faecium*
2.	Ped-2	*Weissella cibaria*
3.	Lab 11.2	*Enterococcus faecium*
4.	Lab 11.1	*Lactobacillus farciminis*
5.	A19	*Weisella cibaria*
6.	Lab 9	*Pediococcus pentosaceus*
7.	Lab 10	*Weisella cibaria*
8.	A21	not identified
Isolated from fermented tea (kombucha)		
1.	S1	*Pediococcus pentosaceus*
2.	S2	*Pediococcus pentosaceus*
3.	S3	*Pediococcus pentosaceus*
4.	L3	*Pediococcus pentosaceus*
5.	L5	*Pediococcus acidilactici*
6.	F1	nd
7.	F2	nd

The culture medium used to cultivate the lactic bacteria was MRS at pH 6.5 ± 0.2.
The lactic bacteria strains source was the frozen stock belonging to the Microbiology Laboratory of the Faculty of Biotechnologies of the University of Agronomic Sciences and Veterinary Medicine of Bucharest. Each strain was cultivated as follows: 200 µL of bacterial suspension were taken from the Eppendorf tube containing the strain and were placed in a test tube with 10 mL of MRS medium and cultivate at 37ºC for 24 hours. From this parent culture, a dilution was performed: 1 mL of bacterial suspension was sown in test tubes with 10 mL of MRS culture medium. The revitalization method is described in the literature (Terpou et al., 2019), but has been slightly modified.
The inhibitory tests employed two different methods: transversal and radial strike. There are data in the literature that propose the two methods for testing the antimicrobial activity of lactic bacteria for rapid test results and efficient results. The good results seem to be due to the production of metabolites and good diffusion of metabolites produced by the lactic bacteria tested by the two methods (Coman et al., 2014; Balouiri et al., 2016).
The strains of lactic bacteria were inline seeded on Petri dishes with MRS medium. They were allowed to grow at 27°C for 48 hours and subsequently were perpendicularly inoculated to the *Candida* strains (*C. albicans*, *C. guillermondii*, *C. krusei*, *C. parapsilosis*).
In the case of the radial streak method, 10 µl of LAB suspension was inoculated on spot in Petri dishes containing MRS solid culture medium. After a 48 h incubation at 37°C, the plates radially incoluated with suspension of *Candida* species. After other 24 h incubation at 37°C, antimicrobial activity was observed. All tests have been done in triplication. Inhibitory activity was calculated by decreasing the diameter of the circle of the spread area of the lactic bacterium strain from the diameter of the observed inhibition zone.
In the case of the transversal method, the inhibitory activity was calculated by measuring the inhibition area observed between lactic bacteria and the pathogenic strain.
The study on the antimicrobial activity was followed by testing the inhibitory activity of mixtures of lactic strains by modified diffusion method (Olaru & Popa, 2019). Each strain was cultivated as follows: 200 µL of bacterial suspension were taken from the Eppendorf tube containing the strain and were placed in a test tube with 10 mL of MRS medium. From this parent culture, a dilution was done: 1 mL of bacterial suspension was sown in test tubes with 10 mL of MRS medium. After incubation at 37°C for 24 hours, they were used according to the modified diffusion method.
Each potential probiotic strain was inoculated in 10 µL volume spots and incubated for 48h at

37°C. The cultivation of lactic strains was carried out both individually and in 1:1 mixture with suspension 10^7 UFC/mL. Pathogenic reference strains were inoculated in an MRS 1% agar environment, melted and maintained at 45°C. After pouring over the spots containing the developed lactic strains, they were incubated at 37°C for 24 h. After incubation, the inhibition region was observed. It was worked in duplicate, using both MRS 1% agar with Tween 80.

RESULTS AND DISCUSSIONS

The inhibitory activity of LAB strains isolated from cocoa beans can be observed in Figure 1, while Tables 3 and 4 present the measurement results of the inhibitory activity on *Candida* strains for lactic bacteria isolated from cocoa beans.

The data indicates a good inhibition of *Candida* strains by Lab 11.1 (*Lactobacillus farciminis*), Lab 9 (*Pediococcus pentosaceus*) and A19 (*Weisella cibaria*) confirmed by both used methids, radial and transversal. In the case of Lab 11.1 (*Lactobacillus farciminis*) the highest inhibition activity was noticed against *C.parapsilosis*, followed by *C. guiliermondii* and *C. albincans*. No inhibitory activity was observed against *C. krusei.*

In the case of Lab 9 (*Pediococcus pentosaceus*) the inhibitory activity was almost the same against *C. parapsilosis* and *C. guilliermondii*, while low inhibition was obtained against *C. albicans.* A different situation was found in the case of A19 (*Weisella cibaria*); the highest inhibitoy activity was found against *C. albicans*; however, the results obtained by both tested method are only partially reproductibl, which is athe same in the case of the non-identified strain A21.

Among all *Candida* specie, the strain *Candida krusei* 2016 MI 41 shows resistance to all strains of lactic bacteria isolated from cocoa beans, while *Candida parapsilosis* ATCC 20019 has the highest sensitivity to lactic bacteria isolated from cocoa beans.

Aspects of the inhibitory activity of Kombucha LAB strains can be observed in Figure 2, while the measurements results are in Tables 5 and 6.

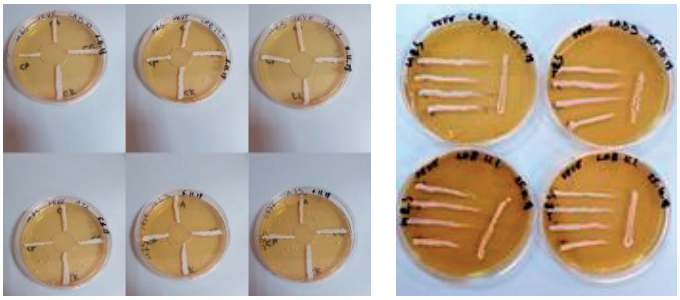

Figure 1. Inhibitory activity of lactic bacteria isolated from cocoa beans on *Candida* species tested by radial streak method (left) and of Lab 9 and Lab 11.1 tested by transversal method (right)

Table 3. Calculation of the inhibition zone of lactic bacteria isolated from cocoa beans on pathogenic strains tested by radial streak method

Lactic bacteria strain	*Candida albicans*	*Candida guilliermondii*	*Candida krusei*	*Candida parapsilosis*
Lab 11.1	2.00±0.00	9.5±0.70	nd	11.5±4.94
Lab 11.2	nd	nd	nd	nd
Lab 10	nd	nd	nd	nd
Lab 9	1.00±1.41	2.5±3.53	nd	4±5.65
Ped 2	nd	Nd	nd	nd
Ped 3	nd	nd	nd	nd
A19	8.5±12.0	5±7.07	nd	2.5±3.53
A21	nd	2.5±3.53	nd	2.5±3.53

*Inhibition area measured in mm

Table 4. Calculation of the inhibition zone of lactic bacteria isolated from cocoa beans on pathogenic strains tested by transversal method

Lactic bacteria strain	*Candida albicans*	*Candida guilliermondii*	*Candida krusei*	*Candida parapsilosis*
Lab 11.1	3±0	7.5±0.70	1±11.3	9.5±3.55
Lab 11.2	nd	nd	nd	nd
Lab 10	nd	nd	nd	nd
Lab 9	1±0.2	5.5±4.0	0.5±10.2	7±4.22
Ped 2	nd	nd	nd	nd
Ped 3	nd	Nd	nd	1±0.7
A19	1.5±13.0	0.5±4.27	nd	nd
A21	nd	nd	nd	nd

*Inhibition area measured in mm

Table 5. Calculation of the inhibition zone of lactic bacteria isolated from Kombucha on pathogenic strains tested by radial streak method

Lactic bacteria strain	*Candida albicans*	*Candida guilliermondii*	*Candida krusei*	*Candida parapsilosis*
S1	nd	4±0.82	6±1.41	5.5±0.94
S2	nd	5.5±0.47	4.5±0.47	3.5±0.94
S3	nd	4±0.82	6.5±0.94	3.5±0.94
L3	13.5±2.87	5.5±4.0	10±0.82	11.5±2.05
L5	17±1.41	14.5±0.94	14±3.56	4.5±0.47
F1	3.5±2.87	5±0.82	4.5±0.94	5.5±4.03
F2	nd	4.5±0.94	5±0	4.5±0.94

Figure 2. Inhibitory activity of lactic bacteria isolated from Kombucha on *Candida* species tested by radial streak method (left) and of L3 and L5 strains by transversal method

All strains of isolated lactic bacteria in kombucha shows anti-*Candida* activity. Although the transversal method mean inhibition is high for most lactic strains, the average standard deviation is high. Clearer results were obtained by radial streak method. Strains of lactic bacteria L3 (*Pediococcus pentosaceus*) and L5 (*Pediococccus acidilactici*) had the most promising results, inhibiting in varying proportions all tested pathogens. L3 presented a high inhibitory action against *C. albicans*, *C. krusei* and *C. parapsilosis* and lower against *C. guilliermondii*. L5 presented a high inhibitory action against *C. albicans*, *C. krusei* and *C. guilliermondii* and lower against *C. parapsilosis*. Following the preliminary results on the inhibitory activity of individual strains, in the next step have been tested the anti-*Candida* potential of mixed suspensions of positive tested Lab strains. Different combinations of L3 (*Pediococcus pentosaceus*), L5 (*Pediococcus acidilactici*) and Lab 11.1 (*Lactobacillus farciminis*) have been employed in a 1:1 ratio: L3 + L5, L3 + Lab 11.1, L5 + Lab 11.1 and Lab 11.1 + L3 + L5 (1:1:1). As seen in Figure 3, the inhibitory activity is kept in different mixture, but the inhibition zone (halo formation) of the single strains is not different than the mixture. By spot method, the highest inhibition activity, in single or mixed strains, was noticed in the case of *C. guilliermondii*, followed equally by *C. parapsilosis* and *C. albicans.*

Our results are confirming and/or completing other reports on anti-*Candida* activity of LAB strains. For instance, strains of *Pediococcus acidilactici* and *Pediococcus pentosaceus*, isolated from honey, have been reported to have significant anti-*Candida* activity (Bulgasem et al., 2013) against *C. krusei*, *C. glabrata* and *C. albicans.* Also, Kim and Kang (2019) have reported that ell-free supernatants of a probiotic *Pediococcus acidilactici* isolated from malt has inhibitory activity on *C. albicans.* Such strains, of *Pediococcus acidilactici* have been recently reported to be used as bread preservative (Bustos et al., 2018). No clear data, on our knowledge, have been reported in regard to *Lactobacillus farciminis* potential to inhibit the *Candida* and candidiasis.

Table 6. Calculation of the inhibition zone of lactic bacteria isolated from kombucha on pathogenic strains tested by transversal method

Lactic bacteria strain	*Candida albicans*	*Candida guilliermondii*	*Candida krusei*	*Candida parapsilosis*
S1	6.5±0.94	5.5±2.49	5.5±1.41	4±6.48
S2	8±4.32	6.5±2.62	12±0.82	3.5±2.05
S3	8±2.87	5.5±2.87	8±5.89	4±2.94
L3	7±1.41	7.5±0.94	15.5±1.89	8.5±1.25
L5	9±0	6.5±1.25	16±2.83	5±2.45
F1	8.5±2.36	5.5±2.49	9±6.48	5.5±4.11
F2	10.5±5.44	5.5±5.19	7±9.90	12.5±11.59

*Inhibition area measured in mm

Figure 5. Inhibitory activity of LAB mixtures on *C. albicans*, *C. guilliermondii*, *C. parapsilosis* and *C. krusei* (from left to right)

CONCLUSIONS

Different LAB strains isolated from fermented cocoa beans and microbial consortium of Kombucha beverages have tested for their anti-*Candida* activity.

Significant inhibition was shown by strains belonging to the following species *Lactobacillus farciminis, Pediococcus pentosaceus, Pediococcus acidilactici* and *Weisella cibaria*.

Mixed supernatant of cultivated LAB haven't increased the inhibitory activity and the reasons are to be discussed and found.

The most inhibited specie among the tested one, were, in order of their inhibition, *C. guilleirmondii, C. parapsilosis* and *C. krusei*.

The LAB strains will be further tested for their probiotic potential as well as for their antioxidative activity and will be subject of new pharmaceutical/cosmetic novel products development.

REFERENCES

Balouiri, M., Sadiki, M., Ibnsouda, S. K. (2016). Methods for *in vitro* evaluating antimicrobial activity: A review. *Journal of Pharmaceutical Analysis*, 6(2), 71–79.

Bulgasem, Y., Mohd, N. L., Zaiton, H., Wan, M. W. Y., Sumaya, G. F. (2016). Antifungal Activity of Lactic Acid Bacteria Strains Isolated from Natural Honey against Pathogenic *Candida Species*. Mycobiology, 44(4), 302–309.

Bustos, A.Y., Font de Valdez, G., Gerez, C. L. (2018). Optimization of phenyllactic acid production by Pediococcus acidilactici CRL 1753. Application of the formulated bio-preserver culture in bread. Biological Control, 123, 137–143.

Coman, M. M., Verdenelli, M. C., Cecchini, C., Silvi, S., Orpianesi, C., Boyko, N., Cresci, A. (2014). *In vitro* evaluation of antimicrobial activity of *Lactobacillus rhamnosus* IMC 501®, *Lactobacillus paracasei* IMC 502® and SYNBIO® against pathogens. *Journal of Applied Microbiology*, 117, 518–527.

Ionescu, A. D., Casarica, A., Boca, E., Nita, S., Rasit, I., Vamanu, A., Vamanu, E. (2013). Studies concerning the characterisation of a probiotic preparation,

containing a mixture of Lactobacillus pure biomass and media with pollen and honey. *AgroLife Scientific Journal*, 2(1), 113–116.

Kim, H., Kang, S. S. (2019). Antifungal activities against *Candida albicans*, of cell-free supernatants obtained from probiotic *Pediococcus acidilactici* HW01. Archives of Oral Biology, 99, 113–119.

Olaru, D., Popa, E. M. (2019). In vitro research on the inhibitory effects of fennel, sage and seabuckthorn essenmtial oils on some food spoilage fungi. *Scientific Bulletin. Series F. Biotechnologies*, XXIII, 87–90.

Sanguinetti, M., Posteraro, B., Lass-Flörl, C. (2015). Antifungal drug resistance among *Candida* species: mechanisms and clinical impact. *Mycoses*, 58(2), 2–13.

Sardi, J. C. O., Scorzoni, L., Bernardi, T., Fusco-Almeida, A. M., Mendes, G. M. J. S. (2013). *Candida* species: current epidemiology, pathogenicity, biofilm formation, natural antifungal products and new therapeutic options. *Journal of Medical Microbiology*, 62, 10–24.

Silva, M. P., Rossoni, R. D., Junqueira, J. C., Jorge, A. O. C. (2016). Probiotics for Prevention and Treatment of Candidiasis and Other Infectious Diseases: *Lactobacillus* spp. and Other Potential Bacterial Species. *Probiotics and Prebiotics in Human Nutrition and Health*, 12, 241–262.

Spampinato, C., Leonardi, D. (2013). *Candida* Infections, Causes, Targets, and Resistance Mechanisms: Traditional and Alternative Antifungal Agents. *BioMed Research International*, 204237, 1–13.

Terpou, A., Mantzourani, I., Galanis, A., Kanellaki, M., Bezirtzoglou, E., Bekatorou, A., Koutinas, A. A., Plessas, S. (2019). Employment of *L. paracasei* K5 as a Novel Potentially Probiotic Freeze-Dried Starter for Feta-Type Cheese Production. *Microorganisms*, 7(3), 1–17.

Yapar, N. (2014). Epidemiology and risk factors for invasive candidiasis. *Therapeutics and Clinical Risk Management*, 10, 95–105.

Vamanu, E., Voica, A. (2017). Total phenolic analysis, antimicrobial and antioxidant activity of some mushroom tinctures from medicinal and edible species, by *in vitro* and *in vivo* tests. *Scientific Bulletin. Series F. Biotechnologies*, XXI, 318–324.

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COMPARISON OF CHEMICAL COMPOSITION AND ANTIOXIDANT ACTIVITY OF SOME ESSENTIAL OILS FROM ROMANIAN MARKET

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Abstract

Essential oils have been used in aromatherapy for centuries because they are easily found in plants and they are usually extracted through a popular method named water distillation. The present study investigates the chemical composition and antioxidant activity of some essential oils. The investigated oils are Basil oil (Ocimum basilicum), Rosemary oil (Rosmarinus officinalis) and Peppermint oil (Mentha piperita and Mentha arvensis). They were purchased from Romanian market and they are produced by four different manufacturers. The main volatile constituents of these essential oils are analyzed by Gas Chromatography coupled with Mass Spectrometry Detector (GC-MSD) and Gas Chromatography coupled with a Flame Ionized Detector (GC-FID). The main compounds detected in Basil essential oil were Estragol and β-Linalool, in Rosemary oil were Eucalyptol (1.8-Cineole), d-Camphor and α-Pinene and in Peppermint oil were Menthan-1-ol and I-Menthone. The antioxidant activity of these oils is evaluated using DPPH method. The highest value for antioxidant activities of Basil essential oil is IC_{50} = 49.17 mg/ml, for Rosemary essential oil is IC_{50} = 14 mg/m and for Peppermint essential oil is IC_{50} = 69.65 mg/ml.

Key words*: essential oils, aromatherapy, chemical compounds, GC-MS, DPPH.*

INTRODUCTION

Essential oils have been used in aromatherapy for centuries. The geographic locations and environmental conditions influence the composition of essential oils, which can vary. Essential oils are obtained from the plant most active parts, such as flowers, seeds, leaves, herbs and roots, and it can be obtained by various extraction methods.

Nowadays, water distillation is the most used extraction method for commercial production. This extraction method is applied to the plant material using selective solvents and standard procedures (Badal & Delgoda, 2016).

Lamiaceae family is one of the most distinguished and largest families of aromatic plants. It includes about 235 genera and 7 000 species worldwide (Andrade et al., 2018). Basil (*Ocimum basilicum),* Peppermint (*Mentha piperita and Mentha arvensis)* and Rosemary (*Rosmarinus officinalis)* belong to this family. These flowered plants are cultivated for their aromatic properties and their commercial use. For example, these herbs are used in gastronomy as seasonings; as flavours in juices; essential oils are used in aromatherapy and in relaxing massage; sanitary articles like tooth paste; in food supplements like sore throat tablets and intestinal tympanites (Dunning, 2013).

Essential oils are consisting of a mixture of phenols, iridoids esters, unsaturated hydrocarbons, saturated hydrocarbons, ethers, ketones, alcohols, aldehydes and terpenes. Essential oils are colorless and they have a pleasant characteristic odour (Ziosi et al., 2013).

The present study investigates the chemical composition and antioxidant activity of some commercially available essential oils: Basil oil, Rosemary oil and Peppermint oil.

MATERIALS AND METHODS

P_1-Basil oil (*Ocimum basilicum*), P_2-Rosemary oil (*Rosmarinus officinalis*) and P_3-Peppermint oil (*Mentha piperita* and *Mentha arvensis*) were purchased from Romanian market and they are produced by four different manufacturers.

Essential oil composition depends on the plant genotype and the environmental factory like soil properties, altitude, wind, sun, humidity, plant age and harvesting methods and on the used extraction method.

The tested oils were produced by four manufacturers named in this article as A, B, C and D and the essential oils are named as: P_1 for Basil essential oil, P_2 for Rosemary essential oil and P_3 for Peppermint essential oil.

The main volatile constituents of these essential oils are analyzed by Gas Chromatography coupled with Mass Spectrometry Detector (GC-MSD) and Gas Chromatography coupled with a Flame Ionized Detector (GC-FID).

Gas Chromatography coupled with a Flame Ionized Detector (GC-FID) is a quantitative method. Gas Chromatography coupled with Mass Spectrometry Detector (GC-MSD) is a qualitative method.

The Gas Chromatography (GC) method was performed on Agilent GC 7890A chromatograph using PHENOMENEX capillary column named ZEBRON (ZB)-5ms, an inlet temperature at 250ºC, a 50:1 split ratio and a 0.8 mL/min Helium flow rate (Deleanu et al., 2018). Temperature of the GC column was kept at 50ºC for 1 min. Then, the temperature gradually increased with the gradients of 8ºC/min up to 100ºC and was kept constant for 2 minute, 2°C/min up to 110°C was kept constant for 2 minute, 5°C/min up to 185ºC and 30°C/min up to 280°C was kept constant for 10 minute. Mass Spectrometry Detector (GC-MSD) is a qualitative method. The Mass Spectrometry (MS) detection was performed on Triple Quad MS Agilent 7000A detector and ZEBRON (ZB)-5 ms plus (30 m × 0.25 i.d. mm, 0.25 film thickness μm) from Phenomenex, capillary column (Deleanu et al., 2018).

The Electron Ionization (EI) is a direct process where the energy is transferred, by different methods like collision, from electrons to the sample molecules (Mirosław et al., 2015).

The Electron Ionization used was 70 eV (electron Volt). Transfer line temperature was 280°C, the source temperature was 230°C and the quadrupole temperature was 150°C. Chromatographic peaks were identified according to National Institute of Standards and Technology (NIST) database.

The antioxidant activity of these oils is evaluated using DPPH (2.2-diphenyl-1-picryl-hydrazyl radical) method. We use a 0.3 mM DPPH ethanol solution mixed with 125 μL of the diluted oils in a 96-well clear microplate (Deleanu et al., 2018).

The control used at this analysis is a 50 μL DPPH solution mixed with 125 μL ethanol. The microplate with the oil samples and the control sample were incubated in dark, at room temperature, for 30 minutes. The plates were read at 518 nm at TECAN M200 INFINITE PRO MICROPLATE READER. The antiradical activity (AA) of the oil samples were determined using the formula:

$$AA\% = 100 - \left(\frac{(A_{SD} - A_{SWD})\ x\ 100}{A_C}\right)$$

where:
AA = Antiradical Activity;
A_{SD} = Absorbance of the oil sample with DPPH;
A_{SWD} = Absorbance of the oil sample without DPPH;
A_C = Absorbance of the control sample.

RESULTS AND DISCUSSIONS

The chemical composition of these essential oils was analyzed by Gas Chromatography coupled with Mass Spectrometry Detector (GC-MSD) and Gas Chromatography coupled with a Flame Ionized Detector (GC-FID).

The compounds concentrations of the studied essentials oils and the Retention Time (RT) are presented in Tables 1-3. The GC-MSD and GC-FID chromatograms of these essential oils are presented in Figures 1-12.

Twenty chemical compounds, representing 99.85% of P_1A, were identified as major like Estragole (75.54%), β-Linalool (17.86%), 1.5.9.9-Tetramethyl-1.4.7-cycloundecatriene (1.87%), and minor compounds like citral (0.7%), α-Bergamotene(0.59%), Caryophyllene (0.51%), β-Citral (0.46%), β-Cubebene (0.37%), Isomenthol (0.36%), cis-β-Farnesene (0.25%), Humulene (0.24%), 3-Carene (0.16%), β-Pinene (0.15%), D-Limonene (0.14%), Eucalyptol (0.14%), l-Menthone (0.12%), 3-Methoxycinnamaldehyde (0.11%), 2.7-Dimethyl-2.6-octadien-1-ol (0.1%), α-Pinene (0.09%) and β-Bisabolene (0.09%).

Table 1. The chemical composition of Basil (*Ocimum basilicum*) essential oils

No	Compound	RT min	P_1A%	P_1B %	P_1C %	P_1D%
1	α-Pinene	7.87	0.09	0.07	0.10	0.08
2	β-Pinene	8.86	0.15	0.25	0.15	0.16
3	2-Ethyl-1-hexanol	9.87	-	0.12	0.60	-
4	Cymene	9.92	-	0.06	-	-
5	D-Limonene	10.05	0.14	0.06	0.28	0.22
6	Eucalyptol	10.17	0.14	0.18	0.10	0.10
7	3-Carene	10.40	0.16	0.15	0.13	0.11
8	β-Linalool	12.0	17.8	18.0	17.6	18.7
9	l-Menthone	14.19	0.12	-	-	0.09
10	Isomenthol	15.05	0.36	0.19	0.13	0.48
11	Estragole	16.1	75.5	76.2	76.4	75.4
12	β-Citral	17.31	0.46	0.39	0.33	0.37
13	2.7-Dimethyl-2.6-octadien-1-ol	17.69	0.1	0.07	-	-
14	Citral	18.37	0.7	0.61	-	0.55
15	Caryo-phyllene	23.4	0.51	0.41	0.35	0.40
16	α-Bergamotene	23.7	0.59	0.49	0.49	0.47
17	cis-β-Farnesene	24.2	0.25	-	0.25	0.19
18	Humulene	24.5	0.24	0.19	0.18	0.19
19	β-Cubebene	25.1	0.37	0.35	0.24	0.28
20	β-Bisabolene	25.8	0.09	0.08	-	-
21	1.5.9.9-Tetramethyl-1.4.7-cyclounde-catriene	26.6	1.87	1.60	1.43	1.56
22	3-Methoxycinnam aldehyde	27.4	0.11	0.21	0.38	0.19
23	Caryo-phyllene oxide	27.8	-	0.06	0.11	0.07
Total		-	**99.85**	**99.88**	**99.32**	**99.77**

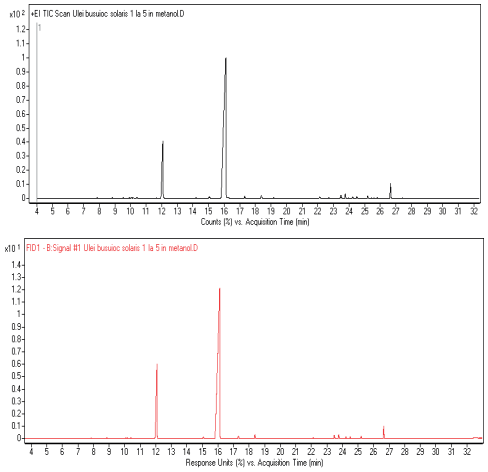

Figure 1. GC-MS and GC-FID chromatogram of P_1A

Twenty-one chemical compounds, representing 99.88% of P_1B, were identified as major like Estragole (76.26%), β-Linalool (18.08%), 1.5.9.9-Tetramethyl-1.4.7-cycloundecatriene (1.6%) and minor compounds like Citral (0.61%), α-Bergamotene (0.49%), Caryophyllene (0.41%), β-Citral (0.39%), β-Cubebene (0.35%), β-Pinene (0.25%), 3-Methoxycinnamaldehyde (0.21%), Isomenthol (0.19%), Humulene (0.19%), Eucalyptol (0.18%), 3-Carene (0.15%), 2-Ethyl-1-hexanol (0.12%), β-Bisabolene (0.08%), α-Pinene (0.07%), 2.7-Dimethyl-2.6-octadien-1-ol (0.07%), D-Limonene (0.06%), Cymene (0.06%) and Caryophyllene oxide (0.06%).

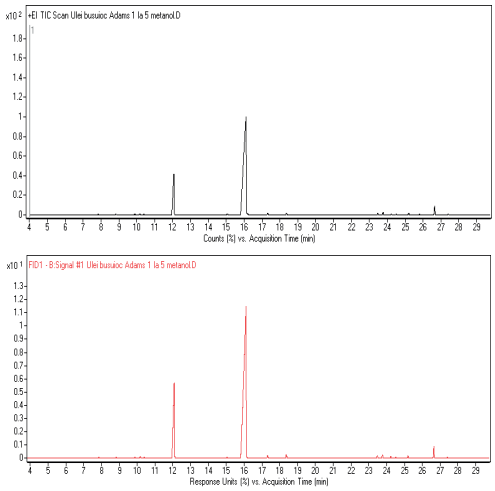

Figure 2. GC-MS and GC-FID chromatogram of P_1B

Seventeen chemical compounds, representing 99.32% of P_1C, were identified as major like Estragole (76.41%), β-Linalool (17.66%), 1,5,9,9-Tetramethyl-1,4,7-cycloundecatriene (1.43%) and minor compounds like 2-Ethyl-1-hexanol (0.6%), α-Bergamotene (0.49%), 3-Methoxycinnamaldehyde (0.38%), Caryophyllene (0.35%), β-Citral (0.33%), D-Limonene (0.28%), cis-β-Farnesene (0.25%), β-Cubebene (0.35%), Humulene (0.18%), β-Pinene (0.15%), 3-Carene (0.13%), Isomenthol (0.13%), Caryophyllene oxide (0.11%) and Eucalyptol (0.1%).

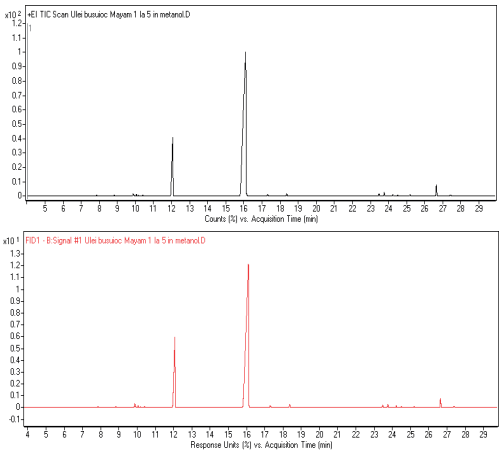

Figure 3. GC-MS and GC-FID chromatogram of P_1C

Nineteen chemical compounds, representing 99.77% of P_1D, were identified as major like Estragole (75.48%), β-Linalool (18.78%) and minor compounds like Citral (0.55%), Isomenthol (0.48%), α-Bergamotene (0.47%), Caryophyllene (0.4%), β-Citral (0.37%), β-Cubebene (0.28%), D-Limonene (0.22%), cis-β-Farnesene (0.19%), Humulene (0.19%), 3-Methoxycinnamaldehyde (0.19%),%), β-Pinene (0.16%),3-Carene (0.11%), Eucalyptol (0.1%), l-Menthone (0.09%), α-Pinene (0.08%) and Caryophyllene oxide (0.07%).

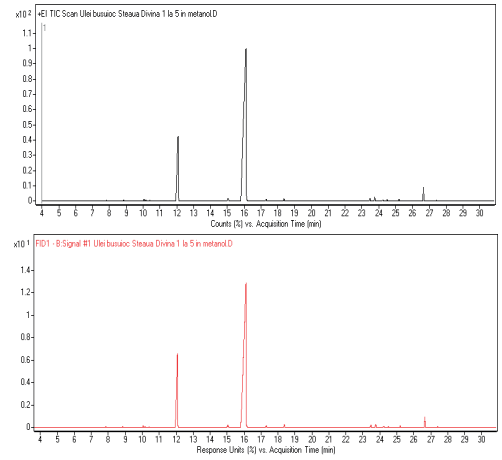

Figure 4. GC-MS and GC-FID chromatogram of P_1D

According to the GC-MS and GC-FID results obtained for the Basil essential oil, the P_1-Basil (*Ocimum basilicum*) essential oil compounds are representing approximately 99% of total composition. P_1B has twenty-one chemical compounds P_1A has twenty, P_1D has nineteen and P_1C has seventeen chemical compounds. The main compounds detected in P_1-Basil essential oil were: Estragol and β-Linalool. P_1C (76.41%) has the most Estragole, then P_1B (76.26), P_1A (75.54%) and P_1D (75.48%), followed by the second largest component Linalool with more than 17%.

Avetisyan et al. (2017) has reported that the main chemical constituents of Basil essential oil of aromatic plant origin include monoterpenes like Estragole/Methyl chavicol (more than 57.3%), β-Linalool (more than 18%), sesquiterpenes like α-Bergamotene, Humulene and their oxygenated derivatives.

Stanojevic et al. (2019) found in his study that the most abundant component in Basil essential oil was β-Linalool (39.9 %).

According to Abbasy et al. (2015) a total of thirty six chemical constituents were identified in Basil essential oil and Linalool (69.87%) was found to be the major constituent. Other main identified constituents included Geraniol (9.75%), 1.8-Cineole (4.90%) and α-Bergamotene (2.36%).

Table 2. The chemical composition of Rosemary (*Rosmarinus officinalis*) essential oils

No	Compound	RT min	P_2A %	P_2B %	P_2C %	P_2D %
1	Tricyclene	7.64	0.58	1.68	0.32	-
2	α-Pinene	7.87	7.54	24.7	14.5	2.45
3	d-Camphene	8.19	-	1.88	-	0.32
4	Camphene	8.24	1.69	6.61	4.80	1.82
	β-Phellandrene	8.68	4.78	-	-	-
5	Isocamphane	8.72	-	0.2	0.21	-
6	L-β-Pinene	8.86	2.41	4.79	5.28	0.63
7	β-Pinene	8.95	1.98	2.36	1.25	0.20
8	α-Phellandrene	9.01	0.53	0.13	-	0.22
9	3-Carene	9.55	-	4.57	0.14	-
10	Isocineole	9.66	-	0.11	-	2.19
11	α-Terpinene	9.73	0.84	0.11	0.43	5.13
12	Cymene	9.92	0.89	2.90	1.26	10.02
13	D-Limonene	10.0	3.98	4.75	2.05	21.68
14	Eucalyptol	10.17	36.07	19.55	41.67	23.09
15	γ-Terpinene	10.8	0.62	0.19	0.63	0.20
16	Terpinolene	11.6	-	0.78	0.29	
17	Fenchone	11.8	-	0.52	-	
18	β-Linalool	12.0	1.10	2.60	0.87	1.38
19	d-Camphor	13.9	22.4	12.5	15.4	19.03
20	Isoborneol	14.5	0.74	3.57	-	1.83
21	endo-Borneol	14.8	0.50	0.48	2.91	3.06
22	Terpinen-4-ol	15.2	1.76	-	0.63	-
23	α-Terpineol	15.7	5.01	2.55	2.07	2.79
24	γ-Terpineol	15.8	-	0.73	-	1.03
25	Borneol acetate	19.1	-	0.95	0.44	0.96
26	safrole	19.2	5.62	-		
27	Caryophyllene	23.2	-	0.24	3.88	1.69
28	(±)-trans-Nerolidol	27.1	0.95	-	-	-
Total		-	**99.99**	**99.57**	**99.06**	**99.72**

Twenty chemical compounds, representing 99.99% of P_2A, were identified as major like Eucalyptol (36.07%), d-Camphor (22.4%), α-Pinene (7.54%), safrole (5.62%), α-Terpineol (5.01%), β-Phellandrene (4.78%), D-Limonene (3.98%), L-β-Pinene (2.41%), β-Pinene (1.98%), Terpinen-4-ol (1.76%), Camphene (1.69%), β-Linalool (1.1%) and minor compounds like (±)-trans-Nerolidol (0.95%), Cymene (0.89%), α-Terpinene (0.84%), Isoborneol (0.74%), γ-Terpinene (0.62%), Tricyclene (0.58%), α-Phellandrene (0.53%) and endo-Borneol (0.5%).

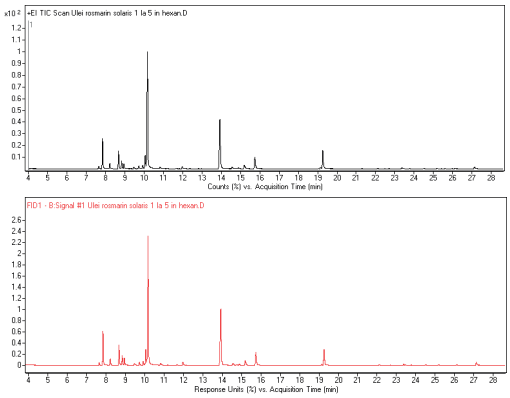

Figure 5. GC-MS and GC-FID chromatogram of P_2A

Twenty-five chemical compounds, representing 99.57% of P_2B, were identified as major like d-Camphene (24.76%), α-Terpineol (19.55%), Terpinen-4-ol (12.56%), 3-Carene (6.61%), Fenchone (4.79%), Safrole (4.75%), Caryophyllene (4.57%), Camphene (3.57%), α-Pinene (2.9%), β-Pinene (2.6%), Cymene (2.55%), γ-Terpineol (2.36%), Isocamphane (1.88%), Tricyclene (1.68%) and minor compounds like Isoborneol (0.95%), D-Limonene (0.78%), α-Terpinene (0.73%), L-β-Pinene (0.52%), β-Linalool (0.48%), α-Phellandrene (0.24%), Terpinolene (0.2%), β-Phellandrene (0.19%), Borneol acetate (0.13%), Eucalyptol (0.11%) and d-Camphor (0.11%).

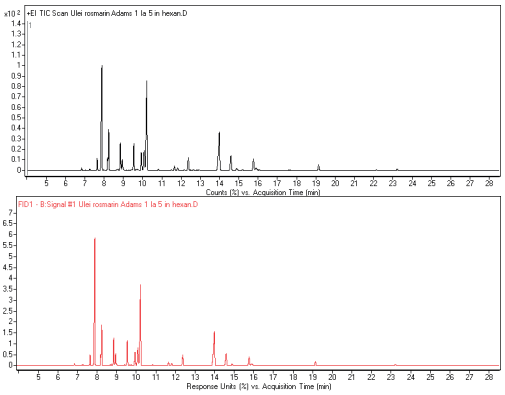

Figure 6. GC-MS and GC-FID chromatogram of P_2B

Twenty chemical compounds, representing 99.06% of P_2C, were identified as major like Eucalyptol (41.67%), d-Camphor (15.4%), α-Pinene (14.53%), L-β-Pinene (5.28%), Camphene (4.8%), Caryophyllene (3.88%), endo-Borneol (2.91%), α-Terpineol (2.07%), D-Limonene (2.05%), Cymene (1.26%), β-Pinene (1.25%) and minor compounds like β-Linalool (0.87%), γ-Terpinene (0.63%), Terpinen-4-ol (0.63%), Borneol acetate (0.44%), α-Terpinene (0.43%), Tricyclene (0.32%), Terpinolene (0.29%), Isocamphane (0.21%) and 3-Carene (0.14%).

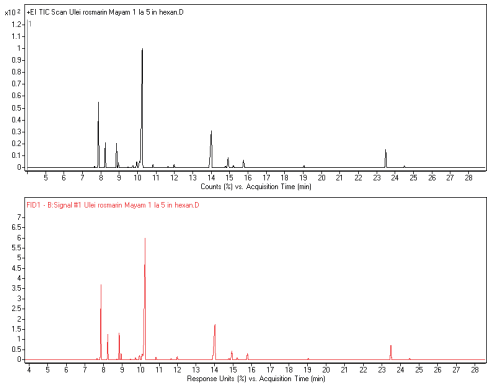

Figure 7. GC-MS and GC-FID chromatogram of P_2C

Twenty chemical compounds, representing 99.72% of P_2D, were identified as major like Caryophyllene (23.09%), Camphene (21.68%), β-Pinene (19.03%), L-β-Pinene (10.02%), α-Pinene (5.13%), γ-Terpinene (3.06%), Borneol acetate (2.79%), d-Camphene (2.45%), d-Camphor (2.19%), β-Linalool (1.83%), α-Phellandrene (1.82%), 3-Carene (1.69%), Cymene (1.38%), α-Terpinene (1.03%) and minor compounds like Terpinolene (0.96%), Isoborneol (0.63%), β-Phellandrene (0.32%), (±)-trans-Nerolidol (0.22%), γ-Terpineol (0.2%) and endo-Borneol (0.2%).

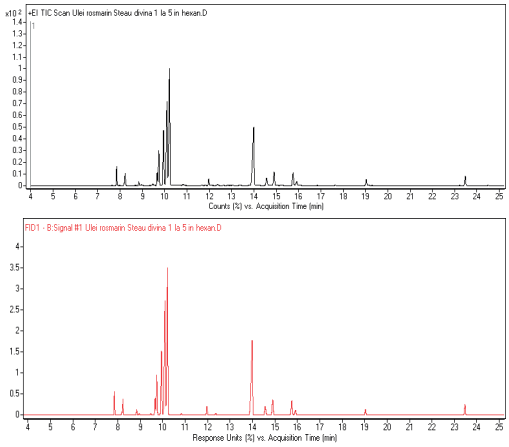

Figure 8. GC-MS and GC-FID chromatogram of P_2D

P_2-Rosemary (*Rosmarinus officinalis*) essential oil compounds are representing approximately 99% of total composition. P_2B has twenty-five chemical compounds, P_2A, P_2C and P_2D has twenty chemical compounds. The main compounds detected in P_2-Rosemary essential oil were: Eucalyptol (1.8-Cineole), d-Camphor and α-Pinene. P_2C (41.67%) has the most Eucalyptol, then P_2A (36.07%), P_2D (23.09%) and P_2B (19.55%).

Jiang et al. (2011) reported that Rosemary essential oil has twenty-two chemical constituents like 1.8-Cineole (26.54%) and α-Pinene (20.14%). Since 2007, Djeddi et al. reported that it can be distinguished two major types of Rosemary oil: the one with over 40% of 1.8-cineole, which is characteristic of oils from Morocco, Tunisia, Turkey, Greece, Serbia, Italy, and France, and oils with approximately equal ratios (20-30%) of 1.8-Cineole, α-Pinene, and Camphor (oils from France, Spain, Italy, Greece, Bulgaria). This difference could be due to the different climates between south Europe and North Africa Mediterranean areas: higher percentage mean monthly temperatures and longer sunshine duration.

Silveira et al. (2012) found that the essential oils of Basil and Rosemary have a great potential for utilization as natural antimicrobial agents in foods.

Table 3. The chemical composition of Peppermint (*Mentha piperita* and *Mentha arvensis*) essential oils

No	Compound	RT min	P_3A %	P_3B %	P_3C %	P_3D %
1	α-Pinene	7.87	3.77	-	0.61	2.46
2	Camphene	8.24	0.55	-	-	0.26
3	β-Phellandrene	8.68	1.11	-	0.24	0.37
4	L-β-Pinene	8.86	5.46	-	0.83	1.99
5	β-Pinene	8.95	2.19	-	0.20	0.54
6	3-octanol	9.11	1.32	-	0.22	1.25
7	2-Menthene	9.35	0.75	-	-	-
8	3-Carene	9.55	2.53	-	-	-
9	α-Terpinene	9.73	0.91	-	-	-
10	Cymene	9.92	9.91	0.04	0.19	0.50
11	D-Limonene	10.05	17.53	2.15	2.61	4.23
12	Eucalyptol	10.17	5.67	0.13	4.77	0.23
13	γ-Terpinene	10.82	6.73	-	-	-
14	Terpinolene	11.65	3.01	-	-	-
15	Fenchone	11.81	0.36	-	-	-
16	Isopulegol	13.93	0.38	0.05	-	1.45
17	I-Menthone	14.23	4.12	28.5	24.98	21.05
18	O-Menthone	14.45	-	0.10	1.05	0.29
19	D-Menthone	14.59	1.67	10.3	3.81	9.93
20	Neo-menthol	14.75	1.13	2.09	3.69	4.56
21	p-Menthan-1-ol	15.2	28.2	50.1	44.50	41.79
22	Isomenthol	15.68	-	0.96	1.35	0.63
23	(±)-Pulegone	17.43	0.35	0.04	0.45	1.06
24	Isomenthol acetate	19.19	0.90	3.49	5.94	3.83
25	Caryophyllene	23.22	-	1.08	3.61	0.86
26	Germa-crene D	25.19	-	-	0.18	0.30
27	Caryo-phyllene oxide	27.85	-	0.10	-	-
Total		-	**98.62**	**99.31**	**99.23**	**97.58**

Twenty-two chemical compounds, representing 98.62% of P_3A, were identified as major like p-Menthan-1-ol (28.27%), D-Limonene (17.53%), Cymene (9.91%), γ-Terpinene (6.73%), Eucalyptol (5.67%), L-β-Pinene (5.46%), I-Menthone (4.12%), α-Pinene (3.77%), Terpinolene (3.01%), 3-Carene (2.53%), β-Pinene (2.19%), D-Menthone (1.67%), 3-octanol (1.32%), Neo-menthol (1.13%), β-Phellandrene (1.11%) and minor compounds like α-Terpinene (0.91%), Isomenthol acetate (0.90%), 2-Menthene (0.75%), Camphene (0.55%), Isopulegol (0.38%), Fenchone (0.36%) and (±)-Pulegone (0.35%).

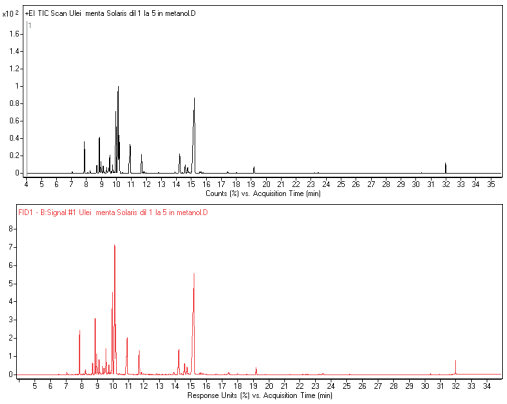

Figure 9. GC-MS and GC-FID chromatogram of P_3A

Fourteen chemical compounds, representing 99.31% of P_3B, were identified as major like p-Menthan-1-ol (50.19%), I-Menthone (28.57%), D-Menthone (10.32%), Isomenthol acetate (3.49%), D-Limonene (2.15%), Neo-menthol (2.09%), Caryophyllene (1.08%) and minor compounds like Isomenthol (0.96%), Eucalyptol (0.13%), O-Menthone (0.10%), Caryo-phyllene oxide (0.10%), Isopulegol (0.05%), (±)-Pulegone (0.04%) and Cymene (0.04%).

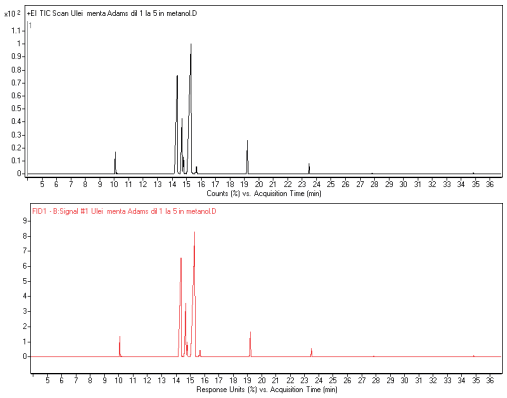

Figure 10. GC-MS and GC-FID chromatogram of P_3B

Eighteen chemical compounds, representing 99.23% of P_3C, were identified as major like p-Menthan-1-ol (44.50%), I-Menthone (24.98%), Isomenthol acetate (5.94%), Eucalyptol (4.77%), D-Menthone (3.81%), Caryophyllene (3.61%), Neo-menthol (3.69%), D-Limonene (2.61%), Isomenthol (1.35%), O-Menthone (1.05%) and minor compounds like L-β-Pinene (0.83%), α-Pinene (0.61%), (±)-Pulegone (0.45%), β-Phellandrene (0.24%), 3-octanol (0.22%), β-Pinene (0.20%), Cymene (0.19%), Germa-crene D (0.18%).

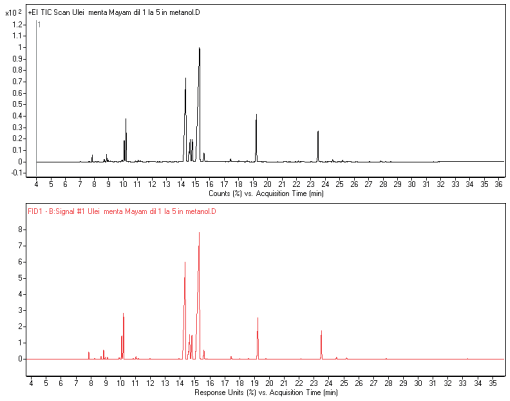

Figure 11. GC-MS and GC-FID chromatogram of P_3C

Twenty chemical compounds, representing 97.58% of P_3D, were identified as major like p-Menthan-1-ol (41.79%), I-Menthone (21.05%), D-Menthone (9.93%), Neo-menthol (4.56%), D-Limonene (4.23%), Isomenthol acetate (3.83%), α-Pinene (2.46%), L-β-Pinene (1.99%), Isopulegol (1.45%), 3-octanol (1.25%), (±)-Pulegone (1.06%) and minor compounds like Caryophyllene (0.86%), Isomenthol (0.63%), β-Pinene (0.54%), Cymene (0.50%), β-Phellandrene (0.37%), Germa-crene D (0.30%), O-Menthone (0.29%), Camphene (0.26%), Eucalyptol (0.23%).

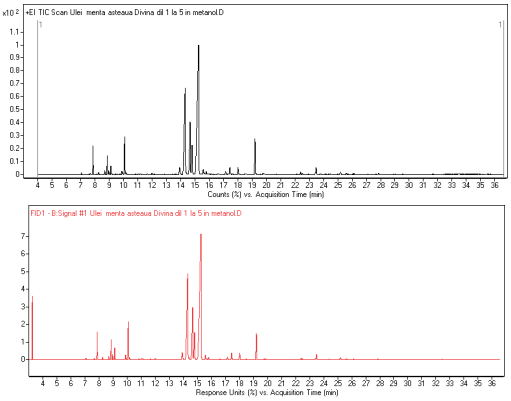

Figure 12. GC-MS and GC-FID chromatogram of P_3D

P_3-Peppermint (*Mentha piperita and Mentha arvensis*) essential oil compounds are representing 97-99% of total composition. P_3A has twenty-two chemical compounds, P_3D has

twenty, P_3C has eighteen and P_3B has fourteen chemical compounds. The main compounds detected in P_3-Peppermint essential oil were: Menthan-1-ol and I-Menthone. *Mentha arvensis* P_3B (50.19%) has the most Menthan-1-ol, then P_3C (44.50%), P_3D (41.79%) and P_3A (28.27%). *Mentha arvensis* from P_3B (28.57%) has the most I-Menthone, then P_3C (24.98%), P_3D (21.05%) and P_3A (4.12%). *Mentha arvensis* has more Menthan-1-ol and I-Menthone than *Mentha piperita.*

Beigi et al. (2018), reported that the main volatile compounds detected in the Peppermint essential oil were Menthol (44.39%), Menthone (15.36%), Menthofuran (10.27%), 1.8-Cineole (5.81%), Menthyl acetate (4.78%), Neoisomenthol (2.37%), and Limonene (1.87%). According to Pino et al. (2018) the major constituents in Peppermint grown in Jalisco (Italy) were identified to be Menthol (35.4%), Menthofuran (18.2%), Menthone (15.4%), and Menthyl acetate (12.4%).

Stanojevic et al. (2019) found in his study that the most abundant component in Peppermint oil was Menthol (45.4 %).

Dorin et al. (2017) identified in Peppermint oil high concentrations of Menthol (33%), Menthone (17%), Limonene (2%), Pulegone (1.8%) and Izomentil acetate (5%). Among sesquiterpene was determined β-Caryophyllene at a concentration of 1.2%

The antioxidant activity of essential oils is expressed as the Inhibitory Concentration (IC_{50}). This IC_{50} is defined as the concentration expressed in mg/ml, of the studied essential oils, required to inhibit the formation of DPPH radicals by 50%.

The antioxidant activities of P_1-Basil essential oils are presented in Figures 13 and 14. P_1A (IC_{50} = 49.17 mg/ml) exhibited a higher scavenging ability for DPPH radicals than the other three essential oils: P_1B (IC_{50} = 52.11 mg/ml), P_1C (IC_{50} = 61.15 mg/ml) and P_1D (IC_{50} = 75.17 mg/ml).

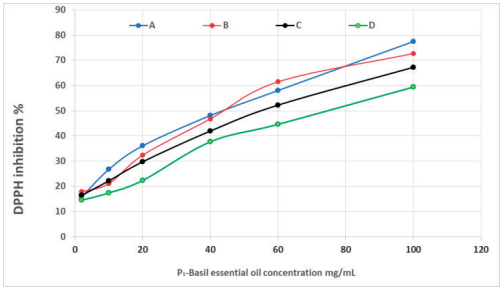

Figure 13. DPPH activity of P_1 in increasing concentrations

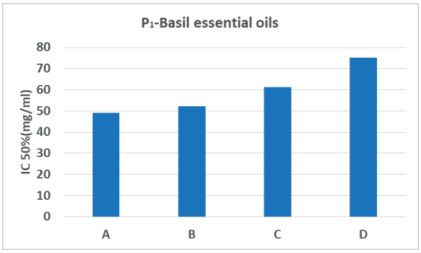

Figure 14. Inhibitory Concentration of P_1

The antioxidant activities of P_2-Rosemary essential oils are presented in Figures 15 and 16. P_2A (IC_{50} = 14 mg/ml) exhibited a higher scavenging ability for DPPH radicals than the other three essential oils: P_2B (IC_{50} = 224.93 mg/ml), P_2C (IC_{50} = 247.55 mg/ml) and P_2D (IC_{50} = 306.4 mg/ml).

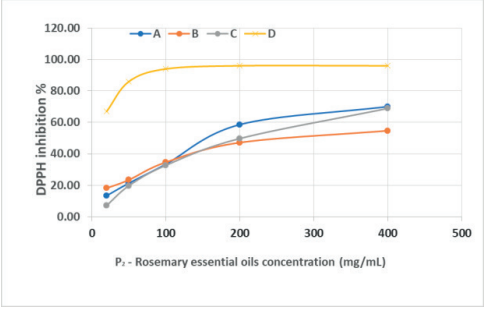

Figure 15. DPPH activity of P_2 in increasing concentrations

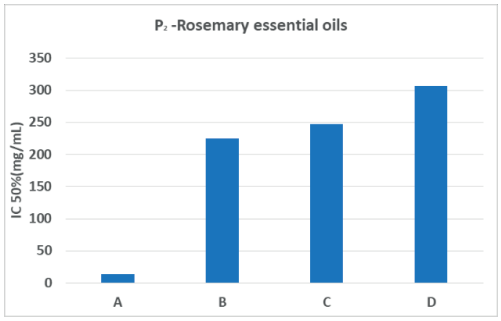

Figure 16. Inhibitory Concentration of P_2

The antioxidant activities of P_3-Peppermint essential oils are presented in Figures 17 and 18. P_3C (IC_{50} = 69.65 mg/ml) exhibited a higher scavenging ability for DPPH radicals than the other three essential oils: P_3D (IC_{50} = 82.62 mg/ml), P_3A (IC_{50} = 134.97 mg/ml) and P_3B (IC_{50} = 413.91 mg/ml).

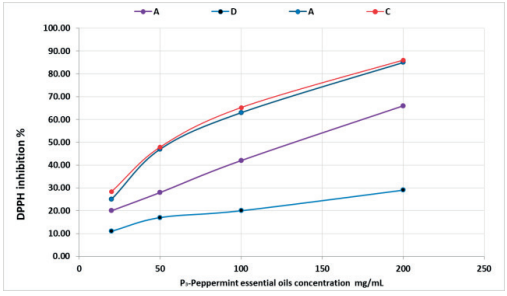

Figure 17. DPPH activity of P_3 in increasing concentrations

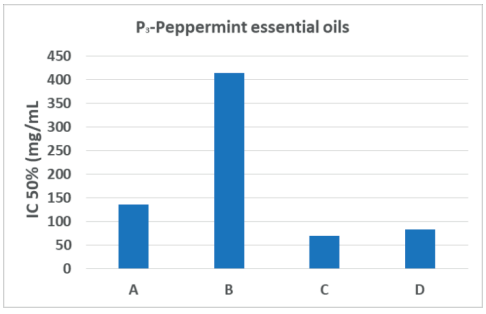

Figure 18. Inhibitory Concentration of P_3

The antioxidant activities of P_1-Basil essential oils is higher for P_1A (IC_{50} = 49.17 mg/ml) than for P_1B (IC_{50} = 52.11 mg/ml), P_1C (IC_{50} = 61.15 mg/ml) and P_1D (IC_{50} = 75.17 mg/ml).
The antioxidant activities of P_2-Rosemary essential oils is higher for P_2A (IC_{50} = 14 mg/ml) than for P_2B (IC_{50} = 224.93 mg/ml), P_2C (IC_{50} = 247.55 mg/ml) and P_2D (IC_{50} = 306.4 mg/ml).
The antioxidant activities of P_3-Peppermint essential oils is higher for P_3C (IC_{50} = 69.65 mg/ml) than for P_3D (IC_{50} = 82.62 mg/ml), P_3A (IC_{50} = 134.97 mg/ml) and P_3B (IC_{50} = 413.91 mg/ml).

CONCLUSIONS

In conclusion, the Basil essential oil with the most Estragole, the Rosemary essential oil with the most Eucalyptol and the Peppermint oil with the most Menthan-1-ol are produced by "C manufacturer". *Mentha arvensis* from "B manufacturer" has more Menthan-1-ol and I-Menthone than *Mentha piperita* from "C manufacturer". The antioxidant activities of Basil and Rosemary essential oils are higher for "A manufacturer". The antioxidant activities of Peppermint essential oils is higher for "C manufacturer". So, it is obviously that many extrinsic factors are influencing bioactivity of various essential oils available on market.

REFERENCES

Abbasy, D.W., Pathare, N., Al-Sabahi, J.N., Khan, S.A. (2015). *Chemical composition and antibacterial activity of essential oil isolated from Omani basil (Ocimum basilicum* Linn.*)*. Asian Pacific Journal of Tropical Disease, Vol. 5, Issue 8, pp. 645-649.

Andrade, J.M., Faustino, C., Garcia, C., Ladeiras, D., Reis, C.P., Rijo, P. (2018). *Rosmarinus officinalis* L.*: an update review of its phytochemistry and biological activity*. Future Science OA. Feb 1;4(4).

Avetisyan, A., Markosian, A., Petrosyan, M. (2017). *Chemical composition and some biological activities of the essential oils from basil Ocimum different cultivars*. BMC Complement Altern. Med. 17, 60.

Badal, McCreath S., Delgoda, R. (2016). *Pharmacognosy: fundamentals, applications and strategies*. Academic Press; London, UK.

Beigi, M., Torki-Harchegani, M., Pirbalouti, A.G. (2018). *Quantity and chemical composition of essential oil of peppermint (Mentha × piperita* L.*) leaves under different drying methods.* International Journal of Food Properties, Vol. 21, Issue 1, pp. 267-276.

Deleanu, M., Popa, E.E., Popa, M.E. (2018). *Composition and active property evaluation of ginger (Zingiber officinale-roscoe) and wild oregano (Origanum vulgare) essential oils*, Revista de Chimie (Bucharest), vol.69(8), pp.1927-1933

Djeddi, S., Bouchenah, N., Settar, I., Skaltsa, H.D. (2007). *Composition and antimicrobial activity of the essential oil of Rosmarinus officinalis from Algeria.* Chemistry of Natural Compounds, Vol. 43, No. 4.

Dorin, C., Dragomir, C., Popescu, C., Iancu, T., Stanciu, S., Luca, R., Hadaruga, N., Moatar, M., Nistor, E., Nicolae, I., Sala, F. (2017). *Study regarding the influence of fertilization on some physiological index and biochemical composition of peppermint oils (Mentha piperita* L*.).* Rom Biotechnol Lett. 2019; 24(4): 676-683.

Dunning, T. (2013). *Aromatherapy: Overview, safety and quality issues.* OA Alternative Medicine, vol. 1, pp. 6.

Jiang, Y., Wu, N.,Fu, Y.J., Wang, W., Luo, N., Zhao, C.J., Zu, Y.G., Liu, X.L. (2011). *Chemical composition and antimicrobial activity of the essential oil of Rosemary.* EnvironmentalToxicology and Pharmacology, Vol. 32, Issue 1, pp 63-68.

Mirosław, A.H., Skalicka-Woźniak, K., Świeboda, R., Niemiec, M., Stępak, K., Waksmundzka-Hajnos, M., Hawrył, A., Szymczak, G. (2015). *GC-MS fingerprints of mint essential oils,* Open Chemistry, vol. 13, issue 1

Pino, J.A., Borges, P.,Martinez, M.A., Vargas, M., Flores, H. (2018). *Essential Oil* of *Mentha piperita L.grown in Jalisco*. Journal of Essential Oil Research, 14(3), pp.189-190.

Silveira, S.M., Cunha Jr., A., Scheuermann, G.N., Secchi, F.L., Vieira, C.R.W. (2012). *Chemical composition and antimicrobial activity of essential oils from selected herbs cultivated in the South of Brazil against food spoilage and foodborne pathogens.* Cienc. Rural, Vol. 42 , no.7.

Stanojevic, L.P., Stanojevic, J.S., Savic, V. Lj., Cvetkovic, D. J., Kolarevic, A., Marjanovic-Balaban, Z., Nikolic, L.B. (2019). *Peppermint and Basil Essential Oils: Chemical Composition, in vitro Antioxidant Activity and in vivo Estimation of Skin Irritation*. Journal of Essential Oil Bearing Plants 22:4, pages 979-993.

Ziosi, P., Manfredini, S., Vertuani, S., Ruscetta, V., Radice, M., Sacchetti, G. (2013). *Evaluating essential oils in cosmetics: antioxidant capacity and functionality,* Asian Pacific Journal of Tropical Biomedicine, vol. 5, pp. 601-611.

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PRELIMINARY STUDY ON THE ANTIMICROBIAL POTENTIAL OF *PHALAENOPSIS* ORCHIDS METHANOLIC EXTRACTS

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Abstract

This paper aims to provide preliminary data about the antimicrobial potential of an ornamental flower, respectively Phalaenopsis orchid. Products from different parts of the orchids, like roots, leaves or flowers, are already in use for different purposes. The orchid plants used in the study were declared florists' waste, and they were carried out tests with methanolic extracts made of different parts of these orchids (roots, leaves, stems). Using the spot technique, it was tested the inhibitory activity of different methanolic extracts on potential pathogens, respectively four Candida spp and on four different Staphylococcus spp. Due to their high diversity, the bioactive compounds from plants are of great interest results have shown promising inhibitory activity of orchid extracts against Candida krusei.

Key words*: waste, orchids, antimicrobial, activity.*

INTRODUCTION

The *Orchidaceae* family, despite all beliefs, is one of the most widespread plant families in the world. The total number of species counts almost 28,484 worldwide (KewWCSP, 2017). In Romania, although that there are identified almost 58 species and hybrids, these flowers are mainly used for their ornamental purpose. Almost all the studies concerning the Romanian orchids aimed to identify the species if they need to be preserved and their threats. Most of them are spontaneous species. (Irimescu L. S. et al., 2019). The orchids species are mainly known as ornamental, but many of these are used for their healing properties, as well as a safe source for the food and perfume industry. Due to the fact that they are widespread, orchids have various uses in multiple cultures also because of the strong aphrodisiac effects, and, in other countries, they are being considered a treatment for gastrointestinal tract, diarrhea and bilious diseases (Singh A., Duggal S., 2009). The phytochemicals produced by orchids are alkaloids, anthocians, flavonoids, sterols and carotenoids, but it is still more to discover about their biological function (Irimescu L.S. et al., 2019). *Phalaenopsis* spp. is known to be the most commonly used orchid from a commercial and economic point of view, and some parts of the plant are often left unused, which can lead to waste management and further ecological problems. Until present times, reports on phytochemical analyses have been available especially for endangered orchids (Minh T.N. et al., 2016).

Although used for their remarkable beauty, orchids are found in cosmetics and have proven useful in creating herbal medicines. In the cosmetic industry, plants from the *Orchidaceae* family are evaluated for their potential anti-aging and skin depigmentation on Japanese female skin (Tadokoro T. et al., 2010). Also, orchids are used for their therapeutic properties. Most of them have been listed in traditional medicine.

Other medicinal properties of orchids have been reported, such as tonic in hysteria, spasm, insanity and epilepsy, rheumatic treatments, tuberculosis, body aches, eczema, headache and fever, aphrodisiac and heart, respiratory, and nervous disorders (Bijaya P., 2013). However, any new developed drug need to be tested before being marketed. Testing, in the beginning, takes the form of experiments performed to evaluate toxicity, and studies that follow the experiments

to evaluate important factors such as: efficacy and adverse effects.

The present study was performed on florists' waste of epiphytic orchids from the species *Phalaenopsis*.

Their phytochemical screening would help to discover new sources of economically important materials such as tannins, oils, gums, resins, quinones etc. In this study we have tried to highlight that the most beloved apartment plant, has important ethnomedicinal values, not only ornamental.

A first step in our research it was to test the antimicrobial potential of methanolic extracts made of orchid waste, respectively from dried leaves, stems and roots of *Phalaenopsis* species. The search of new antimicrobial agents in the field of ethnopharmacology, to isolate compounds with proven antimicrobial activity, that can be used for an effective treatment of the human diseases, with lowered therapeutic doses of antibiotics (Nicolcioiu M.B., et al., 2017)

MATERIALS AND METHODS

Collection of plant material

The flowers waste of *Phalaenopsis* orchids have been harvested from Tria's Flower Shop, Greenhouse Băneasa, Romania. The plants have been cultivated under special conditions and have been donated by the manufacturer.

Preparation of extracts

The leaves, stems and roots of *Phalaenopsis* orchid species have been chopped in small pieces and shredded. The roots were washed with water to remove bark or other impurities which could have contaminated the final sample. After cutting, the tissue was dried. The drying process was carried out in rooms with a constant temperature of 37°C for 7 days. For the preparation of the crude extract, the powdery vegetal material (leaves/roots/stems) was mixed in a stopper bottle (Erlenmeyer) with 80% methanol (ration 1/10) and placed in the microplate mixer at a controlled temperature for 60 minutes at 30°C at 150 rotations/ minute. The crude extracts obtained were then filtered through filter paper.

Test microorganisms used

To test the antimicrobial activity of *Phalaenopsis* extracts, four potentially pathogenic *Staphylococcus* strains and four *Candida* strains were used (Table 1). These pathogenic microorganisms were provided by the Faculty of Biotechnology U.S.A.M.V. of Bucharest.

Table 1. Microorganisms used to test the *Phalaenopsis* spp. antimicrobial activity

Pos.	Microorganisms	Origin
Yeast		
1.	*Candida albicans* ATCC 10231	American Type Culture Collection
2.	*Candida parapsilosis* ATCC20019	American Type Culture Collection
3.	*Candida guilliermondii* MI 40	Collection of Faculty of Biotechnologies, Bucharest, Romania
4.	*Candida krusei* 2016 MI 41	Collection of Faculty of Biotechnologies, Bucharest, Romania
Bacteria		
5.	*S. aureus* ATCC6538	American Type Culture Collection
6.	*S. aureus* ATCC43300 MRSA	American Type Culture Collection
7.	*S. epidermidis* ATCC 51625	American Type Culture Collection
8.	*S. epidermidis* ATCC12228	American Type Culture Collection

Culture media

The culture media used for *Candida* was PDA (Potato Dextrose Agar) and for *Staphylococcus* TSA (Tryptic Soy Agar).

The media were autoclaved at 121°C for 15 minutes.

Testing antimicrobial activity by spot method

The drop-diffusion test method, was used to measure the inhibition zones, in order to determinate the antimicrobial activity (Diguta C. et al., 2014). For the drop diffusion test, the inoculum was prepared in fresh culture, in liquid media. Strains in fresh culture were prepared by inoculation on liquid media, TSA for *Staphylococcus* bacteria, PDA for *Candida*.

For a period of 24-48 hours, the microorganisms were cultivated at 30°C for fungi and 37°C for bacterial strains; the final cells concentration was adjusted to 10^8 CFU/ml for *Staphylococcus* spp. and 10^6 CFU/ml for *Candida* spp.

According to the working method, the spread technique is used for inoculation of pathogenic strains on the surface of the culture media distributed in Petri dishes. After about 1 hour, 5 µL of each sample was added as a spot into the Petri dish. As control, has been used 5 µL of 80% Methanol solution and antibiotic. The next step is to incubate for 24-48 hours the cultures at temperatures of 30-37°C. By measuring the area of inhibition that appeared in the immediate proximity of the antagonist, the degree of sensitivity was determined (Figure 1).

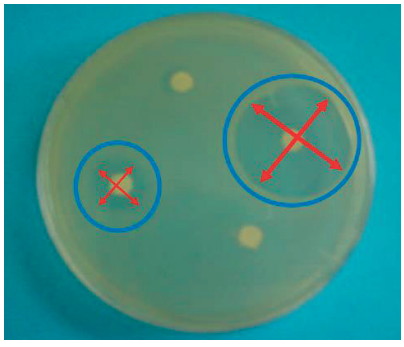

Figure 1. Spot method used to test antimicrobial activity of the extracts

Taking into account the size of the halos, 4 thresholds have been established indicating the level of antimicrobial activity as follows:

- = no antimicrobial activity (no inhibition reaction is observed)

+ = low activity: 0.1-0.9 mm (indicates possible reactivity, halo size close to unobserved)

++ = average activity: 1.0-1.9 mm (visible reactivity, small halo size)

+++ = increased activity: > 2 mm (large hall size, indicates increased reactivity).

RESULTS AND DISCUSSIONS

The microbial cultures can be examined using a variety of techniques; in our case the simplest was the measurement of formed halos diameters.

After incubation, the inhibitory effect on the test organism is indicated by a clear area (halo) around the test substance, in this case, the extracts obtained from different parts of the *Phalaenopsis* orchids. The general image of the inhibitory activity registered by spot method is presented in Table 2.

Clear inhibition halo (medium inhibitory activity) was observed in the case of methanolic stem extract on *C. parapsilosis* and *C. guilermondi*. In the case of methanolic root extract, high inhibitory activity has been noticed against *C. krusei. Staphyloccus aureus* has been inhibited in different degrees by the stem extract, while *Staphyloccus epidermidis* has been moderately inhibited by the root extract table 2. An important finding is that the methanolical extract obtained from the stem has high inhibitory activity on the MRSA (methicillin resistant) strain ATCC43300 of *Staphyloccus aureus*.

Such inhibitory activity may be linked to reports that revealed the presence of phytochemicals such as phenols, tannins, flavonoids, saponins, glycosides, steroids, terpenoids and alkaloids in the orchid extracts (Kumari R. et al., 2017).

Table 2. The inhibitory activity of *Phalaenopsis* extracts on *Candida* and *Staphylococcus* pathogens

Pathogen	Inhibitory effect		
	Leafs - 1	Stems - 2	Roots - 3
Candida albicans	-	-	-
Candida quilliermondii	-	++	-
Candida parapsilosis	-	++	-
Candida krusei	-	-	+++
Staphylococcus aureus 43300 MRSA	-	+++	-
Staphylococcus aureus 6538	-	+	-
Staphylococcus epidermidis 12228	-	-	++
Staphylococcus epidermidis 51625	-	-	-

Legend: -: non inhibitory halo; +: low inhibitory activity (0.1-0.9 mm); ++: moderate inhibitory activity (1.0-1.9 mm); +++: high inhibitory activity (: > 2 mm).

Tannins (commonly called tannic acid) are polyphenols, known as antimicrobial agents that precipitate proteins and prevent the development and growth of microorganisms, in this way, making nutritional protein unavailable to microbes. The growth of large numbers of fungi, yeasts, bacteria and viruses has been inhibited by tannins (Stahl, E., 1988).

The results may suggest a new valuable resource to replace the use of antibacterial drugs. Because of the abuse of antibiotics on a very large scale, as a panaceum, infections caused by multidrug-resistant strains are gaining resistance to antimicrobial drugs, due to their natural mechanisms, therefore antimicrobial agents of plant origin are needed to be use for the treatment of infections caused by multidrug-resistant strains. Considering that nowadays among the florists the orchids' waste, especially of *Phalaenopsis*, are registering increased quantities, their use to develop added-value pharmaceutical or cosmetics new products with antimicrobial activity can be a viable solution in an economy context.

CONCLUSIONS

This preliminary study has revealed that methanolic extracts made of stems and roots of *Phalaenopsis* wastes have potential antimicrobial activity, with an inhibitory effect on pathogens like methicillin resistant *Staphylococcus aureus* and different *Candida* species.

The antimicrobial activity of leafs extract against all tested microorganisms was very low, while in the case of stems and roots extracts, further antimicrobial activity should be performed.

In addition, other tests should be performed to prove any antioxidant potential of such extracts for a higher value of new products.

REFERENCES

Atanassova M., Georgieva S., Ivancheva K. (2011). Total phenolic and total flavonoid contents, antioxidant capacity and biological contaminants in medicinal herbs, *Journal of the University Chemical Technology and Metallurgy*, 46:81-88.

Bijaya, P. (2013). Medicinal orchids and their uses: Tissue culture a potential alternative for conservation, *Afr. J. Plant. Sci*.

Bulpitt C. (2005). The uses and misuses of orchids in medicine. *QJM: monthly journal of the Association of Physicians* 98(9):625-31. Retrieved from: https://www.researchgate.net/publication/7737067_The_uses_and_misuses_of_orchids_in_medicine.

Choma I.M., Olszowy M., Studziński M., Gnat S. (2019). *Determination of chlorogenic acid, polyphenols and antioxidants in green coffee by thin-layer chromatography, effect-directed analysis and dot blot - comparison to HPLC and spectrophotometry methods*. Retrieved from: https://onlinelibrary.wiley.com/doi/abs/10.1002/jssc.201801174

Diguta, C., Cornea C.P., Ionita L., Branduse, E., Farcas, N., Bobit D., Matei F. (2014). Studies on antimicrobial activity of *Inula helenium* L. Romanian cultivar. *Rom. Biotechnol. Letter*, Vol. 19, No.5, p.9699-9704.

Havsteen B. (1983). Flavonoids, a class of natural products of high pharmacological potency. *Biochem Pharmacol.* Apr 1; 32(7):1141-8.

Irimescu L.S., Babeanu N., Matei F. (2019). Mapping geographical distribution of Romanian orchids and their biological active substances. *Scientific Bulletin. Series F. Biotechnologies, XXIII,* 154-163.

Kumari, R., Shakula, K. (2017). Debasish, S., Extraction and screening of bioactive compounds of some

common hydrophyticand wetland plants from East Singbhum, *IOSR Journal of Pharmacy, Jharkhand, India.*

KewWCSP World checklist of selected plant families (2017). Retrieved from: https://wcsp.science.kew.org/incfamilies.do

Minh T.N., Tuyen P.T., Khang D.T., Quan N.V., Ha P.T.T., Quan N.T., Andriana Y., Xinyan F., Van T.M., Khanh T.D., Xuan T.D. (2017). Potential Use of Plant Waste from the Moth Orchid (Phalaenopsis Sogo Yukidian "V3") as an Antioxidant Source, *US National Library of Medicine National Institutes of Health*

Minh T.N., Khang do T., Tuyen P.T., Minh L.T., Anh L.H., Quan N.V., Ha P.T., Quan N.T., Toan N.P., Elzaawely A.A., Xuan T.D. (2016). Phenolic Compounds and Antioxidant Activity of Phalaenopsis Orchid Hybrids, *MDPI Open Access Journals*. Retrieved from: https://doi.org/10.3390/antiox5030031

Nicolcioiu M.B., Popa G., Matei F**.** (2017). Antimicrobial activity of dried biomass ethanolic extracts from mushroom mycelia developed in submerged culture. *Scientific Bulletin. Series F. Biotechnologies, XXI*, p.159-164.

Orłowska M., Kowalska T., Sajewicz M., Jesionek W., Choma I.M., Majer-Dziedzic B., Szymczak G., Waksmundzka-Hajnos M. (2015). A Comparison of Antibacterial Activity of Selected Thyme (Thymus) Species by Means of the Dot Blot Test with Direct Bioautographic Detection, *Journal AOAC International*. Retrieved from: https://www.ncbi.nlm.nih.gov/pubmed/26268965

Singh, Amritpal and Duggal, Sanjiv (2009) "Medicinal Orchids - An Overview," *Ethnobotanical Leaflets*: Vol. 2009: Iss. 3, Article 3.

Stahl, E. (1988). *Thin layer chromatography, 2nd edition*, Springer-Verlag Berlin, Reprint

Tadokoro, T., Bonte, F., Archambault, J. C., Cauchard, J.H., Neveu, M., Ozawa, K., Noguchi, F., Ikeda, A., Nagamatsu, M., Shinn, S. (2010). Whitening efficacy of plant extracts including orchid extract on Japanese female skin with melisma and lentigo senilis. *The Journal of Dermatology*, 37(6): 522-530. doi: 10.1111/j.1346-8138.2010.00897.x PMID: 20536665.

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EFFECTS OF DIRECT-FEED MICROBIAL *BACILLUS* SUPPLEMENTATION ON PIGLET'S MICROBIOTA

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Abstract

Direct-fed microbial (DFM) supplementation in piglet's nutrition may offer high benefits to the young animals by diminishing the bacteria pathogens from the gastrointestinal tract and environment. In this study, we evaluated the efficiency of Bacillus subtilis ATCC 6051a (BS, 1.6 x 10^9 CFU/mL) on the piglet's microbiota in the weaning crisis. A total of 60 piglets, 30 days ± 3 days of age, were allotted in 3 homogeneous groups (C, E1 and E2, 10 piglets/6 pens, 2 replicates/group) supplemented with BS 1% (E1), respectively 3% (E2)/kg feed. At the end of the trial (46 ± 3 d-old), 6 piglets were slaughtered (2 piglets/group) and intestinal content (ileum and cecum) were collected for evaluating the microbiota and intestinal pH values. The piglet's faces were collected for microbial analysis on 1-d, 8d, and 16 d. The numbers of lactic acid bacteria (LAB), Coliforms bacteria, Enterococcus spp., Clostridium spp. and Bacillus spp. from intestinal content were modified at the addition of BS, whereas, from faeces samples, the microbiota was insignificant (P ≥ 0.05). The addition of BS 1% and 3% decreased the numbers of Escherichia coli biotype β-hemolytic from piglet's intestinal content (P ≤ 0.05) and faces vs. C group. Salmonella spp. was not present. The intestinal pH from the ileum and cecum segment was observed to be lower in E1 + BS 1%, while in E2 + BS 3% the pH was higher vs. C group. In conclusion, 1% supplementation of Bacillus subtilis ATCC 6051a in piglets feed had a positive effect during the post-weaning period on endogenous microbiota, fecal microbial count and intestinal pH evolution.

Key words*: probiotic, piglets health status, intestinal microbiota, intestinal pH.*

INTRODUCTION

Direct-fed microbials (DFM) present the capacity to modify gastrointestinal microflora, morphology and immunity after weaning (Mingmongkolchai and Panbangred, 2018).

The feed addition of lactic acid bacteria, yeast and *Bacillus* as DFM (Liu et al., 2018) can bring a number of benefits to animals microbial flora as balancing the health status and maintaining the intestinal ecosystem (Dumitru et al., 2019). Generally, the bacteria used as source of probiotic products are part of the intestinal (commensal) flora of the host (Scharek et al., 2007) and as live microorganisms, can improve the intestinal microbial stability of young animals (He et al., 2017), avoiding's the disadvantage of antibiotics use (Isolauri et al., 2004). According to Casula and Cutting (2002), the presence of *Bacillus* spores and their capacity to resist under exhibits gastrointestinal conditions (thermostability and the capacity to tolerate the low pH values and bile salts), make them to germinate in significantly numbers along to the animal gut. Furthermore, Bacilli as Gram-positive bacteria are present in substantial numbers in agricultural soils and other environment conditions (Cornea et al., 2003), being responsible for the enzymatic process by enhanced animal digestion.

Addition of *Bacillus* as DFM to piglets' diets may improve gut health, by modifying the microflora, thus controlling and protect from pathogenic bacteria, increase nutrient digestibility and feed efficiency, and also, to perform the growth performance of piglets. Due to their stability, *Bacillus* are ideally suitable to produce a variety of enzymes which intensify the digestion process (Merchan et al., 2011). Supplementation piglets' diet with various strains of *Bacillus* involve positive results on body weight gain, feed conversion ratios, lower mortality with a reduction of diarrhea incidence in weaning crisis (Taras et al., 2005). It was reported that *Bacillus subtilis* (*BS*) improves the animal health status by stabilizes the gastrointestinal tract after weaning (Liu et al., 2017). Currently, many of the researches into

probiotics have directed on the protection against pathogens. Scharek et al. (2007) reported that administration of *Bacillus* spp. diminished the intestinal counts of enterotoxigenic *Escherichia coli*, diarrhoea incidence, and morbidity in weaned piglets. Furthermore, Du et al. (2019) confirmed that oral feeding with *BS* in concentration of 1.5 x 10^{10} CFU/mL was able to protect the newborn piglets by inhibiting the pathogenic *E. coli* which is responsible for infection, severe intestinal disorders and death. Their stability to form endospores, make *Bacillus* species to remain viable at higher temperatures during animal feed pelleting and stable for long-term storage (Baker et al., 2013). The aim of the current study was to evaluate the effects of dietary *Bacillus subtilis* ATCC 6051a as source of probiotic, by inclusion in piglets diets, on microbiota from intestinal content (ileum and cecum), faecal and the evolution of pH values.

MATERIALS AND METHODS

The experimental protocol was approved by the ethic guideline Research Committee of INCDBNA Balotesti, Romania.

Bacterial strain, culture medium and growth conditions

Bacillus subtilis ATCC 6051a (*BS*), a bacterial strain used as DFM was purchased from the American Tissue Culture Collection (ATCC) in the form of freeze-dried. The probiotic properties of *BS* were analyzed *in vitro* and presented in a previous study (Dumitru et al., 2019). The bacterial strain was incubated aerobically in the nutrient medium, in a shaker-incubator (200 rev min^{-1}) at 37°C for 24 h. The strain concentration used in this study was 1.6 x 10^9 CFU per mL g^{-1} feed.

Piglets, experimental treatments and diets

A total of 60 piglets Topigs hybrid [♀ Large White × Hybrid (Large White × Pietrain) × ♂ Talent, mainly Duroc] with average body weight (BW) of 8.41 ± 0.92 kg. (30 d ± 3 d of ages) where used in a 16-d experiment. Piglets were randomly allotted to 3 groups distributed in 10 piglets/6 pens, two replicates per group: negative control (C) and 2 experimental groups with the addition of *Bacillus subtilis* (*BS*) in a dose of 1% (E1+*BS* 1%), respectively 3% (E2+*BS* 3%). The concentration of *BS* was 1.6 x 10^9 UFC/mL g^{-1} feed. The probiotic product was added after grinding the raw material and then mixed them uniformly. All piglets were housed in an environmentally controlled room, equipped with water nipples. Feed and water were available *ad libitum* throughout the duration of the experiment and was administrated in the flour from, two meal per day. The feed structure is shown in Table 1. The room temperature was approximately 25 ± 2°C. At the end of the trial (46 ± 3 d-old), 6 piglets were selected randomly and slaghtered (2 piglets per group). Intestinal content (ileum and cecum) were collected for evaluating the microbiota and intestinal pH values.

Sample collection and microbial analyses: intestinal and faecal content

Two piglets per group were selected and euthanized for assessing the gastrointestinal microbial populations from ileum and cecum content. Intestinal content was removed immediately after killing and aseptically collected in sterile plastic bags on ice. From those content, 1 g of sample (ileum and cecum) per capita from two piglets per group were homogenized with 7 ml BHI (Brain Heart Infusion, Oxoid) broth with 2 ml glycerol, and immediately stored at - 20°C until testing (Sorescu et al., 2019). Similar, fresh faeces samples were collected randomly from each group (on 1st d, 8 d, and 16 d) and stored in the same conditions until bacterial analysis was done (no more three months). After defrost, one gram of the composite intestinal content, respectively faecal samples were supposed to decimal dilutions in 9 mL PBS (Phosphate Buffered Saline, Oxoid) solution and then very well homogenized. Microbial flora was assessed for *Lactobacillus* spp. [LABs on MRS agar (Man, Rogosa and Sharpe)], *Escherichia coli* biotype β-haemolytic [Trypticase soy agar (TSA, Sanimed) + 5% sheep blood (w/v), Dumitru et al., 2018], *Salmonella* spp. (Salmonella-Shigella agar, Oxoid), *Clostridium* spp. (Reinforced Clostridial agar, Oxoid), Coliforms (MacConkey agar, Oxoid), *Bacillus* spp. (nutrient agar) and *Enterococcus* spp. (Slanetz-Bartley agar, Oxoid). The LABs, *Clostridium* and *Enterococcus* were cultured in

anaerobic conditions (Oxoid jar with Anaerogen 2.5 L). Bacterial counts from all samples were determined by plate counting method and were $\log_{10}$ CFU transformed before statistical analysis (Vamanu et al., 2013)

Intestinal pH values

The same slaughtered piglets were used for measurement the intestinal pH (ileum and cecum). 1 g intestinal content of each piglet was collected aseptically in 9 mL distilled water (1:10 dilution) and pH values were determined by using a digital Portable meter (Waterproof, pH 7+DHS, Italy).

Statistical analysis

The analytical data were compared using variance analysis "ANOVA" with STATVIEW for Windows (SAS, version 6.0). The results were expressed as mean values and standard error of the mean (SEM), the differences between means considered statistically.

RESULTS AND DISCUSSIONS

During the experiment, the concentration of *Bacillus subtilis* ATCC 6051a used in piglets diet as source of DFM, was prepared in liquid form and contained in average 1.6 x 10^9 CFU/mL.

Table 1. Compositions of basal diet of piglets during weaning crisis

Items %	Control
Maize	33.48
Sorghum	25
Peas	17
Soybean meal	13
Maize gluten	3
Milk replacer	5
DL methionine	0.1
L- Lysine	0.21
Calcium carbonate	1.6
Phytase	0.01
Monocalcium phosphate	0.4
Salt	0.1
Premix choline	0.1
Vitamin-mineral premix*	1
Chemical composition % (g⁻ feed)	
Metabolizable energy (EM, Kcal/ kg)	3237.31
Crude protein (CP)	18.23
Lysine	1.2
Methionine + Cystine	0.59

*ME was calculated based on feed composition and theoretical coefficients.
*The vitamin-mineral premix contained (/kg feed): 10 000 IU vitamin A; 2 000 IU vitamin D3; 30 IU vitamin E; 3 mg vitamin K3; 2 mg vitamin B1; 6 mg vitamin B2; 20 mg vitamin B3; 13.5 mg vitamin B5; 3 mg vitamin B6; 0.06 mg vitamin B7; 0.8 mg vitamin B9; 0.05 mg vitamin B12; 10 mg vitamin C; 30 mg Mn; 110 mg Fe; 25 mg Cu; 100 mg Zn; 0.38 mg I; 0.36 mg Se; 0.3 mg Co; 60 mg antioxidant.

E1+*BS* 1% and E2+*BS* 3% experimental groups received the same diet feed, the difference consisting in the percentage of DFM-probiotic product (*BS*), respectively 1% and 3% (v/w g^{-1} feed).

Intestinal and faecal microbiota of piglets

Ileum *Lactobacillus* spp. increased in piglets fed E1+*BS* 1% with 3.35% compared to C group, whereas *E. coli* concentration decreased with 26.22% at the administration of *BS* 1% *vs.* C, respectively with 2.70% in piglets diet E2+*BS* 3% (Figure 1). A critical role in animal nutrition, performance, health and the quality of the product produced is occurred by the intestinal microbiota.

It was reported that the utilization of *Bacillus* spp. reduce the intestinal count of *Escherichia coli* which is an enterotoxigenic bacteria, responsible of diarrhoea incidence, and in the last form determining the piglet's mortality (Poulsen et al., 2018). Moreover, the *Clostridium* counts along the ileum of 46 ± 3 d-old piglets was reduced by *BS* supplementation, which decreases the pathogens around 10% in E1, and proximately with 1.5% in E2 *vs.* C group. Similar, the addition of *BS* 1% influence *Enteroccocus* spp. which are present in low counts in E1 *vs.* C group.

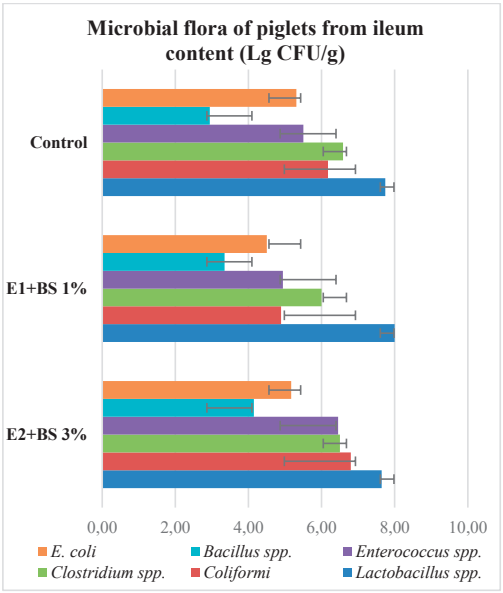

Figure 1. Microbiota from ileum piglets content with *BS*

Compared with C group, E1 and E2 shown an abundance in *Bacillus* counts (more than 13.60% to 41.15%); the coliforms grown was influenced by the concentration of *BS,* a decrease with 26.4% in low dose, and 10.0% in E2. Alternatively, the piglets diet supplemented with *BS* influenced the colonization of lactic acid bacteria between 18% to 25% in the cecum content, with a slow decrease of *E. coli* in the experimental groups (Figure 2).

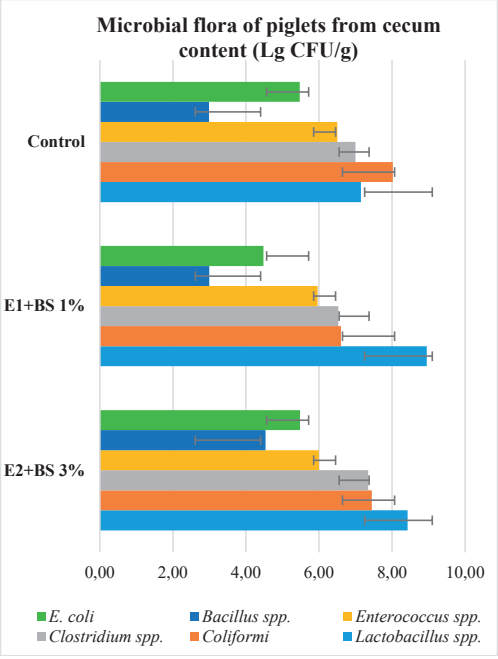

Figure 2. Microbiota from cecum piglets content with *BS*

The counts of Coliforms in the piglets cecum content were diminished between 22% (E1) and around 8% (E2) ad *BS* addition. The data present in the current study, indicate an improvement of the intestinal microbial flora of piglets in E1+*BS* 1% *vs.* E2+*BS* 3%.

According to the results of Alexopoulos et al. (2004), an important aspect of *Bacillus* is their efficacity on health status during piglets weaning crisis. Furthermore, DFM improved the number of lactic acid bacteria, decreasing the number of Gram-negative bacteria from the *Enterobacteriaceae* family, results that can be observed and in our study. The piglets age is an important factor that influences the gastrointestinal microbiota (Slifierz et al., 2015); the microbial flora on 46 ± 3 days was furthermore clearly different from that on 30 ± 3.

The modifications in diet, environment conditions, stress complicates can influence the interpretation of microbial flora in the early piglets weaning (Poulsen et al., 2018).

DFMs are implied in enhancing the gastrointestinal health by increasing the growth of helpful bacteria such as lactobacilli (Giang et al., 2010) and Bifidobacteria, through reducing the growth of harmful bacteria from the general family of Gram-negative *Enterobacteriaceae* (Liu et al., 2018; Bajagai et al., 2016). The decrease of pathogenic bacteria and equilibrium of intestinal microbiota may correspond to the animal ability to digest and ferment nutrients (Kenny et al., 2011), in our case young animals knowing that the enzymatic system is not very well developed (Habeanu et al., 2015). The presence of spores, as *Bacillus subtilis* can produce extracellular enzymes (cellulase, protease, amylase etc.) which can increase the gastrointestinal activity of piglets (Bajagai et al., 2016).

According to He et al. (2017), the addition of *Bacillus subtilis* in piglet diets determines a decrease of bacterial diversity which is associated with gastrointestinal disorders responsible for the development of diarrhoea incidences; the administration of probiotic in piglets diet could protect and ameliorate the intestinal disturbances characteristic periods of weaning (Huang et al., 2004; Prieto et al., 2014). Probiotics are supposed to improve the health of animals by preventing gut microbiota imbalance and improving gut health by adjusting the intestinal bacteria (Veizaj-Delia and Pirushi, 2012; Lescheid, 2014). Mackie et al. (1999) affirmed that the gut of piglet in utero is sterile, and after birth, the bacteria will colonize it, received from the sow and sow feces. Additionally, Baker et al. (2014) reported that utilization of DFM in sow, can be a potential source to reduce the environmental pathogens as *Clostridium* populations and their decrease from piglets gastrointestinal tract.

Table 2 shows the relative abundance of bacteria in piglets faces samples collected on day 1, 8 and 16 following the experimental protocol. On the first day of the experiment, the *Lactobacillus* spp. did not register significant differences (P = 0.2103), comparatively with the second period

were the bacteria counts tended to decrease in E1+*BS* 1%, respectively E2+*BS* 3% (P = 0.0107). Furthermore, on 16-d of the experimental trial, the lactic acid bacteria were not affected when piglets were fed with the diet that contained different levels of *BS* (P = 0.5257).

Table 2. Effect of *Bacillus subtilis* ATCC 6051a on fecal microbiota in weaning piglets (16-d)

Item		C	E1+*BS* 1%	E2+*BS* 3%	*SEM*	*P*
***Lactobacillus* spp., log CFU/g**	I	8.20	8.47	9.04	0.19	0.2103
	II	9.01^{ab}	7.78^{ab}	7.58^{b}	0.22	0.0107
	III	8.44	8.00	8.76	0.26	0.5257
Coliforms, log CFU/g	I	6.23^{a}	7.00^{a}	6.63	0.11	0.0184
	II	6.12	5.79	5.92	0.14	0.6663
	III	6.01	6.82	6.29	0.17	0.1675
***Clostridium* spp., log CFU/g**	I	6.33	6.61	6.66	0.11	0.4443
	II	5.93^{a}	6.36^{b}	7.60^{ab}	0.26	0.0184
	III	6.05	6.37	6.20	0.11	0.5576
***Enterococcus* spp., log CFU/g**	I	5.71^{ab}	7.93^{a}	7.71^{b}	0.26	0.0001
	II	5.33	4.71	5.41	0.23	0.4407
	III	5.41	5.84	5.81	0.14	0.4072
***Bacillus* spp., log CFU/g**	I	5.69	5.57	5.28	0.10	0.2843
	II	4.45	4.79	4.40	0.16	0.5958
	III	4.18	4.18	3.94	0.09	0.5024
***Salmonella* spp., log CFU/g**	I	abs	Abs	abs	nd	nd
	II	abs	Abs	abs	nd	nd
	III	abs	Abs	abs	nd	nd
***E. coli*, log CFU/g**	I	nd	Nd	nd	nd	nd
	II	5.36	4.94	5.02	0.13	0.4576
	III	>4.42	3.78	3.91	0.18	0.3288

*Where: I: 1-d of the experiment, without BS (30±3 days); II: 8-d of the experiment, with BS (38±3 days); III: 16-d of the experiment, with BS (47±3 days); Abs - absent; BS: *BS - Bacillus subtilis* ATCC 6051a (1% and 3%) in a dose of 1.6 x 10^9 UFC/mL/ g^{-1} feed; nd: not applied (*E. coli* < 1 x 10^3 UFC/g, absent); Experimental groups: C (Control), E1+*BS* 1%; E2+*BS* 3%; SEM: standard error of the means; ^{a,b}Means in the same row with the same common superscript are significantly different ($P \leq 0.05$).

The genus *Lactobacillus* as a Gram-positive bacteria is not involved a significantly growing in the presence of *BS* (P = 0.5257).

Lactobacillus is considered a beneficial bacterium for the equilibrium of intestinal microbiota, due to its healthfulness effects such as the prevention of diarrhoea and intestinal infections (Hu et al., 2014). Previous studies have found that the fecal *Lactobacillus* abundances' where not affected at *Bacillus subtillis* addition in piglets diet, and their abundance are in opposition with the *E. coli* concentrations (Konstantinov et al., 2006).

Coliforms were insignificantly ($P > 0.05$) on 8-d and 16-d of the experiment, whereas the *Clostridium* spp. counts significantly increased (P = 0.0184, on 8-d) at the addition of *BS* 1% and 3% *vs.* C group. Furthermore, DFM-product used as a source of probiotic in piglets' diets, did not influence the concentration of *Enterococcus* spp. and *Bacillus* spp. among dietary treatments. Vanhoutte et al. (2006) have been reported that an increase of *Lactobacillus* bacteria is in a relative decrease with *Clostridium* and Coliforms.

The administration of *BS* involves a decrease of *E. coli* on 16-d, but no significant differences were observed in the relative densities of total colonies bacteria ($P > 0.05$). Vanhoutte et al., 2006). *E. coli* is one of the major sources of intestinal pathogens, and some strains can produce serious illnesses, including diarrhoea. In the post-weaning, piglets fed supplementation with probiotics is essential for the prevention of diarrhoea, which is usually caused by β-hemolytic enterotoxigenic *E. coli* strains (García-Meniño et al., 2018).

Salmonella spp. was absent in all experimental groups, respectively analysis of microbial intestinal content and faces of piglets (30 ± 3 days) with and without *BS* supplementation.

Piglets intestinal pH

Along the gastrointestinal tract, administration of *BS* in piglets feed as probiotic treatment affected the pH level (Table 3. Merchant et al. (2011) affirmed that the pH in the small intestine of piglets is around 6 to 7, which is optimal interval for spores of *Bacillus* to germinate, grow and to act efficiently. *Bacillus* spp. due to

their capacity to survive to the gastrointestinal conditions, they are able to resist feeding processing and digestion of the stomach.

Table 3. The intestinal pH values from piglets in the weaning crisis fed control diet or supplemented with DFM for 16-day experimental period

Segment	C	E1+*BS* 1%	E2+*BS* 3%
Ileum	6.75 ± 0.91	6.62 ± 0.50	7.78 ± 0.59
Cecum	6.46 ± 0.33	5.26 ± 0.30	7.10 ± 1.01

Experimental groups: C (Control); E1+*BS* 1%; E2+*BS* 3%.

Interestingly, the ileum pH in the C group was around 6.8 while in E1+*BS* 1% registered 6.63, respectively 7.8 in E2+*BS* 3%. In cecum content, the pH of C group was 6.5 *vs*. 5.3 of E1+*BS* 1%, where E2+*BS* 3% registered an average of 7.1.
Weaning as a stressful period of piglets is influenced by numerous factors that contribute to physiological and microbial diversity in the gut (Lalles et al., 2007). The pH of intestinal digests can represent an indicator of the population of pathogens that colonize the gastrointestinal tract of piglets and in the end the intentness of diarrhea process to develop. An acidic environment encourages the proliferation of beneficial bacteria while inhibiting the growth of pathogenic bacteria (Fuller, 1977 cited by Heo et al., 2012).
Our pH values are in concordance with the results reported by Heo et al. (2012), which confirms that the pH of different areas of the gastrointestinal tract of piglets in the weaning crisis is, for example, in range of 6.0 to 7.4 in ileum segment, respectively 5.4 to 6.7 in caecum content. Dumitru et al. (2018; 2019) presents some results of the probiotic properties of *Bacillus* spp. including pH and bile salts resistance, these being significant criteria for selecting a probiotic product for use in animal nutrition.

CONCLUSIONS

Feed supplementation with *Bacillus subtilis* ATCC 6051a probiotic prepared at 1% was shown to have a positive effect during the post-weaning period on endogenous microbiota, fecal microbial count and intestinal pH evolution of piglets. Supplementation of the compound feed with *BS* 1% reduced the multiplication of Coliforms, *Clostridium* spp. and *E. coli* β-hemolytic in the intestinal and faecal contents of piglets.

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REFERENCES

Alexopoulos, C., Georgoulakis, I. E., Tzivara, A., Kyriakis, C. S., Govaris, A., Kyriakis, S. C. (2004). Field Evaluation of the Effect of a probiotic-containing *Bacillus licheniformis* and *Bacillus subtilis* spores on the health status, performance, and carcass quality of grower and finisher pigs. *Journal of Veterinary Medicine*, Series A, 51(6), 306–312.

Bajagai, Y. S., Klieve, A. V., Dart, P. J., Bryden, W. L. (2016). Probiotics in animal nutrition e production, impact and regulation. In: Makkar HPS, editor. *FAO animal production and health paper*, 179. Rome, Italy: Food and Agriculture Organization of the United Nation.

Baker, A. A., Davis, E., Spencer, J. D., Moser, R., Rehberger, T. (2013). The effect of a *Bacillus*-based direct-fed microbial supplemented to sows on the gastrointestinal microbiota of their neonatal piglets. *Journal of Animal Science*, 91(7), 3390–3399.

Casula, G., Cutting, S. M. (2002). *Bacillus* probiotics: Spore germination in the gastrointestinal tract. *Applied and Environmental Microbiology*, 68, 2344–2352.

Cornea, C. P., Grebenisan, I., Mateescu, R., Vamanu, E., Campeanu, G. (2002). Isolation and characterization of new *Bacillus* spp. Strains - useful as biocontrol agents of plant pathogens. *Rom Biotechnol Lett.,* 8(1), 1115–1122.

Du, Y., Xu, Z., Yu, G., Liu, W., Zhou, Q., Yang, D., Cao, Y. (2019). A newly isolated *Bacillus subtilis* strain named ws-1 inhibited diarrhea and death caused by pathogenic *Escherichia coli* in newborn piglets. *Frontiers in Microbiology*, 10.

Dumitru, M., Habeanu, M., Tabuc, C., Jurcoane S., 2019. Preliminary characterization of the probiotic properties of a bacterial strain for used in monogastric nutrition. *Bulletin UASVM, Animal Science and Biotechnologies*, 76(2), 102–108.

Dumitru, M., Sorescu, I., Habeanu, M., Tabuc, C., Idriceanu, L., Jurcoane, S. (2018). Preliminary characterisation of *Bacillus subtilis* strain use as a dietary probiotic bio-additive in weaning piglet. *Food and Feed Research,* 45(2), 203–211.

Dumitru, M., Sorescu, I., Habeanu, M., Tabuc, C., Jurcoane, S. (2019). Preliminary characterization *in vitro* of *Bacillus licheniformis* strain for used as

dietary probiotic. *Scientific Bulletin*. *Series F. Biotechnologies*, 23, 164–172.

Fuller, R. (1977). The importance of lactobacilli in maintaining normal microbial balance in the crop. *British Poultry Science*, 18, 85–94.

García-Meniño, I., García, V., Mora, A., Díaz-Jiménez, D., Flament-Simon, S. C., Alonso, M. P., Blanco, J. (2018). Swine enteric colibacillosis in spain: pathogenic potential of mcr-1 ST10 and ST131 *E. coli* isolates. *Frontiers in Microbiology*, 9.

Giang, H. H., Viet, T. Q., Ogle, B., Lindberg, J. E. (2010). Growth performance, digestibility, gut environment and health status in weaned piglets fed a diet supplemented with potentially probiotic complexes of lactic acid bacteria. *Livest. Sci*. 129, 95–103.

Habeanu, M., Lefter, N., Gheorghe, A., Tabuc, C., Untea, A., Surdu, I., Dragomir, C. (2015). Changes in certain serum and faeces parameters in weaned piglets as a response to nutritional stress. *South African Journal of Animal Science*, 45(2), 164.

He, Y., Mao, C., Wen, H., Chen, Z., Lai, T., Li, L., Wu, H. (2017). Influence of *ad libitum* feeding of piglets with *Bacillus subtilis* fermented liquid feed on gut flora, luminal contents and health. *Scientific Reports*, 7(1).

Heo, J. M., Opapeju, F. O., Pluske, J. R., Kim, J. C., Hampson, D. J., Nyachoti, C. M. (2012). Gastrointestinal health and function in weaned pigs: a review of feeding strategies to control post-weaning diarrhoea without using in-feed antimicrobial compounds. *Journal of Animal Physiology and Animal Nutrition*, 97(2), 207–237.

Hu, Y., Dun, Y., Li, S., Zhao, S., Peng, N., Liang, Y. (2014). *Bacillus subtilis* KN-42 on growth performance, diarrhea and faecal bacterial flora of weaned piglets. Asian-Australasian Journal of Animal Sciences, 27(8), 1131–1140.

Huang, C.H., Qiao, S.Y., Li, D.F., Piao, X.S., Ren, J.P. (2004). Effects of Lactobacilli on the performance, diarrhoea incidence, VFA concentration and gastrointestinal microbial flora of weaning pigs. *Asian Australas. J. Anim.* Sci., 17, 401–409.

Isolauri, E., Salminen, S., Ouwehand, A. C. (2004). Probiotics. *Best Practice & Research in Clinical Gastroenterology*, 18, 299–313.

Kenny, M., Smidt, H., Mengheri, E., Miller, B. (2011). Probiotics - do they have a role in the pig industry? *Animal*, 5:462-470.

Konstantinov, S. R., Awati, A. A., Williams, B. A., Miller, B. G., Jones, P., Stokes, C. R., Akkermans, A. D. L., Smidt, H., De Vos, W. M. (2006). Post-natal development of the porcine microbiota composition and activities. *Environ. Microbiol.*, 8, 1191–1199.

Lalles, J. P., Bosi, P., Smidt, H., Stokes, C. R. (2007). Weaning-A challenge to gut physiologists. *Livest Sci*., 108(1-3), 82–93.

Lescheid, D. W. (2014). Probiotics as regulators of inflammation: a review. *Funct Foods Health Dis*, 4, 299-311.

Liu, Y., Espinosa, C. D., Abelilla, J. J., Casas, G. A., Lagos, L. V., Lee, S. A., Stein, H. H. (2018). Non-antibiotic feed additives in diets for pigs: A review. *Animal Nutrition*, 4(2), 113–125.

Mackie, R. I., Sghir, A., Gaskins, H. R. (1999). Developmental microbial ecology of the neonatal gastrointestinal tract. *Am. J. Clin. Nutr*. 69, 1035S–1045S.

Merchant, H. A., McConnell, E. L., Liu, F., Ramaswamy, C., Kulkarni, R. P., Basit, A. W., Murdan, S. (2011). Assessment of gastrointestinal pH, fluid and lymphoid tissue in the guinea pig, rabbit and pig, and implications for their use in drug development. *European Journal of Pharmaceutical Sciences*, 42(1-2), 3–10.

Mingmongkolchai, S., Panbangred, W. (2018). *Bacillus* probiotics: an alternative to antibiotics for livestock production. *Journal of Applied Microbiology*, 124(6), 1334–1346.

Poulsen, A.-S. R., Jonge, N. de, Nielsen, J. L., Højberg, O., Lauridsen, C., Cutting, S. M., Canibe, N. (2018). Impact of *Bacillus* spp. spores and gentamicin on the gastrointestinal microbiota of suckling and newly weaned piglets. *PLOS ONE*, 13(11), e0207382.

Prieto, M. L., Laurie, O. S., Shiau, P. T., McLoughlin, P., O'Donovan, R. M. C., Kent, R. M., Cassidy, J. P., Hughes, H., Gardiner, G. E., Lawlor, P. G. (2014). Evaluation of the efficacy and safety of a marine-derived *Bacillus* strain for use as an in-feed probiotic for newly weaned pigs. *PLoS One* 9(2), e88599.

Scharek, L., Altherr, B. J., Tölke, C., Schmidt, M. F. G. (2007). Influence of the probiotic *Bacillus cereus* var. toyoi on the intestinal immunity of piglets. Veterinary Immunology and Immunopathology, 120(3-4), 136–147.

Slifierz, M. J., Friendship, R. M., Weese, J. S. (2015). Longitudinal study of the early-life fecal and nasal microbiotas of the domestic pig. *BMC Microbiology*, 15(1).

Sorescu, I., Dumitru, M., Ciurescu, G. (2019). *Lactobacillus* spp. and *Enterococcus faecium* strains isolation, identification, preservation and quantitative determinations from turkey gut content. *Rom. Biotechnol. Lett*., 24(1), 41–49.

Taras, D., Vahjen, W., Macha, M., Simon, O. (2005). Response of performance characteristics and fecal consistency to long-lasting dietary supplementation with the probiotic strain *Bacillus cereus* var. toyoi to sows and piglets. *Arch. Anim. Nutr.*, 59, 405–417.

Vamanu, E., Pelinescu, D., Avram, I., Niță, S., Vamanu, A. (2012). Study of PROBAC product influence on infant microbiota in a single-chamber colonic fermentation model GIS1. *Annals of Microbiology*, 63(3), 1029–1038.

Vanhoutte, T., De Preter, V., De Brandt, E., Verbeke, K., Swings, J., Huys, G. (2006). Molecular monitoring of the fecal microbiota of healthy human subjects during administration of lactulose and *Saccharomyces boulardii*. *Appl. Environ. Microbiol*. 72, 5990–5997.

Veizaj-Delia, E., Pirushi, R. (2012). The utilization of probiotics as a way to improve human and animal gut health. Maced *J Anim Sci*, 2, 175–181.

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DOWN REGULATION OF LIPID ACCUMULATION IN RATS BY ANTIOXIDANT RICH SEED PLANTS SUPPLEMENTATION

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Abstract

Chia and quinoa seeds considered as a powerful medicinal plants and an excellent dietary source of bioactive constituents i.e. flavonoids and phenolic compounds and plenty of omega-3 and omega-6 fatty acids, I hypothesize that chia and quinoa seeds powder and extracts may have a role in lowering lipid accumulation in non-alcoholic fatty liver-induced by high fat high fructose diet. Thirty-six male rats were randomly divided into 6 dietary groups, containing 6 rats in each; normal control group, high fat high fructose diet (HFHFrD) control group, HFHFrD with CS powder 10 g/kg (BW), HFHFrD with QS powder10 g/kg (BW), HFHFrD with CS extract 50 mg/kg (BW), HFHFrD with QS extract 50 mg/kg (BW). After 8 weeks, it was revealed that the consumption of CS & QS extracts were found to normalize many indicators which were shifted to pathological values as a consequence of HFHFrD-induced nonalcoholic fatty liver disorders: there was a significant increase in HDL-C and a significant decrease in total cholesterol, TG, LDL-C and VLDL-C. In addition, level of lipids peroxidation (LP) was reduced as compared to HFHFrD normal group. Thus, these observations suggest that chia and quinoa seeds are potential agents on management of fat accumulation in nonalcoholic fatty liver rats.

Key words*: chia seed, fatty liver disease, flavonoids, lipid peroxidation, phenolic compounds, quinoa seed.*

INTRODUCTION

Unhealthy lifestyle *i.e*. raising the consumption of fast food, refined foods which rich in fructose and limited physical activity considered as risk factors that led to, metabolic syndrome which is associated with the development of obesity, type 2 diabetes, inflammatory and fatty liver diseases (Couturier et al., 2016). Non-alcoholic fatty liver disease (NAFLD) is a rising epidemic worldwide that leads to various liver disease complications such as cirrhosis, hepatocellular carcinoma and liver transplant (Riazi et al., 2019). Nowadays, managing and treatment of NAFLD became a challenge to scientists in different areas. There are various strategies for management of NAFLD include modulation of lifestyle by reduce body weight through diet and physical activity, increasing consumption of healthy foods which rich in antioxidants, lowering saturated fatty acids and refined foods intake (McCarthy & Rinella, 2012). Until now, there is no confirmed drug can be used to treat NAFLD; only some clinical suggestion must be followed for management of NAFLD (El-Abd et al., 2018). There is growing concerns in functional food which contains bioactive components that can provide a therapeutic solution for NAFLD (Albillos et al., 2020).

Plant seeds represents one of the most effective dietary approaches that possess health benefits besides their nutritional values. These seeds contain high levels of bioactive ingredients such as fibre, mineral, omega-3 fatty acids, phenolic compounds which have a key role in prevention and control of diseases. Bioactive compounds present in dietary supplementation possess various biological properties including, hypolipidemic, hypocholesterolemia, anti-inflammatory, anti-atherosclerotic and hepato-protective effects (Mohamed et al., 2019). Chia (*Salvia hispanica*) and quinoa (*Chenopodium quinoa* Willd) seeds are herbaceous plants which possess novel functional and biological activities (Goyat et al., 2018). Appreciably higher levels of nutrient constituents and bioactive compounds make the two plants ideal effective functional foods grains against various diseases. Chia and quinoa seeds used for medicinal purposes for thousands of years (Suri et al., 2016; Mohamed et al., 2019), and this owing to being rich on protein, polyphenols, vitamins, and minerals (Hernández-Ledesma, 2019). Polyphenols, including phenolic acids, flavonoids, and

tannins make up bioactive secondary plant metabolites that contribute to diverse physiological properties, including antimicrobial, antioxidant, anti-inflammatory, antitumor, and anti-carcinogenic effects (El-Abed et al., 2018; Mohamed et al., 2019).

Consequently, the current work aimed to investigate the effect of chia and quinoa seeds powder and extracts on non-alcoholic fatty liver disease induced by high fat high fructose diet in rats.

MATERIALS AND METHODS

Chia (*Salvia hispanica*) and quinoa (*Chenopodium quinoa* Willd) seeds were obtained from Agriculture Research Centre, Giza, Egypt, Fructose was purchased from the International Company for Scientific and Medical Supplies, Cairo, Egypt; Kits used for the measurements of lipid profile were purchased from Diagnosticum Zrt, Budapest and those for measurements of TAC and LPO were obtained from Labor Diagnostika Nord GmbH and Co, Germany. All other chemicals were of analytical grade and thirty six male of Sprague-Dawley rats weighing 140-150 g were used. The animals were obtained from animal house of National Research Centre, Cairo, Egypt. The animals were acclimatized for 1week before dietary manipulation and were housed individually in metallic cages under laboratory healthy conditions.

Preparation of chia and quinoa seeds powder and extracts: chia and quinoa seeds were dried in an air circulated oven at 40°C and then reduced into powder type and stored in airtight containers and kept at 5-7°C till used. The extracts were prepared by using chia and quinoa seed powder (10 g), extracted in (500 ml) redistilled water (12 hrs.) and stored at ~ 2°C until used (Singh et al., 2001).

Proximate chemical analysis of chia and quinoa seeds powder: chia and quinoa seeds powder were sieved through 100-mesh sieve. The powder samples were analysed for water, protein, fat, dietary fibre, ash according to the method of (AOAC, 2012).

Assessment of fatty acids of chia and quinoa seeds: The fatty acid profile of ethanolic extract of chia and quinoa seed oils were determined by gas chromatography as described by (Aldai and Osoro, 2006)

Total phenolic content: chia and quinoa seeds powder were extracted with 80% ethanol twice according to the optimized extraction which described by (Carciochi et al., 2015). Total phenolics were determined using Folin-Ciocalteu UVPC spectrophotometer. The results were expressed as milligrams of gallic acid equivalents (GAE) per 100 g on dry weight basis.

Diets and Animals treatments: Two types of diets were used in this study: 1- basal diet was based on AIN-93 recommendations (Reeves et al., 1993). 2- HFHFrD, consisted of basal diet contain 20% fat (15% beef tallow + 5% corn oil) combined with fructose added in drinking water at a level of 13% w/v which is the concentration range reported for soft drinks (Light et al., 2009). Thirty six rats were divided into six groups, each of six rats. The first groups served as normal control healthy rats which received standard diet. The second named HFHFrD which were fed on high fat high fructose diet. Rats in groups three and four were fed on high fat high fructose diet supplemented with 10 g/kg (BW) chia or quinoa seeds powder, groups five and six were administrated 50 mg/kg (BW) chia or quinoa extracts. Body weight and feed intake were recorded weekly all the period of experiment. After 8 weeks, body weight changes and feed efficiency ratio were calculated according to the method of (Mohamed et al., 2019).

Blood samples were collected after 12 hours fasting at the end of the experiment (8 weeks). Using the retro-orbital by means of a micro capillary glass tubes, blood was collected into a dry clean centrifugal tube and left to clot in a water bath (37°C) at room temperature for half an hour. the blood was centrifuged for 10 minutes at 3000 rpm to separate the serum was carefully aspirated and transferred into clear quit fit plastic tubes and kept frozen at (-2°C) until analysis.

Biochemical analysis: alanine amino transferase (ALT) & aspartate amino transferase (AST) enzymes were measured according to the methods described by (Breuer, 1996). Total bilirubin and total protein were determined according to the methods described by (Henry, 1974; Gowenlock et al., 1988) respectively. Total cholesterol (TC), Triglycerides (TG) and high density lipoprotein

(HDL) were determined in serum according to the methods described by (Allain, 1974; Fassati & Prencipe, 1982; Burstein, 1970) respectively. Low density lipoprotein (LDL) and very low density lipoprotein (VLDL) were determined according to the method of (Friedewald et al., 1972).

Urea, uric acid and creatinine were determined according to (Patton and Crouch, 1977; Henry, 1974; Jaffe, 1980) respectively. Total antioxidant capacity (TAC) and lipid peroxidation (LPO) were determined in according to (Cao et al., 1993; Ohkawa et al., 1979).

The data statistical Analysis as the mean ±SD. Data for multiple variable comparisons was analysed by one-way analysis of variance (ANOVA). Duncan's test was used for the comparisons of significance between groups as a post hoc test according to the statistical package program (Armitage & Berry, 1987).

RESULTS AND DISCUSSIONS

Proximate composition of chia and quinoa seeds (dry basis %)

Table 1 summarized the chemical composition of chia and quinoa seeds powder. Chia seeds contained high amount of protein (16.5 g/100 g) and fat (30.7 g/100 g) than quinoa seeds. Meanwhile quinoa seeds were found to contain high content of carbohydrate (69.34 g/100 g) than chia seeds which recorded (42.1 g/100 g). The current results are in agreement with the finding of other authors (Vega-Galvez et al., 2010; Halaby et al., 2017**)** which have demonstrated that quinoa seeds are one of the best vegetal protein sources which provides a protein value similar to casein in milk and higher than those present in popular grains. Furthermore, da Silva et al. (2017) reported that chia seed as rich source of dietary fibre. This, unique nutritional composition of chia seed, such as dietary fibre, omega-3 and omega-6 fatty acid and antioxidant properties making its consumption as a perfect choice to increase satiety index and reduce the risk of various diseases (Muñoz et al., 2013; Aktaş & Levent, 2018).The alterations in chemical constituents and antioxidant values of plants depend on several factors such as different genotype, growing condition, agronomic practices employed, season, maturity, post-harvest and storage conditions (Navruz & Sanlier, 2016).

Table 1. Proximate chemical composition of quinoa and chia seeds

Nutrients	Contents	
	Quinoa	Chia
Water (g/100 g)	6.90±0.04	4.6±0.14
Crude Protein (g/100 g)	15.05±0.05	16.5±0.06
Crude Fat (g/100 g)	4.93±0.14	30.7±0.09
Dietary Fiber (g/100 g)	8.92±0.05	30.4±0.14
Ash (g/100 g)	3.78±0.06	4.8±0.04
Carbohydrates*	69.34±0.15	43.4±0.12

Data are expressed as mean ± SD, n = 3, *calculated by difference

Total phenolic and flavonoid contents

The results of the total phenolic and total flavonoid contents of chia and quinoa seed extracts are shown in Table 2.

Table 2. Total phenolic and flavonoid contents of chia and quinoa seeds

Particulars	Chia seeds	Quinoa seeds
Total phenolic (mg/100 g GAE)	35.32 ± 0.22	135.23± 2.28
Total flavonoids (mg/100 g QE)	40.29 ± 1.18	37.33± 1.33

Data are expressed as mean ± SD, n= 3, GAE: Gallic acid equivalents, QE: Quercetin equivalent

Total phenolic of quinoa seed was higher than chia seed (135.23 vs 35.32 mg/100 g GAE). These results are in agreement with Gordillo-Bastidas et al. (2016) they reported that quinoa is a more effective quality food due to its bioactive flavonoids. The total phenolic content of quinoa is 135.23 mg/100g GAE. Gordillo-Bastidas et al. (2016) & Halaby et al. (2017) confirmed these results as quinoa seeds contains more phenols than whole cereals.

The different ranges of total flavonoids and total phenolic contents from previous studies are probably owing to the different origins of quinoa seeds and its extract solvents.

Fatty acids profile

Regarding the fatty acid profile (Table 3), chia and quinoa seeds contained three important fatty acids including α-linolenic acid (C18:3), linoleic acid (C18:2) and oleic acid (C18:1) with the most marked levels. Therefore, the levels of unsaturated fatty acids were more than saturated fatty acids about eight times.

Table 3. Fatty acids composition of quinoa and chia seeds

Fatty acids (g/100 g)	Quinoa	Chia
C12:0	0.12±0.04	N.D.
C14:0	0.41±0.12	0.11 ±0.03
C16:0	9.15±1.32	0.14 ±0.05
C16:1	0.22±0.09	0.14 ±0.05
C18:0	0.84±0.12	0.74 ±0.14
C18:1 (ω-9)	6.01±1.15	8.4 ±2.05
C18:2 (ω-6)	20.63±3.30	22.91 ±3.94
C18:3 (ω-3)	58.94±2.59	54.76 ±4.72
C20:0	0.43±0.08	3.42 ±1.03
C22:0	0.78±0.14	N.D.
C22:1	1.36±0.20	0.65 ±0.20
SFA	11.95	4.55
MUFA	7.37	9.05
PUFA	79.57	77.67
ω-3/ω-6	2.86	2.39

Values reported as means _ SD of three replicate analyses (n = 3).
SFA: saturated fatty acids; MUFA: monounsaturated fatty acids;
PUFA: polyunsaturated fatty acid, N.D.: Not detected

These results are in agreement with Yi et al. (2018) as they reported that chia seed contain high level of α-linolenic acid (ω-3) and consider chia as a good daily supplementation to decline triglyceride and alleviate high density lipoprotein concentrations. They also linked these benefits to α-linolenic acid in chia seeds. Furthermore, fatty acids profile of quinoa seeds provides good functional lipids rich in monounsaturated and polyunsaturated fats. The results were in harmony with those of Halaby et al. (2017) they showed that the unsaturated fatty acids content of quinoa is 70-89.4%.

Effect of chia and quinoa supplementation on final weight, weight gain, FER and liver ratio

The results of feed intake and body weight gain of HFHFrD-induced fatty liver rats treated with chia and quinoa seed powders and extracts with two doses each are shown in Table 4, revealed that HFHFrD group showed significant increase in, final weight, weight gain and FER compared to normal control (NC) group.

Table 4. Effects of chia and quinoa supplementation on nutritional indicators

Indicators	NC	HFHFrD	CS	QS	CSE	QSE
Initial weight (g)	142.05± 9.70 a	147.00± 6.16 a	148.05± 5.62 a	149.00± 3.09 a	147.25± 3.10 a	148.25± 3.10 a
Final weight (g)	251.75± 10.35 cd	297.00± 14.52 a	279.75± 12.30 a	269.00± 11.91 b	263.04± 11.11 bc	255.04± 10.41 c
Weight gain (g)	109.25± 10.06 c	149.50± 14.82 a	131.25± 13.89 a	120.00± 12.42 b	116.56± 11.92 c	107.56± 10.92 cd
Feed efficiency ratio(FER)	0.073± 0.001b	0.078± 0.002a	0.093± 0.001b	0.086± 0.002a	0.077± 0.001 b	0.071± 0.001 b
Liver Ratio (%)	4.30± 0.43 b	5.23± 0.38 a	4.26± 0.27 b	4.40± 0.42 ab	4.46± 0.20 ab	4.27± 0.19 b

Data represented as mean ± SD, n = 6, values in each raw having different superscript (a, b, c, d ,e) are significantly different at ($p<0.05$), NC: Normal Control, HFHFrD: High fat high fructose diet, CS: Chia seed, QS: Quinoa seed, CSE: Chia seed extract, QSE: Quinoa seed extract.

Effect of chia and quinoa supplementation on lipid profile and renal function parameters

Data in Table 5 showed the parameters of lipid profile of rats groups that were fed on HFHFrD with treatments of chai and quinoa seeds and extracts for 8 weeks. Significant differences of TC, TG, HDL-C, LDL-C and VLDL-C levels were observed between HFHFrD group and the normal control (NC) group. HFHFrD group show a significant rising in TC, TG, LDL-C and VLDL-C levels, meanwhile a significant decline in HDL-C level comparing with normal control (N.C) group. Similarly, Korish & Arafah (2013) and El-Abed et al., (2018) found significant alleviation in lipid profile and oxidative stress parameters in nonalcoholic fatty liver rats induced by high fat high fructose administration. Generally, all treated groups with chia and quinoa seed powder and extracts showed a significant reduction in TC, TG LDL-C and VLDL-C levels and a significant increase of HDL-C in comparison with HFHFrD group. High levels of LDL-cholesterol are directly linked with cardiovascular disease development in humans (Rasheed & Cummins, 2018). Consumption of chia seed has shown promising results in reducing lipids levels, since it has good levels of unsaturated omega-3 fatty acids and dietary fiber (da Silva et al., 2017). As, omega-3 fatty acids supplementation has a basic role in promoting some markers in liver and kidney of

rats (Valenzuela et al., 2014; Mañán et al., 2018). Furthermore, these results are in parallel with de Souza Ferreira et al. (2015) who observed a significant enhancement in lipid profile on rats fed a sucrose-rich diet in a long-term which treated with chia seed compared to a sucrose-rich diet group. In addition, it has been indicated that the protein of chia has a key role in blocking markers of cholesterol synthesis (Coelho et al., 2018). In addition, Kumar et al. (2016) due the effect of chia seed to bioactive dietary elements which responsible for its therapeutic properties. In another study of Berti et al. (2005) they evaluate the safety of quinoa seed consumption of 50 g quinoa/day for 6 weeks to celiac patients and reported a reduction in TG, and total, LDL cholesterol. Moreover, Foucault et al. (2011) demonstrated consumption of quinoa caused a reduction in triglycerides (TG) and total and low density lipoproteins (LDL) cholesterol levels in rats fed enriched-fructose diet, declined the negative impact of fructose on high density lipoproteins (HDL). Also, Zevallos et al. (2014) and Halaby et al. (2017) as they reported that high cholesterol diet fortified with quinoa seed powder at 40% improved levels of cholesterol and triglycerides levels.

Table 5 indicated that fructose group showed a significant increase in serum urea and creatinine concentration as compared to that in the control group.

Table 5. Effects of chia and quinoa supplementation on lipid profile and renal parameters.

Indicators	NC	HFHFrD	CS	QS	CSE	QSE
T C mg/dl	63.80± 7.34 f	107.55± 12.75 a	84.31± 7.43 d	80.02± 6.41 d	70.02± 7.22 e	68.02± 6.92 e
TG mg/dl	75.77± 9.49 e	119.38± 11.61 a	97.07± 10.21 b	89.51± 7.53 c	87.51± 7.53 c	83.51± 7.53 c
HDL-c mg/dl	39.57± 3.71 a	26.15± 2.43 e	32.02± 1.75 d	34.12± 2.42 c	38.12± 2.74 a	38.12± 2.74 a
LDL-c mg/dl	22.17± 2.04 e	40.30± 3.92 a	30.32± 2.66 c	22.77± 2.88 e	22.97± 2.88 e	22.53± 2.88 e
VLDL-c mg/dl	15.15± 1.90 d	24.68± 2.32 a	19.41± 2.04 b	17.50± 1.51 c	17.50± 1.51 c	16.50± 1.51 c
Creatinine mg/dl	0.85± 0.02 d	2.74± 0.21 a	1.94± 0.11 b	0.88± 0.12 d	0.94± 0.04 c	0.70± 0.15 e
Uric acid mg/dl	2.12± 0.33 c	4.44± 0.33 a	3.08± 0.19 b	3.35± 0.45 b	2.44± 0.28 c	2.18± 0.19 c
Urea mg/dl	24.80± 1.26 d	46.10± 3.08 a	27.85± 2.17 c	24.05± 1.75 d	24.75± 1.19 d	27.85± 2.17 c

Data represented as mean ± SD, n = 6, values in each raw having different superscript (a, b, c, d ,e) are significantly different at ($p<0.05$),TC: Total cholesterol, TG: Triglycerides, HDL-C: High density lipoprotein cholesterol, LDL-C: Low density Lipoprotein Cholesterol, VLDL-C: very Low density Lipoprotein Cholesterol, NC: Normal Control, HFHFrD: High fat high fructose diet, CS: Chia seed, QS: Quinoa seed, CSE: Chia seed extract, QSE: Quinoa seed extract.

Quinoa seed (QS) group showed no significant changes in serum creatinine and urea concentration as compared to normal control (NC) group. Consumption of chia or quinoa extracts with high fat high fructose diet showed no significant difference in serum urea and creatinine concentration as compared to N.C group. Fructose is popular food ingredient and has potential influence to alleviate oxidative stress. Study finding showed that high fat high fructose diet administration in HFHFrD control group showed a significant increase of creatinine, uric acid and urea concentration as compared to control group.

These results come in parallel with the finding that suggest that increase of fructose consumption consider as key factor of metabolic syndrome and consequently to increase chronic renal disease (Abdel-Kaw et al., 2016).

These finding was in harmony with Halaby et al. (2017) they reported that quinoa seed fortified diet can enhancing creatinine, uric acid and urea, levels. The results of improvement in renal function may be linked with bioactive components that promote the biological functions by their antioxidants activities (Abderrahim et al., 2015).

Effect of chia and quinoa supplementation on liver function activities

With regard to liver activities (Figures 1, 2 and 3), AST, ALT and total bilirubin levels were significantly alleviated in the untreated fatty liver (HFHFrD) group compared to the normal control (NC), CS, QS, CSE and QSE groups. On the other hand, total portein significantly decreased in the HFHFrD group compared to the NC, CS, QS, CSE and QSE groups which was in agreement with (Charlton et al., 2011; Korish & Arafah, 2013; El-Abd et al., 2018).

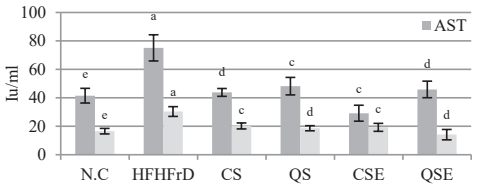

Figure 1. Changes of AST and ALT activities on different rats groups. Data represented as mean ± SD, n = 6, values in each bar having different superscript (a, b, c, d ,e) are significantly different at ($p<0.05$), AST: Aspartate aminotransferase, ALT: alanine aminotransferase, NC: Normal Control, HFHFrD: High fat high fructose diet, CS: Chia seed, QS: Quinoa seed, CSE: Chia seed extract, QSE: Quinoa seed extract

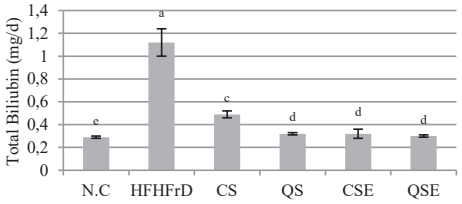

Figure 2. Changes of total bilirubin activity on different rats groups. Data represented as mean ± SD, n = 6, values in each bar having different superscript (a, b, c, d ,e) are significantly different at $p<0.05$, NC: Normal Control, HFHFrD: High fat high fructose diet, CS: Chia seed, QS: Quinoa seed, CSE: Chia seed extract, QSE: Quinoa seed extract

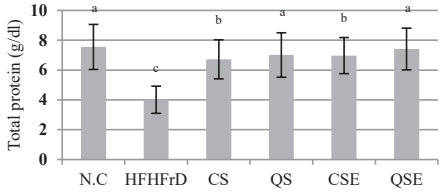

Figure 3. Changes of total bilirubin activity on different rats groups. Data represented as mean ± SD, n = 6, values in each bar having different superscript (a, b, c, d ,e) are significantly different at $p<0.05$, NC: Normal Control, HFHFrD: High fat high fructose diet, CS: Chia seed, QS: Quinoa seed, CSE: Chia seed extract, QSE: Quinoa seed extract

As can be seen, the activities of AST, ALT and total bilirubin decreased with treating of chia and quinoa suppementation. treatment of HFHFrD rats with CS, QS, CSE and QSE decreased AST enzyme by about (41.69%, 35.82%, 61.14% and 38.89%), ALT by about (33.42%, 38.72%, 36.61% and 53.53%) and total bilirubin by about (56.25%, 71.42%, 71.42% and 73.21%) and caused an increase in total portein by about (67.58%, 74.81%, 73.81% and 84.78%) comparing with HFHFrD untreated group. From these results, it could be conculded that, treating HFHFrD rats with chia and quinoa supplementation improved liver enzymes activities.

In addition, Saxena et al. (2017) confirmed these results as quinoa seed powder have a positive effect in improvement of liver enzymes and oxidative stress markers and considered quinoa seed as hepatoprotective agent.

Oxidative stress parameters

The alterations occurring in total antioxidant capacity in different groups of rats are shown in (Figure 4).

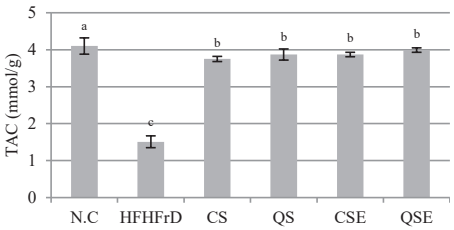

Figure 4. Changes of TAC (Total Antioxidant capacity) concentration on different rats groups. Data represented as mean ± SD, n = 6, values in each bar having different superscript (a, b, c) are significantly different at $p<0.05$, NC: Normal Control, HFHFrD: High fat high fructose diet, CS: Chia seed, QS: Quinoa seed, CSE: Chia seed extract, QSE: Quinoa seed extract

The total antioxidant capacity concentration is decreased significantly ($p<0.05$) in HFHFrD group compared to normal control (NC) group. Treatment of HFHFrD rats with CS, QS, CSE and QSE had a very high significant influence in total antioxidant capacity ($p<0.05$) comparing with HFHFrD (untreated) rats. It can observed that high fat and high fructose induced depletion in the TAC concentration from 4.10 mmol in normal control (NC) group to 1.51 mmol (HFHFrD group) ($p<0.05$). There was a highly significant increase in TAC level at all supplemented groups in comparison to control (HFHFrD) group. These results

confirmed by the finding of Foucault et al. (2011) who indicated the protective effects of quinoa seed consumption against oxidative stress by enhancing the antioxidant capacity concentration and declining lipid peroxidation level in plasma and tissues of rats. It is well established that these supplements play a basic role as an indirect antioxidant and alleviate TAC level. Hence, it seems that treatment with chia and quinoa seeds powder and extracts supplements may decline the damage HFHFrD-induced nonalcoholic fatty liver in rats via enhancing antioxidant activity (Mohamed et al., 2019). High fat high fructose diet-induced significant increase in lipid peroxidation activity in liver (Figure 5) shows the concentration of LPO in all groups.

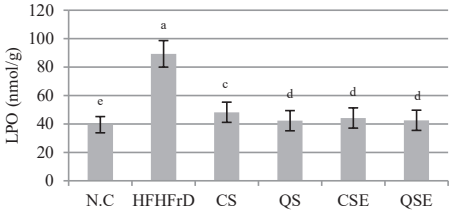

Figure 5. Changes of LPO activity on different rats groups. Data represented as mean ± SD, n = 6, values in each bar having different superscript (a, b, c, d ,e) are significantly different at $p<0.05$, NC: Normal Control, HFHFrD: High fat high fructose diet, CS: Chia seed, QS: Quinoa seed, CSE: Chia seed extract, QSE: Quinoa seed extract

After 8 consecutive weeks significant increase in LPO concentration was observed in untreated fatty liver (HFHFrD) group (89.31± 9.33 nmol/g) compared to N.C group (39.46±5.71 nmol). These results in the same line of (Crescenzo et al., 2018) who indicated that lipid oxidative damage induced by fructose rich diet that cause metabolic liver impairment. Hence, the LPO concentration decreased from 89.31 nmol for untreated (HFHFrD) group to 42.28 and 42.56 nmol in QS and QSE groups. Induction fatty liver by high fat high frcutose diet caused highly allevation in lipid peroxidation activity in liver. HFHFrD group exhibited a significant elevation of oxidative stress indicators (TAC & LPO) in addition to significant decline of the serum level of TAC in comparison with the NC group. These results in parallel with those studies of El-Abed et al. (2018) as they related this reduction to high fat diet consumption that alleviate free radicals which caused oxidative stress that plays avital role in the progression of NAFLD. Additionally, reactive oxygen species production activate lipid peroxides, thus caused the hepatic membranes damage (Li et al., 2014). In addition, Serviddio et al. (2013) reported that the increase in lipid peroxidation and the reduction antioxidants status have been observed in NAFLD patients and animals models.

CONCLUSIONS

It was concluded that dietary supplementation, is an alternative research area for the discovery of new functional ingredients for the control and management of fatty liver disease by diminishing lipids accumulation. Chia and quinoa seeds are considered as rich sources of bioactive compounds such as phenols, flavonoids, omega-3 & omega-6 constituents which abolished lipids accumulation and possess a protective influence against fatty liver disease. So, these herbal seeds can be used as functional dietary supplements in course of fatty liver disease through reducing lipid accumulation and enhancing lipids parameters, liver and renal functions.

ETHICS STATEMENT

The study experiment was performed in accordance with laws and regulation of the Medical Research Ethics Committee of National Research Center.

REFERENCES

Albillos, A., Gottardi, A., Rescigno, M. (2020). The gut-liver axis in liver disease: Pathophysiological basis for therapy. *J. Hepatol*., 72, 558-577.

Abdel-Kawi, S.H., Kamel, M.A. H., Khalid S. H. (2016). The effect of high dietary fructose on the kidney of adult albino rats and the role of curcumin supplementation: A biochemical and histological study. *Beni-suef university journal of basic and applied sciences*, 5, 52-60.

Abderrahim, F., Huanatico, E., Segura, R., Arribas, S., Gonzalez, M.C., Condezo-Hoyos, L. (2015). Physical features, phenolic compounds, betalains and total antioxidant capacity of coloured quinoa seeds (Chenopodium quinoa Willd.) from Peruvian Altiplano. *Food Chem*, 183, 83-90.

Aktaş, K., Levent, H. (2018). The effects of chia (Salvia hispanica L.) and quinoa flours on the quality of rice flour and starch based-cakes. *GIDA,* 43(4), 644-654.

Aldai, N., Osoro, K., Barrón, L.J.R., Nájera, A.I. (2006). Gas-Liquid Chromatographic Method for Analysing Complex Mixtures of Fatty Acids Including Conjugated Linoleic Acids (*cis*9*trans*11 and *trans*10*cis*12 Isomers) and Long- Chain (n-3 or n-6) Polyunsaturated Fatty Acids: Application to the Intramuscular Fat of Beef Meat. *Journal of Chromatography A*, 1110, 133-139.

Allain, C., Poon, L., Chan, C. (1974). Enzymatic determination on total serum cholesterol. *Clin. chem*., 20, 470-475.

AOAC. (2012). Official Methods of Analysis of the Association of Official Analytical Chemists, Washington, DC,USA.

Armitage, P., Berry, G. (1987). Statistical method in medical research. Blackwell, Oxford, UK, 93-213.

Ayerza, R., Coates, W. (2005). Ground chia seed and chia oil effects on plasma lipids and fatty acids in the rat. *Nutr Res.*, 25, 995-1003.

Barakat, L., (2011). Hypolipidemic and Antiatherogenic Effects of Dietary Chitosan and Wheatbran in High Fat- High Cholesterol Fed Rats. *Australian Journal of Basic and Applied Sciences,* 5(10), 30-37.

Berti, C., Riso, P., Brusamolino, A., Porrini, M. (2005). Effect on appetite control of minor cereal and pseudocereal products. *Brit J Nutr*, 94, 850-858.

Breuer, J. (1996). Report on the symposium drug effects in clinical chemistry methods. Eur. *J. Clin. Chem. Clin. Biochem.,* 34, 385-386.

Burstein, M. (1970). Rapid method for isolation of lipoproteins from human serum by precipitation with poly-anion. *J. lipid Research,* 11,583- 588.

Cao, G., Alessio, H.M. Cutler, R.G. (1993). Oxygen-radical absorbance capacity assay for antioxidants. *Free Radical Biology and Medicine*, 14, 30-311.

Carciochi, R.A., Manrique, G.D., Dimtrov, K. (2015). Optimization of antioxidants phenolic compounds extraction from quinoa (chenopodium quinoa) seeds. *J.Food Sci. Technol*., 52, 4396-4404.

Charlton M., Krishnan, A., Viker, K., Sanderson, S., Cazanave, S., McConico, A., Masuoko, H. Gores, G. (2011). Fast food diet mouse: Novel small animal model of NASH with ballooning, progressive fibrosis, and high physiological fidelity to the human condition. *Am. J. Physiol.Gastrointest. Liver Physiol*., 301 (5), 825-834.

Coelho, M. S., Soares-Freitas, R. A., Areas, J. A., Gandra, E. A., Salas-Mellado, M., de las, M. (2018). Peptides from chia present antibacterial activity and inhibit cholesterol synthesis. *Plant Foods for Human Nutrition*, *73*, 101-107.

Couturier, K., Hininger, I., Poulet, L., Anderson, R.A., Roussel, A.M., Canini, F., Batandier, C. (2016). Cinnamon intake alleviates the combined effects of dietary-induced insulin resistance and acute stress on brain mitochondria. *J. of Nutr. Biochem.*, 28, 183-190.

Crescenzo, R., Cigliano, L., Mazzoli, A., Cancelliere, R., Carotenuto, R., Tussellino, M., Liverini, G., Iossa, S. (2018) Early Effects of a Low Fat, Fructose-Rich Diet on Liver Metabolism, Insulin Signaling, and Oxidative Stress in Young and Adult Rats. *Front. Physiol*., 9,411.

da Silva, B. P., Anunciacao, P. C., Matyelka, J. C. da. S., Della Lucia, C.M., Martino, H. S. D., Pinheiro-Sant'Ana, H. M. (2017). Chemical composition of Brazilian chia seeds grown in different places. *Food Chemistry*, *221*, 1709-1716.

de Souza Ferreira, C., dd Sousa Fomes, L. de F., da Silva, G. E. S., Rosa, G. (2015). Effect of chia seed (Salvia hispanica L.) consumption on cardiovascular risk factors in humans: A systematic review. *Nutricion Hospitalaria*, 32(5), 1909–1918.

El-Abd, M.M., Abdel-Hamid, M., Ahmed-Farid, O.A., El-demerdash, M.E., Mohame, Z.F.A. (2018). Protective Effect of Synbiotic Fermented Camel Milk on Non Alcoholic Fatty Liver Disease in Rats. *Curr. Sci. Int*., 7(4), 466-480.

Fassati, P., Prencipe, L. (1982). Triglycerides determination after enzymatic hydrolysis. *clin. chem.*, 28, 2077.

Foucault, A.S., Mathe, V., Lafont, R., Even, P., Dioh, W., Veillet, S., Tome, D., Huneau, J.F., Hermier, D., Quignard-Boulangé, A. (2011). Quinoa extract enriched in 20-hydroxyecdysone protects mice from diet induced obesity and modulates adipokines expression. *Obesity*, 20, 270-277.

Friedewald, T., Levy, R. Fredrichsor, D. (1972). Estimation of the concentration of low-density lipioprotein cholesterol in plasma without use of the preparative ultracentrifuge. *Clin Chem*., 18, 499-502.

González-Mañán, D., Pettinelli, P., Tapia, G. (2018). Vegetable Oils as a Source of Alpha-Linolenic Acid and Tocopherols: Potential Effects in the Treatment of Chronic Diseases, *Clin Pharmacol Transl Med*, 2(1), 75-83.

Gordillo-Bastidas, E., Díaz-Rizzolo, D.A., Roura, E., Massanés, T., Gomis, R. (2016) Quinoa (*Chenopodium quinoa* Willd), from Nutritional Value to Potential Health Benefits: An Integrative Review. *J Nutr Food Sci*, 6, 497.

Goyat, J., Passi, S.J., Suri, S., Dutta, H. J. (2018). Development of Chia (*Salvia hispanica* L.) and Quinoa (*Chenopodium quinoa* L.) Seed Flour Substituted Cookies- Physicochemical, Nutritional and Storage Studies. *Curr. Res. Nutr Food Sci Jour*., 6(3), 757-769

Halaby, M.S., Abdel-Rahman, M.K., Hassan, R.A. (2017). Protective Influence of Quinoa on Hypercholesterolemia in Male Rats. *Current Science International*, 6(1), 259-270.

Henry, R., Cannon, D., Winkelman, J. (1974). Clinical Chemistry Principles and Techniques, *Harper and Row. New York*, 1440-1452.

Hernández-Ledesma, B. (2019). Quinoa (*Chenopodium quinoa* Willd.) as a source of nutrients and bioactive compounds: a review. *Bioactive Compounds in Health and Disease*, 2(3), 27-47.

Jaffe, M. (1980). Determination of creatinine in serum. *Phys. Chem*., 10, 391.

Korish, A.A., Arafah, M.M. (2013). Camel milk ameliorates steatohepatitis, insulin resistance and lipid peroxidation in experimental non-alcoholic fatty liver disease. BMC Complementary and *Alte. Medi*., 13, 264.

Kumar, D.G., Perumal, P.C., Kumar, K., Muthusami, S., Gopalakrishnan, V.K. (2016). Dietary Evaluation, Antioxidant and Cytotoxic Activity of Crude Extract from Chia Seeds (*Salvia hispanica* L.) against Human Prostate Cancer Cell Line (PC-3). *International Journal of Pharmacognosy and Phytochemical Research*, 8(8), 1358-1362.

Li, C., Nie, S.P., Zhu, K.X., Ding, Q., Li, C., Xiong, T., Xie, M.Y. (2014). *Lactobacillus plantarum* NCU116 improves liver function, oxidative stress and lipid metabolism in rats with high fat diet induced non-alcoholic fatty liver disease. *Food Funct.,* 5, 3216-3223.

Light, H. R., Tsanzi, E., Gigliotti, J., Morgan, K., Tou, J.C. (2009). The Type of Caloric Sweetener Added to Water Influences Weight Gain, Fat Mass, and Reproduction in Growing Sprague-Dawley Female Rats. Exp. *Biol. Med.,* 234, 651-666.

Masi L.N., Martins, A.R., Crisma, A.R., Amaral, C.L., Davanso, M.R., Afonso, T.D., Serdan, R.D.C., Cunha de Sá, M.M., Cruz, M.I.C., Alonso-Vale, R.P., Torres, J.M., Filho, J.N.P., Pereira, M.M.S., Righetti, E.A., Liberti, S.M., Hirabara. R.C. (2017). Combination of a high-fat diet with sweetened condensed milk exacerbates inflammation and insulin resistance induced by each separately in mice. *Scientific Report*., 7(1), 3937.

McCarthy, E.M., Rinella, M.E. (2012). The role of diet and nutrient composition in nonalcoholic fatty liver disease. *Journal of the Academy of Nutrition and Dietetics*, 112(3), 401-409.

Mohamed, D. A., Fouda, K.A., Mohamed, R.S. (2019). In vitro anticancer activity of quinoa and safflower seeds and their preventive effects on non-alchoholic fatty liver. *Pak.J.Biol.Sci*, 22, 383-392.

Muñoz, L.A., Cobos, A., Diaz, O., Aguilera, J.M. (2013). Chia seed (*Salvia hispanica*): an ancient grain and a new functional food. *Food Rev Int,* 29, 394-408.

Navruz, V., Sanlier, N. (2016). Nutritional and health benefits of quinoa (*Chenopodium quinoa* Willd.). *Journal of Cereal Science.,* 69, 371-376.

Ohkawa, HOhishi, N., Yagi, K. (1979). Assay for lipid peroxides in animal tissues by thiobarbituric acid reaction. *Anal Bio.*, 95, 351-358.

Patton, C., Crouch, S. (1977). A Colorimetric method for the determination of blood urea concentration. *J. Anal. Chem*., 49, 46-469.

Poudyal, H., Campbell, F., Brown, L. (2010). Olive leaf extract attenuates cardiac, hepatic, and metabolic changes in high carbohydrate-high fat-fed rats. *J Nutr.*, 140(5), 946-53.

Rasheed, A., Cummins, C. (2018). Beyond the foam cell: The role of LXRS in preventing atherogenesis. *International Journal of Molecular Sciences*, *19*(8), 2307.

Reeves, P.G., Nielsen, P.M., Fahmy, G.G. (1993). AIN-93. Purified diets for laboratory rodents: I;inal report of the American Institute of Nutrition adhoc wriling committee on the reformulation of the AIN-76 A Rodent diet. *J. Nutrition*, 123, 1939-151.

Saxena, S., Shahani, L., Bhatnagar, P. (2017). Hepatoprotective effect of *Chenopodium quinoa* seed against CCL_4 –induced liver toxicity in swiss albino male mice. *Asian J Pharm Clin Res*, 10(11), 273-276.

Serviddio, G., Bellanti, F., Vendemiale, G. (2013). Free radical biology for medicine: learning from nonalcoholic fatty liver disease. *Free Radic Biol Med* 65, 952-968.

Singh, B., Saxena, A.K., Chandan, B.K., Gupta, D.K., Bhutani, K.K.,Anand, K.K. (2001). Adaptogenic activity of a novel withanolide-free aqueous fraction from the roots of Withania somnifera Dun. *Phytother. Res.*, 15, 31-318.

Suri, S., Passi, S.J., Goyat, J. (2016). Chia seed (*Salvia hispanica* L.)-A new age functional food. *Int. J. Adv. Technol. Eng. Sci.*, 4, 286–299.

Valenzuela, R., Barrera, C., González-Astorga, M., Sanhueza, J., Valenzuela, A. (2014). Alpha linolenic acid (ALA) from *Rosa canina*, sacha inchi and chia oils may increase ALA accretion and its conversion into n-3 LCPUFA in diverse tissues of the rat. *Food Funct.*, 5, 1564-1572.

Vega-Gálvez, A., Miranda, M., Vergara, J., Uribe, E., Puente, L., Martínez, E.A. (2010). Nutrition facts and functional potential of quinoa (*Chenopodium quinoa* Willd.), an ancient Andean grain: a review. *J Sci Food Agric*, 90, 2541-2547.

Yi, D., Hui-Wen, L., Yi-Ling, L., Deng-Jye, Y., Yu-Shan, Y., Jr-Wei, C., Sheng-Yao, W., Yi-Chen, C. (2018). Nutritional composition in the chia seed and its processing properties on restructured ham-like products. *Journal of food and drug analysis*, 26, 124 - 134.

Zevallos, V., Herencia, L., Chang, F., Donnelly, S., Ellis, H., Ciclitira, P. (2014). Gastrointestinal effects of eating quinoa (*Chenopodium quinoa* Willd.) in celiac patients. *Am J Gastroenterol*, 109, 270-8.

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A SHORT REVIEW ON ACETYL XYLAN ESTERASES

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Abstract

Lignocellulose is a resource for renewable organic matter. Between the three main components, hemicellulose is the second most abundant natural polymer on earth, its main constituent being xylan. Acetyl xylan esterases are accessory enzymes involved in biodegradation of xylan, releasing acetic acid from side chains of xylan backbone. After the action of these enzymes, other lignocellulases are able to act on their specific substrate. The main microbial sources for acetyl xylan esterase include Penicillium sp., Thermoanarobacterium sp., Aspergillus sp., Fusarium sp., Streptomyces sp., Phanerochaete sp., Bacillus sp., Trichoderma sp. etc. Screening methods for identification of acetyl xylan esterase microorganisms requires specific substrate for this enzyme such as acetylated xylan or xylooligosaccharides, α and β-naphthyl acetate or p-nitrophenyl acetate. The importance of these enzymes is given by their role in various applications such as biofuel production, pulp and paper biobleaching or food and feed.

Key words*: acetyl, esterase, lignocellulose, xylan, xylanase.*

INTRODUCTION

Plant biomass, mostly represented by lignocellulose, is one of the most abundant biomasses on Earth. Lignocellulose degradation is still a top research subject, due to its potential for biofuel, biodegradable plastics, organic acids (Dumitru et al., 2018; Trulea et al., 2016) or other value-added compounds.

Lignocellulose is comprised mostly of cellulose, hemicellulose and lignin. Between the three main components of lignocellulose, hemicellulose is the second most abundant polymer.

Hemicellulases are enzymes that catalyse hemicelluloses degradation acting either as glycoside hydrolases or carbohydrate esterases (e.g. acetyl xylan esterases) (Chiș et al., 2010).

According to several reports, the potential worldwide market value for hemicellulose was estimated to almost 178 million € (Wysokińska, 2010), with the condition that hemicellulose is depolymerised to pure forms of oligosaccharides or monosaccharide (Sista Kameshwar & Qin, 2018).

Xylan forms hemicellulose and is mainly found in plant cell wall (Ciotea & Popa, 2019). Its depolymerisation requires the combined action of a group of enzymes generally known as xylanases. Among them, acetyl xylan esterase is an accessory enzyme important for deacetylation of xylo-oligosaccharides and xylans.

Acetyl groups increase the plant resistance to the action of lignocellulosic enzymes (Biely et al., 2013). Therefore, acetylation has a crucial role in establishing the physio-chemical properties of the cell wall, such as: water solubility, recalcitrance to degradation and bulk volume of polysaccharide.

Removing the acetyl groups from xylan structure will lead to exposed areas susceptible to hydrolysation by other enzymes such as xylanases and in the end will increase cellulases accessibility (Sista Kameshwar & Qin, 2018).

Although there are several pretreatment methods that can remove acetyl groups from lignocellulosic structures, most of these methods have some disadvantages such as: economic viability (Adesioye et al., 2016), environmental impact or harsh experimental conditions (Diguta et al., 2007). Therefore, there's a necessity for developing a method that can overcome these obstacles, one possibility being the enzymatic hydrolysis of these acetyl groups with acetyl xylan esterases.

CHARACTERISTICS OF ACETYL XYLAN ESTERASES

Acetyl xylan esterase (E.C. 3.1.1.72, AcXE, AXE) catalyses the hydrolysis of acetyl side-chain groups linked to xylan backbone, as seen in Figure 1.

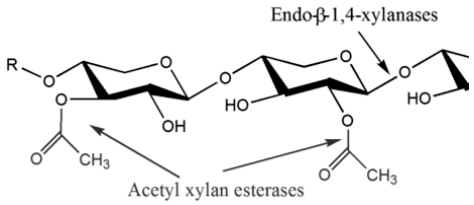

Figure 1. Hydrolysis of xylan by endoxylanases and acetyl xylan esterases (Wu et al., 2017)

The CAZy database integrate acetyl xylan esterases in carbohydrate esterase families CE 1-7 and 16-17. Most of these enzymes need for deacetylation a catalytic triad of Ser-His-Asp, with the exception of CE 4 family that entails a different mechanism, by using a metal-ion dependent hydrolysis (Mai-Gisondi & Master, 2017).

Acetyl xylan esterases were first recognized as part of xylanolytic and cellulolytic systems since 1985 by Biely, described as enzymes able to remove acetyl groups from D-xylopyranosyl residues (Biely & Côté, 2005).

Several studies (Biely & Côté, 2005; Sista Kameshwar & Qin, 2018; Zhang et al., 2011) suggest that the complete degradation of xylan by endoxylanases proceeded faster and with a higher level after deacetylation with acetyl xylan esterases. Also, the synergistic action of cellulase, endoxylanase and AXE resulted in an improved hydrolysis of cellulose, highlighting the intricate structure of acetylated xylan interlinked with cellulose fibrils (Sista Kameshwar & Qin, 2018).

The usual substrates subjected to the action of acetyl xylan esterase are: O-acetyl-4-O-methyl-D-glucurono-D-xylan (acetyl xylan found hardwood hemicellulose), acetylated xylan (Johnson et al., 1988), acetylated glucose, acetylated xylose, alpha-napthyl acetate or p-nitrophenyl acetate. Substrate specificity of acetyl xylan esterases is not well understood yet, due to lack of knowledge regarding the relationship between structure and function (Biely & Côté, 2005).

SCREENING FOR ACETYL XYLAN ESTERASE ACTIVITY

There are different screening methods used to identify the microbial producers that exhibit acetyl xylan esterase activity, most of them using fluorogenic or chromogenic acetyl esterase substrates.

The qualitative screenings of AXE are plate screening methods that are based on cultivating the microbial strain on a minimal agar medium with an unique carbon source such as: acetylated xylan, p-nitrophenyl acetate, α- or β-naphthyl acetate or 4-methylumbelliferyl acetate (Biely & Côté, 2005; Martínez-Martínez et al., 2007). After incubation, AXE activity can be identified as a hydrolysis zone around the microbial colony.

For a quantitative assay of AXE activity, the substrate can be natural or synthesized: p-nitrophenyl acetate (Atta et al., 2011), α-naphthyl acetate, N, N'-diacetylchitobiose, acetylated xylan, cellulose pentaacetate, galactose pentaacetate (Degrassi et al., 2000), 7-amino cephalosporanic acid (Martínez-Martínez et al., 2007).

An easy and highly reproducible assay for AXE activity is based on measuring the hydrolysis of p-nitrophenyl acetate to p-nitrophenol, as suggested by several studies (Atta et al., 2011; Burlacu et al., 2018). The assay mixture containing 1 mL 100 mM sodium phosphate buffer (pH 7.00), 0.9 mL 10 mM p-nitrophenyl acetate and 0.1 mL enzyme sample was incubated at 37ºC and after 10 minutes, the release of p-nitrophenol was measured by reading the absorbance at 410 nm. One unit of acetyl xylan esterase activity was defined as the amount of enzyme that will release one μmol of p-nitrophenol per minute under the specified assay conditions (Atta et al., 2011; Burlacu et al., 2018).

SOURCES OF ACETYL XYLAN ESTERASES

The acetylated glycosyl residues found in lignocellulosic structures protect cellulose and hemicellulose from the action of glycoside hydrolases. Thus, microorganisms were required to secrete several enzymes capable of releasing acetyl groups from these structures

known as carbohydrate esterases (CE), one of them being acetyl xylan esterases.

AXE producing microorganisms have been isolated and characterised from various environments (Adesioye et al., 2016). Microbial production of AXE was preferred to plant or animal sources due to easier genetic modifications or manipulation, availability and structural stability (Atta et al., 2011).

The bacterial strains that are known to exhibit AXE activity are included in Table 2, the main producers belonging to *Bacillus, Fibrobacter, Streptomyces* and *Thermobifida*.

Table 2. Bacterial sources of AXE

Microorganism	Literature
Acidothermus cellulolyticus	Shahid et al. (2018)
Anoxybacillus flavithermus	Eminoğlu et al. (2015)
Bacillus pumilus	Degrassi et al. (2000) Martínez-Martínez et al. (2007)
Bacillus subtilis	Tian et al. (2014), Christov & Prior (1993)
Butyrivibrio proteoclasticus	Till et al. (2013)
Caldanaerobacter subterraneus	Moriyoshi et al. (2013)
Caldicellulosiruptor saccharolyticus	Lüthi et al. (1990)
Chrysosporium lucknowense	Pouvreau et al. (2011)
Clostridium cellulovorans	Kosugi et al. (2002)
Fibrobacter succinogenes	Yoshida et al. (2010)
Flavobacterium johnsoniae	Razeq et al. (2018)
Geobacillus stearothermophilus	Lansky et al. (2014)
Hungateiclostridium thermocellum	Neumüller et al. (2015)
Ruminiclostridium josui	Wang et al. (2018)
Streptomyces sp.	Coman et al. (2013)
Streptomyces flavogriseus	Christov & Prior (1993)
Streptomyces lividans	Biely et al. (2013)
Streptomyces olivochromogenes	Christov & Prior (1993)
Thermoanaerobacterium saccharolyticum	Lorenz & Wiegel (1997)
Thermobifida fusca	Huang et al. (2010) Christov & Prior (1993)
Thermotoga maritima	Drzewiecki et al. (2010)

The most studied xylan degrading fungi (Table 3) were filamentous fungi (*Aspergillus spp., Trichoderma spp.)*, known for their ability to produce a wide range of xylanases.

Table 3. Fungal sources of AXE

Microorganism	Literature
Aspergillus awamori	Christov & Prior (1993) Koseki et al. (2005)
Aspergillus ficuum	Park (2011)
Aspergillus japonicus	Christov & Prior (1993)
Aspergillus luchuensis	Komiya et al. (2017)
Aspergillus nidulans	Mai-Gisondi et al. (2017) Christov & Prior (1993)
Aspergillus niger	Neumüller et al. (2015)
Aspergillus oryzae	Manavalan (2017)
Chrysosporium lucknowense	Pouvreau et al. (2011)
Coprinopsis cinerea	Juturu et al. (2013)
Fusarium oxysporum	Christov & Prior (1993)
Neocallimastix frontalis	Kwon et al. (2016)
Orpinomyces sp.	Comlekcioglu et al. (2014) Neumüller et al. (2015)
Penicillium chrysogenum	Yang et al. (2017)
Phanerochaete chrysosporium	Huy et al. (2013)
Rasamsonia emersonii	Neumüller et al. (2015)
Rhodotorula mucilaginosa	Christov & Prior (1993)
Schizophyllum commune	Biely et al. (2013) Christov & Prior (1993)
Talaromyces purpureogenus	Colombres et al. (2008)
Termitomyces clypeatus	Mukhopadhyay et al. (2003)
Thermothelomyces thermophilus	Kool et al. (2014)
Trichoderma longibrachiatum	Neumüller et al. (2015)
Trichoderma reesei	Biely et al. (2013) Christov & Prior (1993) Neumüller et al. (2015)
Volvariella volvacea	Liu & Ding (2016) Tian et al. (2012)

As observed, there are numerous microorganisms that display acetyl xylan esterase activity from both bacteria and fungi, strains that will secrete various hydrolases for the complete breakdown of cellulose and xylan (Sista Kameshwar & Qin, 2018).

Acetyl xylan esterase production is linked to the type of microbial strain, cultivation media composition and the fermentation protocol. Solid state fermentation (SSF) has an immense potential for AXE synthesis due to its advantages such as: higher productivity, wide variety of matrices, higher concentration and stability of the desired product, low energy consumption, easier control of contamination or less expensive process (Atta et al., 2011).

APPLICATIONS OF ACETYL XYLAN ESTERASES

An important role of acetyl xylan esterases is its synergistic action with xylanases and cellulases in lignocellulose degradation for biofuel (bioethanol) production (Sista Kameshwar & Qin, 2018).

Another application of these enzymes is linked to pulp and paper industry, were their action combined with endoxylanases activity leads to an improved biobleaching process (Sista Kameshwar & Qin, 2018), a protocol that requires less highly toxic chemical pretreatments.

By removing some of the side chains of xylan structure, including acetyl groups, the modified xylan obtained can be directed to form a hydrogel suitable for pharmaceutical use as a drug delivery agent (Van Zyl et al., 2013). Furthermore, some studies suggest that AXE can be used in deacetylation of cephalosporin C and thus in antibiotic production (Benini et al., 2001), such as cephalosporins, penicillins, monobactams and carbapenems (Sista Kameshwar & Qin, 2018).

AXE action on the highly viscous lignocellulose can lead to deacetylated xylo-oligosaccharides that are used as feed additives that will increase digestibility (Ștef et al., 2013). Also, AXE can be use as prebiotics in both food or feed industries (Motta et al., 2013). In addition, the supplementation of cellulases and xylanases, including AXE, to animal feedstock increased milk production of buffaloes and goats (Sista Kameshwar & Qin, 2018).

AXE can be used in food processing applications for clarifying fruit juices along with pectinases (Atta et al., 2011).

CONCLUSIONS

Despite its potential, lignocellulose remains relatively underutilized due to its structural complexity and recalcitrance, demanding a combined action of several various enzymes with specific mechanisms for complete degradation.

Acetyl xylan esterase are responsible for removing acetyl side-chain groups linked to xylan backbone. Deacetylation of xylan can improve cellulase access to cellulose and thus improve depolymerisation of lignocellulose and generate value-added products.

Due to scarcity of microbial producers of AXE, there's a high interest in finding new sources of acetyl xylan esterases by employing different screening protocols.

Although, AXE is considered to be an accessory enzyme, its importance is depicted from its role in different industrial applications such as food, feed, medical, biofuel or pulp and paper.

REFERENCES

Adesioye, F. A., Makhalanyane, T. P., Biely, P., & Cowan, D. A. (2016). Phylogeny, classification and metagenomic bioprospecting of microbial acetyl xylan esterases. *Enz. and microbial technology*, 93, 79-91.

Atta, S., Ali, S., Akhtar, M. N., & Haq, I. (2011). Determination of some significant batch culture conditions affecting acetyl-xylan esterase production by *Penicillium notatum* NRRL-1249. *BMC biotechnology*, 11(1), 52.

Benini, S., Degrassi, G., Krastanova, I., Lamba, D., & Venturi, V. (2001). Purification, crystallization and preliminary X-ray analysis of an acetylxylan esterase from *Bacillus pumilus*. *Acta Crystallographica, Section D: Biological Crystallography*, 57(12), 1906-1907.

Biely, P., & Côté, G. L. (2005). Microbial hemicellulolytic carbohydrate esterases. In C.T. Hou (Ed.), *Handbook of industrial biocatalysis* (pp. 21-24). Taylor and Francis Group, Boca Raton, FL.

Biely, P., Cziszárová, M., Uhliariková, I., Agger, J. W., Li, X. L., Eijsink, V. G., & Westereng, B. (2013). Mode of action of acetylxylan esterases on acetyl glucuronoxylan and acetylated oligosaccharides generated by a GH10 endoxylanase. *Biochimica et Biophysica Acta (BBA)*, 1830(11), 5075-5086.

Burlacu, A., Israel-Roming, F., & Cornea, C. P. (2018). Screening of microorganisms displaying acetyl xylan esterase activity. *Scientific Papers, Series B, Horticulture*, 62, 715-720.

Chiș, A., Fetea, F., Taoutaou, A., & Socaciu, C. (2010). Application of FTIR spectroscopy for a rapid determination of some hydrolytic enzymes activity on sea buckthorn substrate. *Romanian Biotechnological Letters*, 15(6), 5738-5744.

Christov, L. P., & Prior, B. A. (1993). Esterases of xylan-degrading microorganisms: production, properties, and significance. *Enzyme and microbial technology*, 15(6), 460-475.

Ciotea, D., & Popa, M. E. (2019). Trends on pharmaceutical packaging materials. *Scientific Bulletin. Series F. Biotechnologies*, 23, 137-142.

Colombres, M., Garate, J. A., Lagos, C. F., Araya-Secchi, R., Norambuena, P., Quiroz, S., ... & Eyzaguirre, J. (2008). An eleven amino acid residue deletion expands the substrate specificity of acetyl xylan esterase II (AXE II) from *Penicillium purpurogenum*. *Journal of computer-aided molecular design*, 22(1), 19-28.

Coman, G., Georgescu, L., Bahrim, G. (2013). Streptomyces p12-137 endoxylanases characteristics evaluation in order to obtain xylo-oligosaccharides. *Romanian Biotechnological Letters*, 18, 8086-8096.

Comlekcioglu, U., Tutus, A., Cicekler, M., Gunes, M., & Aygan, A. (2014). Application of recombinant xylanase from *Orpinomyces* sp. in elemental chlorine-free bleaching of kraft pulps. *Romanian Biotechnological Letters*, 19(1), 8941-8950.

Degrassi, G., Kojic, M., Ljubijankic, G., & Venturi, V. (2000). The acetyl xylan esterase of *Bacillus pumilus* belongs to a family of esterases with broad substrate specificity. *Microbiology*, 146(7), 1585-1591.

Diguta, C., Jurcoane, S., Israel-Roming, F., Brule, M., Mukengele, M., Lemmer, A., & Oechsner, H. (2007). Studies concerning enzymatic hydrolysis of energy crops. *Romanian Biotechnological Letters*, 12(2), 3203-3207.

Drzewiecki, K., Angelov, A., Ballschmiter, M., Tiefenbach, K. J., Sterner, R., & Liebl, W. (2010). Hyperthermostable acetyl xylan esterase. *Microbial biotechnology*, 3(1), 84-92.

Dumitru, M., Tabuc, C., Sorescu, I., Vasilachi, A., Hăbeanu, M., Petre, S., & Jurcoane, Ş. (2018). Researches concerning the level of fermentable sugars from feed materials in relation with cellulase hydrolysis by carbohydrase enzyme. *Scientific Bulletin. Series F. Biotechnologies*, 22, 205-208.

Eminoğlu, A., Ülker, S., & Sandallı, C. (2015). Cloning, Purification and Characterization of Acetyl Xylane Esterase from *Anoxybacillus flavithermus* DSM 2641 T with Activity on Low Molecular-Weight Acetates. The *Protein Journal*, 34(4), 237-242.

Huang, Y. C., Chen, G. H., Chen, Y. F., Chen, W. L., & Yang, C. H. (2010). Heterologous expression of thermostable acetyl xylan esterase gene from *Thermobifida fusca* and its synergistic action with xylanase for the production of xylooligosaccharides. *Biochemical and biophysical research communications*, 400(4), 718-723.

Huy, N. D., Thayumanavan, P., Kwon, T. H., & Park, S. M. (2013). Characterization of a recombinant bifunctional xylosidase/arabinofuranosidase from *Phanerochaete chrysosporium*. *Journal of bioscience and bioengineering*, 116(2), 152-159.

Johnson, K. G., Fontana, J. D., & MacKenzie, C. R. (1988). Measurement of acetyl xylan esterase in Streptomyces. In Methods in Enzymology. Academic Press. 160, 551-560.

Juturu, V., Aust, C., & Wu, J. C. (2013). Heterologous expression and biochemical characterization of acetyl xylan esterase from *Coprinopsis cinerea*. *World Journal of Microbiology and Biotechnology*, 29(4), 597-605.

Komiya, D., Hori, A., Ishida, T., Igarashi, K., Samejima, M., Koseki, T., & Fushinobu, S. (2017). Crystal structure and substrate specificity modification of acetyl xylan esterase from *Aspergillus luchuensis*. *Applied Environmental Microbiology*, 83(20), e01251-17.

Kool, M. M., Schols, H. A., Wagenknecht, M., Hinz, S. W., Moerschbacher, B. M., & Gruppen, H. (2014). Characterization of an acetyl esterase from *Myceliophthora thermophila* C1 able to deacetylate xanthan. *Carbohydrate polymers*, 111, 222-229.

Koseki, T., Miwa, Y., Fushinobu, S., & Hashizume, K. (2005). Biochemical characterization of recombinant acetyl xylan esterase from *Aspergillus awamori* expressed in *Pichia pastoris*: mutational analysis of catalytic residues. *Biochimica et Biophysica Acta (BBA)-Proteins and Proteomics*, 1749(1), 7-13.

Kosugi, A., Murashima, K., & Doi, R. H. (2002). Xylanase and acetyl xylan esterase activities of XynA, a key subunit of the *Clostridium cellulovorans* cellulosome for xylan degradation. *Applied Environmental Microbiology*, 68(12), 6399-6402.

Kwon, M., Song, J., Park, H. S., Park, H., & Chang, J. (2016). Characterization of heterologously expressed acetyl xylan esterase1 isolated from the anaerobic rumen fungus *Neocallimastix frontalis* PMA02. *Asian-Australasian journal of animal sciences*, 29(11), 1576-1584.

Lansky, S., Alalouf, O., Salama, R., Dvir, H., Shoham, Y., & Shoham, G. (2014). Preliminary crystallographic analysis of a double mutant of the acetyl xylo-oligosaccharide esterase Axe2 in its dimeric form. *Acta Crystallographica, Section F: Structural Biology Communications*, 70(4), 476-481.

Liu, S., & Ding, S. (2016). Replacement of carbohydrate binding modules improves acetyl xylan esterase activity and its synergistic hydrolysis of different substrates with xylanase. *BMC Biotechnology*, 16(1), 73.

Lorenz, W. W., & Wiegel, J. (1997). Isolation, analysis, and expression of two genes from *Thermoanaerobacterium* sp. strain JW/SL YS485: a beta-xylosidase and a novel acetyl xylan esterase with cephalosporin C deacetylase activity. *Journal of bacteriology*, 179(17), 5436-5441.

Lüthi, E., Jasmat, N. B., & Bergquist, P. L. (1990). Overproduction of an acetylxylan esterase from the extreme thermophile "*Caldocellum saccharolyticum*" in *Escherichia coli*. *Applied microbiology and biotechnology*, 34(2), 214-219.

Mai-Gisondi, G., Maaheimo, H., Chong, S. L., Hinz, S., Tenkanen, M., & Master, E. (2017). Functional comparison of versatile carbohydrate esterases from families CE1, CE6 and CE16 on acetyl-4-O-methylglucuronoxylan and acetyl-galactoglucomannan. *Biochimica et Biophysica Acta (BBA)-General Subjects*, 1861(9), 2398-2405.

Mai-Gisondi, G., & Master, E. R. (2017). Colorimetric detection of acetyl xylan esterase activities. In D. Wade Abbott, Alicia Lammerts van Bueren (Ed.), *Protein-Carbohydrate Interactions* (pp. 45-57). Humana Press, New York, NY.

Manavalan, T., Liu, R., Zhou, Z., & Zou, G. (2017). Optimization of acetyl xylan esterase gene expression in *Trichoderma reesei* and its application to improve the saccharification efficiency on different biomasses. *Process Biochemistry*, 58, 160-166.

Martínez-Martínez, I., Montoro-García, S., Lozada-Ramírez, J. D., Sánchez-Ferrer, Á., & García-Carmona, F. (2007). A colorimetric assay for the determination of acetyl xylan esterase or cephalosporin C acetyl esterase activities using 7-amino cephalosporanic acid, cephalosporin C, or acetylated xylan as substrate. *Analytical biochemistry*, 369(2), 210-217.

Moriyoshi, K., Koma, D., Yamanaka, H., Sakai, K., & Ohmoto, T. (2013). Expression and characterization of a thermostable acetyl xylan esterase from *Caldanaerobacter subterraneus* subsp. *tengcongensis* involved in the degradation of insoluble cellulose acetate. *Bioscience, biotechnology, and biochemistry*, 77(12), 2495-2498.

Motta, F. L., Andrade, C. C. P., & Santana, M. H. A. (2013). A review of xylanase production by the fermentation of xylan: classification, characterization and applications. In Anuj Chandel (Ed.), *Sustainable Degradation of Lignocellulosic biomass-Techniques, Applications and Commercialization* (pp. 8815). IntechOpen.

Mukhopadhyay, A., Hazra, P. P., Sengupta, T., Saha, R., Nandi, R., & Sengupta, S. (2003). Protein-Protein Interaction Conferring Stability to an Extracellular Acetyl (Xylan) Esterase Produced by *Termitomyces clypeatus*. *Biotechnology progress*, 19(3), 720-726.

Neumüller, K. G., de Souza, A. C., van Rijn, J. H., Streekstra, H., Gruppen, H., & Schols, H. A. (2015). Positional preferences of acetyl esterases from different CE families towards acetylated 4-O-methyl glucuronic acid-substituted xylo-oligosaccharides. *Biotechnology for biofuels*, 8(1), 7.

Park, S. M. (2011). Acetyl xylan esterase of *Aspergillus ficcum* catalyzed the synthesis of peracetic acid from ethyl acetate and hydrogen peroxide. *Journal of bioscience and bioengineering*, 112(5), 473-475.

Pouvreau, L., Jonathan, M. C., Kabel, M. A., Hinz, S. W. A., Gruppen, H., & Schols, H. A. (2011). Characterization and mode of action of two acetyl xylan esterases from *Chrysosporium lucknowense* C1 active towards acetylated xylans. *Enzyme and microbial technology*, 49(3), 312-320.

Razeq, F. M., Jurak, E., Stogios, P. J., Yan, R., Tenkanen, M., Kabel, M. A., ... & Master, E. R. (2018). A novel acetyl xylan esterase enabling complete deacetylation of substituted xylans. *Biotechnology for biofuels*, 11(1), 74.

Shahid, S., Tajwar, R., & Akhtar, M. W. (2018). A novel trifunctional, family GH10 enzyme from *Acidothermus cellulolyticus* 11B, exhibiting endo-xylanase, arabinofuranosidase and acetyl xylan esterase activities. *Extremophiles*, 22(1), 109-119.

Sista Kameshwar, A. K., & Qin, W. (2018). Understanding the structural and functional properties of carbohydrate esterases with a special focus on hemicellulose deacetylating acetyl xylan esterases. *Mycology*, 9(4), 273-295.

Stef, L., Simiz, E., Drinceanu, D., & Stef, D. (2013). The effects of enzyme supplementation on bio-productive performance, intestinal viscosity, blood parameters and intestinal microflora of broiler chicken fed with Triticale based diets. *Romanian Biotechnological Letters*, 18(6), 7283-7292.

Tian, B., Chen, Y., & Ding, S. (2012). A combined approach for improving alkaline acetyl xylan esterase production in *Pichia pastoris*, and effects of glycosylation on enzyme secretion, activity and stability. *Protein expression and purification*, 85(1), 44-50.

Tian, Q., Song, P., Jiang, L., Li, S., & Huang, H. (2014). A novel cephalosporin deacetylating acetyl xylan esterase from *Bacillus subtilis* with high activity toward cephalosporin C and 7-aminocephalosporanic acid. *Applied microbiology and biotechnology*, 98(5), 2081-2089.

Till, M., Goldstone, D. C., Attwood, G. T., Moon, C. D., Kelly, W. J., & Arcus, V. L. (2013). Structure and function of an acetyl xylan esterase (Est2A) from the rumen bacterium *Butyrivibrio proteoclasticus*. *Proteins: Structure, Function, and Bioinformatics*, 81(5), 911-917.

Trulea, A., Vintila, T., Popa, N., & Pop, G. (2016). Mild alkaline pretreatment applied in the biorefinery of Sorghum biomass for ethanol and biogas production. *AgroLife Scientific Journal*, 5(2), 156-159.

Van Zyl, W. H., Chimphango, A. F. A., & Gorgens, J. F. (2013). U.S. Patent Application No. 13/703,228.

Wang, Y., Sakka, M., Yagi, H., Kaneko, S., Katsuzaki, H., Kunitake, E., ... & Sakka, K. (2018). *Ruminiclostridium josui* Abf62A-Axe6A: a tri-functional xylanolytic enzyme exhibiting α-l-arabinofuranosidase, endoxylanase, and acetylxylan esterase activities. *Enzyme and microbial technology*, 117, 1-8.

Wu, H., Xue, Y., Li, H., Gan, L., Liu, J., & Long, M. (2017). Heterologous Expression of a new acetyl xylan esterase from *Aspergillus niger* BE-2 and its synergistic action with xylan-degrading enzymes in the hydrolysis of bamboo biomass. *BioResources*, 12(1), 434-447.

Wysokińska, Z. (2010). A market for starch, hemicellulose, cellulose, alginate, its salts and esters, and natural polymers, including chitin and chitosan: analysis results. *Fibres & Textiles in Eastern Europe*, 18(6), 83.

Yang, Y., Zhu, N., Yang, J., Lin, Y., Liu, J., Wang, R., ... & Yuan, H. (2017). A novel bifunctional acetyl xylan esterase/arabinofuranosidase from *Penicillium chrysogenum* P33 enhances enzymatic hydrolysis of lignocellulose. *Microbial cell factories*, 16(1), 166.

Yoshida, S., Mackie, R. I., & Cann, I. K. (2010). Biochemical and domain analyses of FSUAxe6B, a modular acetyl xylan esterase, identify a unique carbohydrate binding module in Fibrobacter succinogenes S85. *Journal of bacteriology*, 192(2), 483-493.

Zhang, J., Siika-aho, M., Tenkanen, M., & Viikari, L. (2011). The role of acetyl xylan esterase in the solubilization of xylan and enzymatic hydrolysis of wheat straw and giant reed. *Biotechnology for Biofuels*, 4(1), 6.

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EXTRACTION AND CHARACTERIZATION OF WATERMELON SEED OIL

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Abstract

Oil extracts from a number of many fruits, nuts and seeds are being used in soap making, cooking, pharmaceutical products and other industries. The seeds of numerous fruits are thrown out as waste despite of their nutritional values. Many of them represent an important source of high quality protein and vitamins. Watermelon seed oil has a large concentration of unsaturated fatty acids. The low acid, peroxide and iodine values of the oil together with some favourable functional properties makes the watermelon seed and seed oil suitable for human use rather than as a waste where the crop is grown in abundance. The study was realized using Soxhlet method and micro-Kjeldahl. Citrullus lanatus seed oil has a lot of nutritional compounds. As mineral content is shown that is rich in phosphorus 30.8 mg, magnesium 2.98 mg and sodium 2.3 mg. The fatty acid composition is predominant by linoleic acid 65.7%, polyunsaturated fatty acid 66.03% and saturated fatty acids 17.87%.

Key words*: watermelon seed, oil characteristics, nutritional quality.*

INTRODUCTION

Vegetable oils are essential in satisfying global nutritional requests and are exploid in industrial and food purposes. Although the broad range of sources of vegetable oils, the world comsumption is dominated by sunflower, soya bean and rapeseed oils. These sources are conventional and no longer meet the increasing demands of industrial and domestic sectors. From this point of view, non-conventional seed oils are much approachable to confront with this challenge. It was reported that seeds of some species of *Cucurbitaceae* family can be the edible oil sources to meet the increasing demands for vegetable oil (Stevenson, 2007).

Citrullus lanatus is grown all over the warm parts of the globe and is one of the under-utilized fruits. Watermelon is almost exclusively consumed as a fresh fruit. The pulp can be also found in the composition of juices, jams, salads, jellies and even sauces. In some regions of Middle East and in China seeds are consumed. In other countries the peel is pickled. Some beers have in the ingredients watermelon juice. In India make bread using the seed flour. But in most parts the seed and rind remaining major wastes (Dias, 2010).

The largest producers of watermelon are China with 79.2 million tons, Turkey with 3.9 tons and followed by Iran with 2.8 million tons. Total production volume is aproximative 117.01 million tons.

Watermelon seeds remain intact after removing the pulp and rind making them usable in the food industry.

The seeds consist in 57.1% fat, 31.9% protein, 8.2% fiber, 6.2% ash, 4.4% carbohydrates, 456 mg phosphorus, 130 mg calcium and 7.5 mg iron. It also contains aminoacids as Isoleucine, Leucine, Tryptophan and Valine (Adejumo, 2015).

According to the results obtained by Hassan et al. in 2013 in the oil are predominant high levels of fatty acids (78.35%) such as linoleic acid (59.6%), stearic, palmitic and oleic acids (18.1%). The seeds contain protein (35.66-36.47%) and oil (50.10-51.01%) making them good to use in food formulations.

To extract lipids from seed oil are used organic solvents, but this has some disadvantages because of the possibility of thermal degradation of the functional compounds and the unsaturated fatty acids, depending on the need to eliminate the residues of organic solvent from the oil (Costa de Conto, 2011).

The main objective of this study is to determine the physicochemicals proprieties and the fatty acid composition of the *Citrullus lanatus* seed oil practicing the solvent extraction method.

MATERIALS AND METHODS

A number of 10 pieces of watermelon (*Citrullus lanatus*) were purchased from a local producer (Călărași, Romania) during the summer season of 2019. The selection of the seeds was done in a random way.

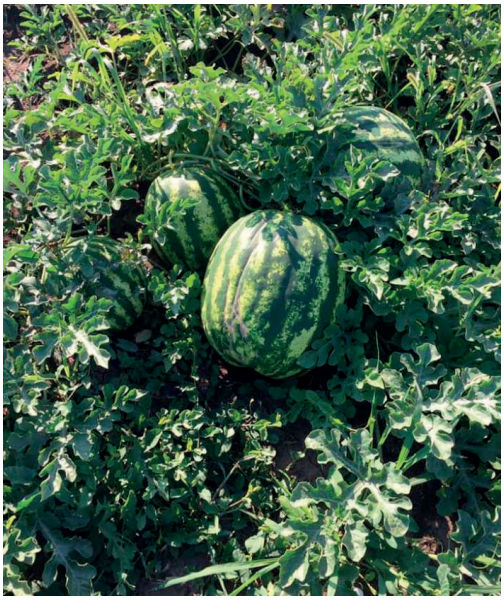

Figure 1. Landscape of watermelon producer

Watermelons were cutted into slices and the seeds were collected using the hands and then washed with distilled water. They were put to dry in the sun at aproximative 28°C for a week. The seeds were peeled manually after being shelled by breaking them with a metal cylinder to remove the kernels. Fine flour of kernels was obtained after they were ground with a coffee grinder and stored in the refrigerator at 6°C until analyses were made.

Extraction

The crude fat was extracted using Soxhlet method and total nitrogen with micro-Kjeldahl. The protein was calculated N x 5.3. The refractive index at 25°C, the acid value Cd 3a-63, the peroxide value Cd 8-53, the saponification value Cd 3-25, the iodine value Cd 1-25 and the unsaponificable matter Ca 6a-40 of the samples of oil were determined.

Using the 743 Rancimat at 110°C was evaluated the oxidative stability of the *Citrullus lanatus* oil. The oil sample weighing 3 g was placed in the Rancimat apparatus and heated under an airflow rate of 4 L/h. After 30 minutes the temperature reached 110°C, the vessels head outlets were connected to the conductivity cells, the air flow rate was increased to 20 L/h so the measurement started.

All analyses were repeated three times and expressed as ± SD.

Using boron trifluoride the methyl ester of crude oil was prepared. A gas chromatograph was used to separate the methyl esters using helium as the carrier gas.

Mineral contents and physicochemical properties were characterised by using various standard methods.

Percentage oil yield

The percentage of oil yield of the seeds was determinated and calculated using the equation:

$$\text{Percentage yield} = \frac{\text{weight of extracted oil}}{\text{weight of seed}} \times 100$$

Specific gravity

The specific gravity of the watermelon oil was identified emplying the ratio weight of the oil to the equivalent weight of water corresponding to the next formula:

$$\text{Specific gravity} = \frac{W1}{\text{W2}}$$

Where: W_1 is noted the weight of the oil and W_2 is weight of equivalent volume of water.

Acid value

For the measurement of acid value some ethanol was heated on a water bath for a few number of minutes to eliminate dissolved gases. After it was then neutralised by including small drops of phenolphtalein and potassium hydroxide till a light pink colour was achieved. A quantity 6 g of oil was weighed into a conical bottle and 50 ml of boiling already neutralized ethanol was supplementary added. The fusion was then titrated with potassium hydroxide solution before the pink colour recurred.

The acid value was calculated using:

$$\text{Acid value} = \frac{\text{titre value(ml)x N x 56.1}}{\text{weight of sample}}$$

Where: N is noted normality of KOH is 0.1M and 56.1 is molar mass of KOH.

Free fatty acid

Oleic acid as the percentage of free fatty acid was calculated by multiplying the acid value with the 0.503.

Saponification value

For saponification value was used 2 g of oil into a conical cilinder and 25 ml of alcoholic KOH was combined. A blank was also processed by setting 25 ml of alcoholic KOH in a identical cilinder. Reflux condensers were implemented to bottles and the constituents were heated in a water bath for exactly 60 minutes, rolling the bottle from time to time. The bottles were then let to cool for awhile and after the condensers washed down with some distillated water. The surplus KOH has been titrated with HCl acid utilizing phenolphthalein indicator. Then was calculated applying the upcoming formula:

$$\text{Saponification value} = \frac{(b-a)\text{x F x } 28.05}{\text{weight of sample}}$$

Where:
b is notted titre value of blank (ml)
a is notted titre value of sample (ml)
F is factor of 0.46 M HCl = 1
28.05 = mg of KOH proportionate to 1 ml of 0.46 M HCl

Peroxide value

Peroxide value of the *Citrullus lanatus* oil was calculated using 2 g of oil sample scaled into a 500 ml conical cylinder and 10 ml of chloroform was mixed to disslolve the sampling. This was succeded by including of 15 ml of acetic acid plus 1ml of freshly processed saturated potassium iodide suspension. The cylinder then was rapidly closed, stirred for 1 minute and stored at room temperature for 10 minutes away from sunlight. A quantity 75 ml of distilled water was mixed to the content of the cylinder and next shaken energetically. Several drops of starch solution were joined as indicator. The solution of liberated iodine was titrated across 0.01 N sodium thiosulphate solution. The alike procedure was applied out for blank and the peroxide value indicated in milliequivalent of active oxygen per kg of sample was notted:

$$\text{Peroxide value} = \frac{V1-V0\ x\ T\ x\ 1000}{M}$$

With V_0 is indicated the volume of the sodium thiosulphate solution needed for blank.
With V_1 is the volume of the sodium thiosulphate solution needed for determination of the sample.
With T is notted normality of the sodium thiosulfate used.
And with M is represented the mass of the test sample in grams.

Iodine value

Iodine value of the watermelon oil was asessed using to the titrimetric method. A value 2 g of oil specimen was weighed into a moistureless 250 ml glass bottle and 10 ml of carbon tetrachloride was mixed with the oil. Exactly 20 ml of Wij's solution was then combined and allowed to stay in the dark for 20 minutes. A quantity of 15 ml potassium iodide (10%) plus 100 ml of water was joined and the resulting blend was then titrated with 0.1 M sodium thiosulphate solution taking starch as indicator just before the final point. A blank determination was made out parallel to the oil samples. The formula is:

$$\text{Iodine value} = \frac{(V2-V1)x\ 1.269}{weight\ of\ sample\ (g)}$$

Where: V_2 is titer value for blank, and V_1 is titer value for sample.

Mineral contents

Determination of mineral contents of oil: The oil sampling were digested individually for mineral analysis by wet digestion method expressed by Oluremi in 2013. Amount of 0.5 g of sample was scaled and moved into 75 ml micro digestion tubes. Concentrated solution of H_2SO_4 in 4 ml quantity and 2 ml H_2O_2 solution were combined delicately. The tubes were warmed in a block digester that was preheated to 270°C for a half an hour. They were then removed out and let to lose heat. Another division of 2 ml H_2O_2 was added extra and warmed more to accomplish complete digestion that was indicated by a clear solution aspect.

Magnesium, copper, zinc, iron and calcium quantities were identified using an Atomic Absorption Spectrophotometer. Sodium and potassium were determined using Flame Photometer while phosphorus using a Spectrophotometer.

RESULTS AND DISCUSSIONS

The physicochemical properties of the *Citrullus lanatus* seed oil tested in this article are featured in Table 1 in correlation with the literature.

Table 1. Physicochemical properties of watermelon seed oil

	Watermelon seed oil	Literature according to Adejumo, 2015
Colour	Orange	Golden yellow
Odour	Fruity	Fruity
State at room temperature	Liquid	Liquid
Specific gravity (g/ml)	0.92±0.10	0.918±0.002
Percentage oil yield	33.02±0.22	30-34%
Acid value (mg KOH/g)	3.93±0.11	2.283
Percentage of free fatty acids (oleic acid)	1.97±0.12	1.15
Saponification value (mg/KOH/g)	65.30±1.00	201
Peroxide value (mg O2/g)	8.51±0.90	3.4
Ester value	62.56±0.20	
Iodine value (GI2/100 g)	5.07±0.10	115

The specific gravity is 0.92 g/ml and in the literature in comparison to Adejumo 0.918 g/ml. Percentage oil yield is is between the range. The acid value in this study is big.

The peroxide value is 8.51 mg O_2/g and according to The Codex Alimentarius Commision the specified permitted maximum peroxide level that does not exceed 10 mequiv of peroxide oxygen/kg of oil, for example coconut oil, soybean and cotton seed.

Minerals are very important for the human health. The body uses minerals for different functions that include hormone production, bones construction and regulation of heart functions. Macrominerals are minerals that the organism needs in big amounts and these include calcium, magnesium, phosphorus, potassium, sodium, chloride and sulfur. The body also needs some small amounts of iron, copper, manganese, cobalt, zinc, selenium and fluoride. The best way of getting these minerals in the body is consuming food that have them in their composition. The daily recommended dose of calcium is 1000 mg, magnesium 310 mg, potassium 4.7 mg. Watermelon seed oil mineral content is presented in Table 2.

Table 2. Mineral content of watermelon seed oil

	mg/100 g
Phosphorus	30.87
Calcium	2.07
Magnesium	2.98
Potassium	0.86
Sodium	2.35
Iron	1.6
Copper	0.75
Zinc	1.26

Table 3 shows the fatty acid composition of the watermelon seed oil.

Table 3. Fatty acid composition for watermelon seed oil

Fatty acid	%
Myristic acid	0.07±0.0
Palmitic acid	10.01±0.11
Palmitoleic acid	0.10±0.00
Stearic acid	6.98±0.01
Oleic acid	15.89±0.15
Linoleic acid	65.73±0.07
Linolenic acid	0.20±0.03
Arachidic acid	0.21±0.00
Gadoleic acid	0.12±0.01
Behenic acid	0.10±0.02
Erucic acid	0.05±0.04
Lignoceric acid	0.05±0.00
Saturated fatty acids	17.87
Monounsaturated fatty acids	15.98
Polyunsaturated fatty acids	66.03

Oleic, palmitic and linoleic acids are the main fatty acids present in the seed oil, with a predominance of linoleic acid (65.73%), similar to the results found by Okunrobo (65.85%). Oleic acid is utilized as an excipient in pharmaceutical products and is also a emulsifying agent. Palmitic and linoleic acids

are used in the cosmetic industry and soap making.

The values however, are an indication that the *Citrullus lanatus* oil has a valuable number of long chain fatty acids in structure.

CONCLUSIONS

The results obtained in this research work in comparison with literature have shown that the percentage yield of the watermelon seed oil is high enough to be commercialized. If harvested, it will reduce the amount of agricultural waste and serve as a valuable source.

The *Citrullus lanatus* oil has a lot of nutritional values and can be consumed and also utilized for soap making because of its physicochemical properties.

Because of the high level of phosphorus in the oil it can be used also in pharmaceutical industry. The peroxide value indicated that is stable to auto-oxidation and can be stored for a long time under normal conditions. It is less susceptible to rancidity.

REFERENCES

Adejumo, B. A., Olorunsogo S. T., Njoku, C. B. (2015). Qualities of watermelon seed oil extracted at different moisture content. *International Journal of advanced enginerrring research and technology*

Banji A., Adebayo O. (2016). Physicochemical properties and mineral compositions of pawpaw and watermelon seed oils. *Pakistan Journal of nutrition.*

Dias R. C. S., Rezende G. M. (2010). Watermelon system production. EMBRAPA, ISSN 1807-0027. In:http://sistemasdeproducao.cnptia.embrapa.br/FontesHTML/Melancia/SistemaProducaoMelancia.

Hasan F. (2013). Research on the possibility of using watermelon juice in fruit juice cocktails. *Gida* 18:369–371

Costa de Conto L., Graganani M. A. L, Ambiel D. M., Chiu M. C., Grimaldi R., Goncalves L. (2011). Characterization of crude watermelon seed oil by two different extraction methods. *Journal of the American oil chemists society.*

Okunrobo O. L., Uwaya O. J., Imafidon E. K., Osarumwense O. P., Omorodion E. J. (2012). Quantitative determination, Metal analysis and Antiulcer evaluation of Methanol seeds extract of *Citrullus lanatus* Thunb (Cucurbitaceae) in Rats. *Asian Pacific Journal of Tropical Disease*, S1261–S1265.

Stevenson, D. G., Eller, F. J., Wang, L., Jane, J. L., Wang, T. and G. E. Inglett (2007). Oil and Tocopherol Content and Composition of Pumpkin Seed Oil in 12 Cultivars. *Journal of Agricultural Food Chemistry*, 55, 4005–4013.

Zahra Moaddabdoost Baboli, Ali Akbar Safe Kordi (2010). Characteristics and composition of watermelon seed oil and solevent extraction parameters effects. *J Am Oil Chem Soc.*

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VALORIZATION OF APPLE PROCESSING BY-PRODUCTS

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Abstract

Apple products are considered to be one of the major fruit products consumed globally. In apple processing industries, the remaining mixture of seeds, peels and pulp are collectively referred to as "apple pomace". Although this waste has a lot of potential for biodegradation, its disposal lead to concerns regarding the impact on the environment. This study is focused on valorization of apple pomace, by using it as a substrate for pectin extraction. Extraction was optimized by varying several factors such as: extraction solvent (HCl, HNO_3, acetic acid or citric acid), temperature (60 or 75°C) or extraction period (1 or 2 hours). The best extraction was registered with citric acid, at 60°C for 2 hours. These results are significant for further studies on pectin extraction from apple pomace.

Key words*: apple, pectin, pomace.*

INTRODUCTION

According to FAO, a third of the food directed for human consumption is lost during earlier stages of production or wasted, which amounts to almost 1.3 billion tons annually (http://www.fao.org/food-loss-and-food-waste/en/). While loss of food happens in low-income countries, waste of food is more encountered in industrialized countries due to bad habits of both consumer and producer.

One of the most consumed fruits are apples, the production rate annually being 70 million tons worldwide (Catană et al., 2018).

Apple processing generates sometimes almost 50% solid waste of raw material such as pomace, core, peel, unripe or damaged fruits etc. (Virk & Sogi, 2004). This mixture, generally known as apple pomace or apple cake is a valuable by-product that can be use as a substrate for different applications such as food, feed, fuel etc. (Toma et al., 2019).

One of the main products recovered primarily from apple wastes is a complex macromolecule known as pectin.

In 1790, Vauquelin was the first who designed the chemical structure of pectin, a complex heteropolysaccharide consisting of D-α-(1-4) anhydro-galacturonic acid (Ziari et al., 2011).

Pectin was first isolated and named by Braconnot in 1824. Pectin is found in higher plants in the primary cell walls and in the middle lamella between plant cells (Sundarraj & Ranganathan, 2017) and acts as a hydration and binding agent for cellulose (Loyola et al., 2011).

Pectin has a very complex structure in higher plants, which gives shape to the soft non-woody parts of the plant (Ziari et al., 2011).

Pectin is widely used in various industrial applications (food, pharmaceutical, cosmetic, biomedical etc.), due to its stabilizing or gelating properties (Venkatanagaraju et al., 2019). Its role includes: thickener, emulsifier, gelling and glazing agent and stabilizer (Ziari et al., 2011; Tiwari et al., 2017). In food industry, pectin is considered to be a safe additive with no specified limit on accepted daily intake (Tiwari et al., 2017).

Pectin can be isolated from various sources (carrots, sugarbeet, sunflower, mango, pomegranate, sweet potatoes etc.) with different extraction protocols such as: hot acidic solution, cold diluted sodium hydroxide, cold and/or hot solutions of chelating agents (EDTA, CDTA, ammonium oxylate, sodium hexa meta phosphate) (Toma et al., 2019; Srivastava & Malviya, 2011; Pereira et al., 2016; Zaidel et al., 2015; Renard & Thibault, 1993; Albu et al., 2019).

An improved extraction can be achieved by optimising several parameters such as: extraction solvent, temperature, pH or extraction time. Due to pectin solubility in

water and insolubility in organic solvents, its extraction requires aqueous solvent followed by precipitation and recovery (Perussello et al., 2017). The solvent should have a low pH (1-3) in order to break protopectin, but not too low in order to obtain a higher quality pectin (Perussello et al., 2017).

For economic reasons, the sources used for extracting pectin commercially are represented by wastes from food industry, such as: peels, pulp, pomaces, rinds, husks etc. (Ziari et al., 2011; Sundarraj and Ranganathan, 2017). Usually, the extraction method used is conducted with acidic solution at elevated temperature (Ziari et al., 2011). The most used pectin sources are citrus peels (25-35% dry basis pectin content) and apple pomace (10-15% dry basis pectin content) (Sundarraj & Ranganathan, 2017).

Pectin composition and properties are conditioned by the source from which is extracted. Therefore, several studies suggested that pectin from apple pomace has superior gelling properties in comparison with pectin from citrus peels (Ziari et al., 2011).

The annually production of pectin worldwide is around 40.000 metric tons (Sundarraj & Ranganathan, 2017) and specialists suggest that it may increase, therefore deriving the importance of establishing an optimized protocol for pectin extraction.

This study is focused on valorization of apple pomace as a substrate for pectin extraction. In order to achieve an improved protocol, several factors were considered such as: temperature, extraction period or optimal aqueous solvent.

MATERIALS AND METHODS

Sample preparation

The materials used were green apples (Golden delicious variety), considered to have a higher pectin content than other varieties (Rascón-Chu et al., 2009).

The apples were washed, cut and pressed for juice extraction, resulting an apple pomace that contained peels, seeds, cores and pulp residues.

Pectin extraction

The fresh apple pomace was subjected to pectin extraction with different aqueous solvents (Figure 1) in fresh sample:solvent ratio of 1:25 (w/v). The extraction solvents used in this study were selected as optimal after reviewing the literature (Perussello et al., 2017; Ziari et al., 2011; Sandarani, 2017; Sayah et al., 2014; Canteri-Schemin et al., 2005; Tiwari et al., 2017): 0.1 N hydrochloric acid, 0.5% nitric acid, 10% acetic acid and 5% citric acid.

After the extraction, the samples were cooled and precipitated with 96% ethanol (Figure 1). After filtration, the precipitates were washed with 60% ethanol and pressed down to remove excess alcohol.

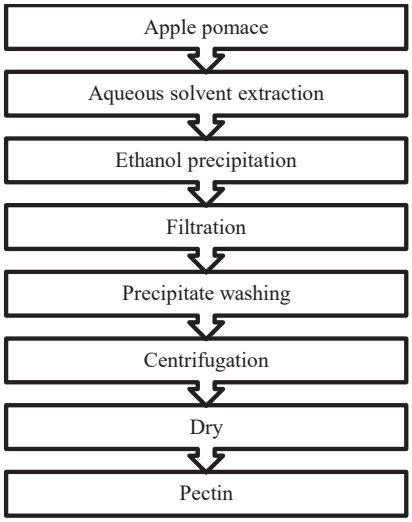

Figure 1. Extraction protocol

The extraction protocol was conducted at different temperatures (60°C and 75°C) for different extraction time: 1 or 2 hours.

Determination of pectin yield

The pectin yield of the samples was assessed gravimetrically after the extraction protocol, by oven drying the washed precipitates (Figure 1) at 40°C until constant weight was obtained.

Pectin yield was calculated after the following formula:

$$\text{Pectin yield (\%)} = \frac{\text{Pectin (g)}}{\text{Dry matter of apple pomace (g)}} * 100$$

The dry matter content was necessary for the assessment of pectin yield. The method involved drying the weighed samples in an oven at 105°C until it reaches constant weight.

The dry matter content was calculated by the next equation:

$$\text{Dry matter (\%)} = \frac{\text{Dried sample (g)}}{\text{Fresh sample (g)}} * 100$$

RESULTS AND DISCUSSIONS

Due to its biodegradability, apple pomace is often discarded near the processing units, which lead to environmental and economic concerns (Sharma et al., 2014). Therefore, an important application for this waste could be pectin extraction.

The dry matter content of the apple pomace was necessary for the comparison of the results of the extraction protocols with different design. For this purpose, 4 samples were weighed and subjected to oven dry at 105°C for several hours until they reached constant weight, as seen in Table 1.

Table 1. Dry matter content of apple pomace

Sample	Fresh apple pomace (g)	Dried apple pomace (g)	Dry matter (%)
1.	6.87	2.579	37.54
2.	6.87	2.577	37.51
3.	6.87	2.578	37.53
4.	6.87	2.579	37.54

Therefore, the average dry matter content was calculated to be 37.53%, similar to the results of other studies (Gullón et al., 2007).

The first design for the extraction of pectin involved selecting the best aqueous solvent between 2 mineral acids (0.1 N hydrochloric acid and 0.5% nitric acid) and 2 organic acids (10% acetic acid and 5% citric acid). Different acid concentrations were selected because of their different acidity constants - pKa (-6 for HCl, -1.32 for HNO_3, 4.75 for acetic acid and 2.92, 4.28 and 5.21 for citric acid).

The solvent + apple pomace mixtures were left to extract for 1 hour, at 60°C in a shaker at 150 rpm, and the results obtained were presented in Table 2.

Following the extraction with the 4 aqueous solvents, it was found that by using mineral acids (HCl and HNO_3) were obtained similar concentrations of pectin 1.57-2.16% pectin of fresh matter (Table 2). Comparable value was also recorded with acetic acid, being even lower than those recorded with nitric acid extraction, as seen in Table 2.

Table 2. Pectin content of apple pomace extracted at 60°C for 1 hour and 150 rpm

Sample	Extraction solvent	Pectin content (% of fresh matter)	Pectin content (% of dry matter)
1.	HCl	1.57	4.19
2.	HNO_3	2.16	5.75
3.	Acetic acid	1.65	4.40
4.	Citric acid	**13.49**	**35.96**

Unexpectedly, citric acid extraction resulted in considerably better results (13.49% pectin of fresh matter and 35.96% pectin of dry matter), about 6-7 times compared to other extractions (Table 2). Developing an efficient extraction using citric acid has a great importance for obtaining pectin intended as a food additive.

The second step in optimising this protocol implied selecting the best temperature for pectin extraction, by conducting a similar experiment but at 75°C. The other parameters remained constant: 1 hour and 150 rpm (Table 3).

Table 3. Pectin content of apple pomace extracted at 75°C for 1 hour and 150 rpm

Sample	Extraction solvent	Pectin content (% of fresh matter)	Pectin content (% of dry matter)
5.	HCl	2.42	6.46
6.	HNO_3	3.47	9.26
7.	Acetic acid	1.58	4.21
8.	Citric acid	**10.69**	**28.50**

The extractions conducted with HCl, HNO_3 and acetic acid resulted in low pectin content related to total dry matter of apple pomace (Table 3).

Similar to the first protocol, citric acid extraction resulted in a higher pectin content, but this time it was 3-4 times higher than the other extractions, reaching 10.69% pectin content of fresh apple pomace (Table 3).

The temperature change from 60°C to 75°C lead to slightly higher pectin content for mineral acid extractions, but for acetic acid

extraction there were no significant differences in pectin content (Tables 2 and 3).
However, by increasing the temperature for citric acid extraction, it was concluded that pectin content decreased by almost 20.74% (Tables 2 and 3). These results indicated that increasing the temperature was not beneficially for all the aqueous solvents, the optimal temperature for citric acid extraction being 60°C.
The final step required for optimizing pectin extraction involved selecting the best extraction period, by using the same solvents at 60°C and 75°C but for 2 hours (Table 4). The experiment was conducted at both 60°C and 75°C, because it was not determined the best temperature optimal for all the aqueous solvents. The results were compared with those recorded in the previous experiments, where the extractions were performed for 1 h (Tables 2 and 3).
The extractions with HCl, HNO_3 and acetic acid at 60ºC for 2 hours lead to lower pectin content in comparison with citric acid extraction (Table 4), which recorded the highest content of 23.06% of fresh matter.
By conducting the experiment at 75ºC for 2 hours, the pectin content was also lower for the first 3 acids but higher for citric acid with a value of 14.38% (Table 4).

Table 4. Pectin content of apple pomace extracted at 60°C and 75°C for 2 hours and 150 rpm

Sample	Extraction solvent	Extraction temperature	Pectin content (% of fresh matter)	Pectin content (% of dry matter)
9.	HCl	60ºC	1.66	4.44
10.	HNO_3	60ºC	3.48	9.29
11.	Acetic acid	60ºC	1.93	5.14
12.	Citric acid	60ºC	**23.06**	**61.45**
13.	HCl	75ºC	2.46	6.55
14.	HNO_3	75ºC	4.80	12.81
15.	Acetic acid	75ºC	2.38	6.35
16.	Citric acid	75ºC	**14.38**	**38.32**

Therefore, it was concluded that pectin extraction with hydrochloric acid was improved significantly by increasing the temperature from 60ºC to 75ºC and it was noted that by increasing the extraction period the results were comparable (Tables 2, 3 and 4). Pectin content of dry matter was variable between 4.19-6.55%, the results being comparable with other studies (Canteri-Schemin et al., 2005).
Pectin extraction with HNO_3 was improved both by increasing the temperature to 75ºC as well as by increasing the extraction period from 1 hour to 2 hours (Tables 2, 3 and 4). Pectin content was registered between 5.75-12.81% of dry matter, higher than the values obtained HCl extraction, but comparable with other studies (Sandarani, 2017).
Organic acid extractions were not significantly improved by increasing the temperature and the extraction period, as noted for the mineral acid extractions (Tables 2, 3 and 4).
Acetic acid extraction lead to low pectin content (4.21-6.35% of dry matter), comparable with citric acid extraction, with the mention that it was observed a slightly higher pectin content by increasing the temperature to 75ºC (Table 4).
The best results in all of the experiments were registered with 5% citric acid extraction, pectin content ranging from 28.5-61.45% of dry matter (Tables 2, 3 and 4), higher than the results of other studies (Canteri-Schemin et al., 2005). Also, it was concluded that increasing the extraction time from 1 hour to 2 hours lead to higher contents of pectin by 34.45-70.88%.
The higher temperature affected pectin extraction, resulting in lower values in all of the experiments. This may be linked with thermal degradation of pectin, as suggested by other researchers (Fraeye et al., 2007; Woo et al., 2010).

CONCLUSIONS

Apple pomace is an important by-product in apple processing, one main application being extraction of valuable compounds such as pectin.
This paper was centred on optimizing pectin extraction protocol from apple pomace derived from pressing fruits for juice.

The experiments lead to selecting the best aqueous solvent for extraction (5% citric acid), its high extraction efficiency being of great interest for food industry. The others factors that positively influenced extraction were temperature (60ºC - optimal for citric acid and 75ºC for the other solvents extractions) and extraction period (2 hours for all the protocols). The results of the research are valuable because they allow for milder extraction conditions, but also that they have a reduced impact on the environment, by using organic acids instead of mineral ones.

REFERENCES

Albu, C. V., Encea, E. S., Diguta, C., Matei, F., Cornea, C. P. (2019). Laccase: macro and microbial sources, production, purification and biotechnological applications. *Scientific Bulletin, Series F. Biotechnologies*, 23, 128-136.

Canteri-Schemin, M. H., Fertonani, H. C. R., Waszczynskyj, N., & Wosiacki, G. (2005). Extraction of pectin from apple pomace. *Brazilian Archives of Biology and Technology*, 48(2), 259-266.

Catană, M., Catană, L., Iorga, E., Lazăr, M. A., Lazăr, A. G., Teodorescu, R. I. & Belc, N. (2018). Achieving of functional ingredient from apple wastes resulting from the apple juice industry. *AgroLife Scientific Journal*, 7(1), 9-17.

Fraeye, I., De Roeck, A., Duvetter, T., Verlent, I., Hendrickx, M., & Van Loey, A. (2007). Influence of pectin properties and processing conditions on thermal pectin degradation. Food Chemistry, 105(2), 555-563.

Gullón, B., Falqué, E., Alonso, J. L., & Parajó, J. C. (2007). Evaluation of apple pomace as a raw material for alternative applications in food industries. *Food Technology and Biotechnology*, 45(4), 426-433.

Loyola, N., Pavéz, P., & Lillo, S. (2011). Pectin extraction from cv. Pink Lady (*Malus pumila*) apples. *Ciencia e investigación agraria*, 38(3), 425-434.

Pereira, P. H. F., Oliveira, T. Í. S., Rosa, M. F., Cavalcante, F. L., Moates, G. K., Wellner, N., ... & Azeredo, H. M. (2016). Pectin extraction from pomegranate peels with citric acid. *International journal of biological macromolecules*, 88, 373-379.

Perussello, C. A., Zhang, Z., Marzocchella, A., & Tiwari, B. K. (2017). Valorization of apple pomace by extraction of valuable compounds. Comprehensive Reviews in Food Science and Food Safety, 16(5), 776-796.

Rascón-Chu, A., Martínez-López, A. L., Carvajal-Millán, E., de León-Renova, N. E. P., Márquez-Escalante, J. A., & Romo-Chacón, A. (2009). Pectin from low quality 'Golden Delicious' apples: Composition and gelling capability. *Food Chemistry*, 116(1), 101-103.

Renard, C. M., & Thibault, J. F. (1993). Structure and properties of apple and sugar-beet pectins extracted by chelating agents. *Carbohydrate Research*, 244(1), 99-114.

Sandarani, M. D. J. C. (2017). A review: Different extraction techniques of Pectin. *Journal of Pharmacognosy & Natural Products*, 3(3), 1-5.

Sayah, M. Y., Chabir, R., El Kandri, Y. R., Chahdi, F. O., Touzani, H., & Errachidi, F. (2014). Optimization of pectin extraction from steam distillated orange peels through an experimental factorial design. International Journal of Innovation and Applied Studies, 7(4), 1642.

Sharma, P. C., Gupta, A., & Kaushal, P. (2014). Optimization of method for extraction of pectin from apple pomace. *Indian Journal of Natural Products and Resources*, 5(2), 184-189.

Srivastava, P., & Malviya, R. (2011). Sources of pectin, extraction and its applications in pharmaceutical industry - An overview. *Indian Journal of Natural Products and Resources*, 2(1), 10-18.

Sundarraj, A. A., & Ranganathan, T. V. (2017). A review-Pectin from Agro and industrial waste. International Journal of Applied Environmental Sciences, 12(10), 1777-1801.

Tiwari, A. K., Saha, S. N., Yadav, V. P., Upadhyay, U. K., Katiyar, D., & Mishra, T. (2017). Extraction and characterization of pectin from orange peels. *International Journal of Biotechnology and Biochemistry*, 13(1), 39-47.

Toma, A., Crăciunescu, O., Tatia, R. (2019). Comparative study on extraction methods of pectin from by-products of juiced carrots. *Scientific Bulletin, Series F. Biotechnologies*, 23, 71-77.

Venkatanagaraju, E., Bharathi, N., Sindhuja, R. H., Chowdhury, R. R., & Sreelekha, Y. (2019). Extraction and Purification of Pectin from Agro-Industrial Wastes. In Martin Masuelli (Ed.), *Pectins-Extraction, Purification, Characterization and Applications*. IntechOpen.

Virk, B. S., & Sogi, D. S. (2004). Extraction and characterization of pectin from apple (*Malus pumila* cv Amri) peel waste. *International journal of food properties*, 7(3), 693-703.

Woo, K. K., Chong, Y. Y., Hiong, S. L., & Tang, P. Y. (2010). Fruit (*Hylocereus potyrhizus*): A Preliminary Study. *Journal of Biological Sciences*, 10(7), 631-636.

Zaidel, D. N. A., Zainudin, N. N., Jusoh, Y. M. M., & Muhamad, I. I. (2015). Extraction and characterization of pectin from sweet potato (*Ipomoea batatas*) pulp. Journal of Engineering Science and Technology, 10, 22-29.

Ziari, H., Ashtiani, F. Z., & Mohtashamy, M. (2011). Comparing the effectiveness of processing parameters in pectin extraction from apple pomace. Afinidad, 67(549), 374-379.

http://www.fao.org/food-loss-and-food-waste/en/

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LIFE CYCLE ASSESSMENT OF *CAMELINA SATIVA* CROP IN A CIRCULAR ECONOMY APPROACH - A MINIREVIEW

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Abstract

Camelina (Camelina sativa) is a flower plant belonging to the family Brassicaceae, originating in Eastern Europe. Camelina oil has many applications in various fields such as cosmetics, pharmaceuticals, animal feed etc. The oil obtained from Camelina seeds has a high content of fatty acids with 50-60% unsaturated fatty acids, 35-40% omega 3-fatty acids and 15-20% omega 6-fatty acids. The main attractive features are: drought and frost tolerance, disease and pest resistance, a considerably high seed oil content, and satisfactory seed yields, in particular under low-input management and in limiting environments. The environmental benefits of the crop and a multipurpose applicability of the oil make Camelina sativa a promising oil-seed crop. Animal feeding has been identified to be a key factor in environmental sustainability. For this reason, by carrying out a life cycle assessment, there was many investigations focused on studying the environmental performance of the production of Camelina sativa in different regions of the world such as United States of America, Spain and France. For all of this utilities, Camelina sativa can be a part of circular economy in future.

Key words*: antioxidants, camelina oil, fatty acids, lecithin, stability, tocopherol.*

INTRODUCTION

Camelina (*Camelina sativa* L. Crantz) has the popular names gold of pleasure and false flax. It is an annual member of the *Brassicaceae* family (Putnam et al., 1995; Zubr., 2003) from south-eastern Europe and south-western Asian steppe region (Zohary & Hopf, Eynck et al., 2013). *Camelina sativa* was grown as an agricultural crop before the Second World War in Russia and a few European countries. In Bronze Age in Western Europe and Scandinavia was found seeds and siliculas of *Camelina sativa* ssp. *C. linicola*. Camelina plants are annual or biennial herbs, the flowers are yellowish colored, hermaphroditic actinomorphic, grouped in racemes, leaves are simple, lanceolate to narrowly elliptic. Their seeds are formed in dehiscent siliques, pubescent annual (height: 3-80 cm). Leaves lanceolate, oblong or linear lanceolate (long: 20-70 mm, wide: 2-10 mm), pubescent, with sagitate or auricular base, with entire margins to subdenticulate, at the acute apex. Flowers with oblong seples (long: 2-3 mm), with obovate and yellow petals (long: 4-5 mm), grouped in terminal raceme.

It is a very good source of animal feed products and human food because *Camelina sativa* has essential fatty acids, particularly n-3 (omega-3) fatty acids (Waraich et al., 2013; Belayneh et al., 2018).

The oil obtained from camelina seeds has a high content of fatty acids with 50-60% unsaturated fatty acids, 35-40% omega 3-fatty acids and 15-20% omega 6-fatty acids. The main attractive features are: drought and frost tolerance, disease and pest resistance, a considerably high seed oil content (Belayneh et al., 2015).

CULTIVATION TECHNOLOGIES FOR *CAMELINA SATIVA*

In the context of drought becoming more and more aggressive in Romania, a less popular culture could be an opportunity for farmers, especially since this plant is not at all pretentious in terms of soil or weather conditions. *Camelina sativa* is the one known mainly because it is used as a raw material in obtaining the kerosene used by the airplanes.

To introduce the plant into the culture, the most important elements of the culture of camelina are: analysis of the composition chemicals of seeds camelina and evaluation profile of fatty acids from chamomile oil by highlighting high content polyunsaturated fatty acids Omega-3 type (Dobre et al., 2014; Toncea et al., 2013, Feussner, 2015), economic efficiency of camelina culture given by reduced costs setting up and maintenance, the possibility to use new practices agricultural for camelina culture, carrying out works minimum soil, organic farming, introduction as double culture (Dobre et al., 2014; Berti et al., 2016).

Camelina has low demands on soil and climate so can be grown on `marginal` soils, but for high yields it is recommended a soil with medium fertility and no weeds. Camelina can be grown without problems on damaged or contaminated soils. Established on barren land, impracticable or even contaminated "it cures" the soil, the plant fertilizes the substrate and prepares it for more demanding crops.

For the sowing of the camelina, it is necessary an advanced ground cutting because the camel seeds are very small. The operations performed before sowing are plowing, debating and rolling. The plowing is done at a depth of 20-23 cm, but the grounding and leveling must be done very well (with the combiner), precisely to avoid the spread of seeds. The experiments carried out in the research institutes in Romania (Dobre et al., 2014) have shown that there is not necessarily a need for a plow, as there are sufficient minimum interventions of double discussion and roll-over. In camelina culture, the vegetation period is very short, somewhere at 3 months and does not require any further maintenance of plants and soil until harvest.

The culture is set up in autumn or spring because the short period of camelina vegetation and its resistance to drought and frost allow the establishment of the crop in both autumn and spring, which recommends it for both rotational and double culture.

The best cultivation technologies with low input energy technology for *Camelina sativa* in Romania is to cultivate as a second crop, after the main crop represented by triticale. Minimal tilling system was used to reduce energy consumption. Originating from Austria, 'Calena' was tested, without fertilizers and water and the climatic conditions were monitored (precipitation and temperature). In conclusion, *Camelina sativa* can be cultivated in Romania as a double crop but is very important to sowing the plant in the late June - early July and after camelina sowing, watering is necessary to stimulate plant emergence (Dobre et al., 2014).

The sowing must be done at least 3-4 weeks prior to the sowing date of this trial: fertilizer application is needed in order to increase camelina yield, watering is mandatory after camelina sowing in order to stimulate plant emergence.

VALORISATION OF *CAMELINA SATIVA*

Camelina is grown for its seeds containing sicativa oil, with the iodine index Ii = 144-155. Oil extracted from camelina seeds is clear, golden and can have multiple uses: from the manufacture of paints and varnishes, to obtaining biopolymers and bioplastics, adhesives, in the pharmaceutical (antioxidant), cosmetic (massage oils, natural cosmetics and aromatherapy products), soaps (potassium soap), or as biofuel for agricultural machines equipped with diesel engines.

The productivity of camelina crop varies depending on the time of sowing, the use or not of fertilizers, soil quality, irrigation or not of the soil, the correct use of herbicides. Depending on these factors the productivity of spring or autumn crops is between 800-2,300 kg/ha. In the case of double crops, the productivity per hectare on irrigated land is 1,100-1,200 kg/ha, while the double crop on non-irrigated land brings a production of 500-800 kg/ha.

The possible industrial applications of camelina include its use in environmentally safe paintings, coatings, cosmetics and low emission biodiesel fuels (Bonjean and Goffic, 1999; Bernardo et al., 2003). Although the presence of polyunsaturated fatty acids make camelina oil susceptible to lipid oxidation but it remains sufficiently stable during storage due to the presence of antioxidants in the seed (Ni Eidhin et al., 2006; Abramovic et al., 2007).

The nutritional deficiency due to the disproportion of poly-unsaturated fatty acids can be alleviated by the addition of n-3 fatty

acid rich oils in the diet. In such a situation camelina oil can be an excellent source of poly-unsaturated fatty acids and n-3 fatty acid in particular. Camelina oil can enhance the biological value of diet by changing the proportion of n-6/n-3 fatty acids (Petre et al., 2015).

Animal feed

Camelina oil cake or meal can be used as a protein rich source in poultry diets (Zubr, 1997). Camelina oil mixed with chicken (*Gallus gallus domesticus*) feed increased the n-3 (omega-3) content in the eggs without any unpleasant flavor, which often comes when flax oil is used (Rokka et al., 2002).

In Finland, the meal was used in the feed of broiler chickens and it was concluded that the high glucosinolate content of Camelina meal was not suitable for broiler feeding (Waraich et al., 2013).

Medicinal value

Because of its beneficial health effects (Ni Eidhin et al., 2003), camelina oil possesses great potential if is used in the production of health promoting supplements. Karvonen et al. (2002) determined cholesterol reducing effect of camelina oil in a test with mildly and moderately hypercholesterolemic subjects.

To obtain the oil, the camelina seeds are cold pressed, and the machine used may be the one used in the case of rapeseed. A small capacity seed press that can process 3 kilograms of seed per hour costs around 2,000 euros.

Camelina oil can be used as a biofuel. The seeds of camelina are very small, half a kilogram contains about 400,000 seeds. The oil content of a seed is 35-38%, which makes them more efficient than soybeans, which contain about 20% oil. In Camelina oil, 55-56% omega 6 fatty acids predominate and 11-12% omega 3 fatty acids predominate (Waraich et al., 2013).

Biodiesel/fuel production

Biodiesel, a low cost renewable fuel made from vegetable oils or animal fat, has recently attracted great attention as one of the more important alternatives for petro-diesel fuel. Biodegradability, lower sulfur and aromatic content, derivation from renewable and waste feedstock, higher cetane number and less emission of carbon monoxide are the main advantages of biodiesel.

Cameline oil can replace diesel with a simple adaptation made according to the scheme of the engineer Paul Dobre from the Mechanization Department of USAMV Bucharest, tractors and diesel engines used in agriculture and not only can be supplied with cameline oil (Dobre et al., 2014).

The simplicity of the works and the low costs of cultivation combined with the increased productivity per hectare and with the many questions of the seeds make *Camelina sativa* an excellent solution both for farmers who want to substantially reduce their fuel costs for agricultural work, and for those who will to diversify their cultures (Moraru et. al., 2013).

LIFE CYCLE ASSESSMENT - STUDY CASES FOR *CAMELINA SATIVA* IN DIFFERENT REGIONS

Life Cycle Assessment (LCA) is a technique used to assess the environmental impacts of a product during its entire life cycle from the "cradle", where raw materials are cultivated, and their products with final application or can be related only to agricultural technology and possibility of using its by products in sustainable application such as animal nutrition in commercial farms (Petre et al., 2013).

The evaluation of the life cycle of a product can also be used to compare two different production processes in terms of use of resources and emissions. As defined by ISO standards and several studies, a correct LCA assessment consists of four major phases, (ISO14040): 1) Goal and Scope Definition; 2) Inventory analysis; 3) Impact assessment; 4) Interpretation.

To demonstrate the effect of reducing greenhouse gases different authors (Krohn & Fripp, 2012; Petre et al., 2015) have evaluated the life cycle of carbon in the production of biofuel using *Camelina sativa* as a raw material and its social implications in the context of meeting the sustainability criteria.

During the agricultural production process, to demonstrate the importance of reducing the environmental burdens, for the highest environmental performance, camelina oil was used as a feedstock for fuel production.

Attaining higher seed yield would dramatically lower environmental impacts associated with camelina seed, oil, and fuel production. The lower GHG emissions and energy consumption associated with Camelina in comparison with

other oilseed derived fuel and petroleum fuel make camelina derived fuel from Canadian Prairies environmentally attractive (Li et al., 2014).
In Romania, camelina meal is very good to be fed as a protein source because feeds and forages are common to be produced by each farm in order not to buy compound feed from feed mills. Camelina cake or camelina meal resulted as a 60-70% from crops crushed, chemical analysis and trials performed worldwide suggest that can be fed to poultry, swine and ruminants. Camelina meal can replace up to 15% of the protein sources from the standard diet of dairy cows, trial for assessing nutritional benefits will start after 2015 harvest will be crushed, results will follow to complete our study. Nutritional profile is favourable for using in daily ratio and safely replace important amount of protein sources in cow's ratio, as an alternative to seasonal prices of the protein sources (Petre et al., 2015).

CIRCULAR BIOECONOMY - PRINCIPLES AND LINK TO ENVIRONMENTAL PROTECTION

In the European Union, circular bioeconomy is very important, accounting for 9 percent of Europe's economy. A circular bioeconomy is an economic system aimed at eliminating waste and the continual use of resources.
Within the environment protection, the total flow of biomass is 25 percent higher compared to the total flows of the last years. The circular bioeconomy is more present compared to the last years (Ronzon et al., 2017). Biomass found in the European Union comes from 63% of agriculture, 36% of forestry and 1% of fishing (Gurria et al., 2017).
Biofuel production is directly proportional to agricultural biomass, using only 2% of it (Gurria et al., 2017).
A principle addressed in the circular economy is related to the transition to unconventional energy systems in Europe. For an extension of the application of renewable energies, it is important not to diminish the areas of arable land to the detriment of photovoltaic/wind farms.
Waste recovery is another criterion that should not be neglected, making a direct reference to food waste and the selective collection of waste on at least four fractions.
In conclusion, to protect the environment, it is essential that the circular bioeconomy focus on major sectoral policies (agriculture, greenhouse gases, transport, waste, green energy) that can deliver tangible and sustainable results.

CAN CAMELINA CROP BE APPROACHED IN A SUSTAINABLE CIRCULAR BIOECONOMY?

Camelina sativa (L.) Crantz (family: *Brassicaceae*) can be approached in a sustainnable bioeconomy because it can be used in different domains such animal food, cosmetics, biofuels, oil for food industry, all of that in a 100% that means can be a fully friendly environment plant.
The main attractive features are: drought and frost tolerance, disease and pest resistance, a considerably high seed oil content, and satisfactory seed yields, in particular under low-input management and in limiting environments. The environmental benefits of the crop and a multipurpose applicability of the oil make *Camelina sativa* a promising oil-seed crop.
Circular bioeconomy can help to reduce competition for land and aquatic resources and thus contribute to the mitigation of climate change and biodiversity loss, but ultimately a coherent perspective on the main policy interventions is necessary.
Animal feeding has been identified to be a key factor in environmental sustainability. For this reason, by carrying out a life cycle assessment, there was many investigations focused on studying the environmental performance of the production of *Camelina sativa* in different regions of the world such as United States of America, Spain and France. For all of this utilities, *Camelina sativa* can be a part of circular economy in future.
A sustainable and circular bioeconomy would keep resources at their highest value for as long as possible through cascading biomass use and recycling, while ensuring that natural capital is preserved.

REFERENCES

Abramovic, H., Butinar, B., Nikolič, V. (2007). Changes occurring in phenolic content, tocopherol composition and oxidative stability of Camelina sativa oil during storage, *Food Chemistry*, 104(3), pages 903–909.

Belayneh, D. H., Wehling, R. L., Cahoon, E., Ciftcia, O. N. (2015). Extraction of omega-3-rich oil from *Camelina sativa* seed using supercritical carbon dioxide. *Journal of Supercritical Fluids*, 104, 153–159.

Belayneh, D. H., Wehling, R. L., Cahoon, E., Ciftci, O. N. (2018). Lipid composition and emulsifying properties of *Camelina sativa* seed lecithin. *Food Chemistry*, 242, 139–146.

Bernardo, A., Howard-Hildige, R., O'Connel, A. (2003). Camelina oil as a fuel for diesel transport engines, *Industrial Crops and Products*, 17(3):191–197, online Journal.

Berti, M., Gesch, R., Eynck, C., Anderson, J. (2016). Camelina uses, genetics, genomics, production and management, *Industrial Crops and Products*, Amsterdam.

Bonjean, A., Le Goffic, J., 1999. *Camelina - Camelina sativa* (L.) Crantz*: an opportunity for European agriculture and industry*, OCL, 6(1): 28–34.

Dobre, P., Jurcoane, S., Matei, F., Stelica, C., Farcas, N., Moraru, A.C. (2014). *Camelina sativa* as a double crop using the minimal tillage system, *Romanian Biotechnological Letters*, Vol. 19, No. 2, Bucharest, Romania.

Eynch, C., Shrestha, D., Vollman, J., Falk, K. C., Friedt, W., Singh, H. P., Obeng, E. (2013). *Sustainable Oil Crops Production*, Biofuel Crop Sustainability, John Wiley & Sons, Inc., 177, 181.

Feussner, I. (2015). Camelina - a promissing oilseed crop to contribute to the growing demand for vegetable oils, *European Journal of Lipid Science and Technology*, ISSN 1438-9312, online Journal.

Gurria, P., Ronzon, T., Tamosiunas, S., Lopez, R., Garcia Condado, S., Guillen, J., Cazzaniga, N. E., Jonsson, R., Banja, M., Fiore, G., Camia, A. and M'Barek, R. (2017). *Biomass flows in the European Union*, JRC Technical Reports, Joint Research Centre, Ispra, Italy.

Karvonen, H. M., Aro, A., Tapola, N. S., Salminen, I., Uusitupa, M. I., Sarkkinen E. S. (2002). *Effect of alpha-linolenic acid-rich Camelina sativa oil on serum fatty acid composition and serum lipids in hypercholesterolemic subjects*, Elsevier Science, USA.

Krohn, B. J., Fripp, M. (2012). A Life Cycle Assessment of Biodiesel Derived from the Niche-Filling Energy Crop Camelina in the USA, *Applied Energy*, onlie Journal.

Li, X., Mupodondwa, E. (2014). Life cycle assessment of camelina oil derived biodiesel and jet fuel in the Canadian Prairies, *Science of the total Environment*, 481(1):17–26, online Journal.

Moraru, A., Jurcoane, S., Dimitriu, D. (2013). Camelina cultivation for biofuels production, *Scientific Bulletin. Series F. Biotechnologies*, Vol. XVII, Bucharest.

Ni Eidhin, D., Burke, J., O'Beirne, D. (2006). Oxidative stability of omega3-rich Camelina oil and Camelina oil-based spread compared with plant and fish oils and sunflower spread, *Journal of Food Science*, Volume 68, Issue 1, online Journal.

Petre, S. M., Jurcoane, S., Dobre, P., Petcu, R., Dimitriu, D. (2013). Life Cycle Assessment: By-Products In Biofuels Production Battle; Rapeseed Vs. *Camelina sativa* L., *AgroLife Scientific Journal*, Vol. II (1), pg. 58–65.

Petre, S. M., Jurcoane, S. (2015). Life cycle assessment and potential of camelina meal for dairy cattle nutrition in romanian commercial farms, *Scientific Bulletin. Series F. Biotechnologies*, Vol. XIX, Bucharest.

Petre, S. M., Moraru, A., Dobre, P., Jurcoane, S. (2015). Life Cycle Assessment of Camelina sativa - Environmental friendly source for biofuels and livestock protein available in Romania? *Romanian Biotechnological Letters*, 20(4):10561–10571, online Journal.

Putnam, D.H., Budin, J.T., Field, L.A., Breene, W.M. (1993). *Camelina: a promising low input oilseed*, J. Janick and J.E. Simon (eds), New Crops. Wiley, New York, 314, 322.

Rokka, T., Alen, K., Valaja, J., Ryhanen, E.L. (2002). The effect of *Camelina sativa* enriched diet on the composition and sensory quality of hen eggs, *Food Research International*, 35(2):253–256, online Journal.

Ronzon, T., Lusser, M., Landa, L., M'barek, R., Giuntoli, J., Cristobal, J., Parisi, C., Ferrari, E., Marelli, L., Torres de Matos, C., Gomez Barbero, M. and Rodriguez Cerezo, E. (2017). *Bioeconomy report*, JRC Scientific and Policy Report EUR 28468 EN, Joint Research Centre, Brussels, Belgium.

Toncea, D., Necseriu, T, Prisecaru, L. N., Balint, M. I., Ghilvacs, M. (2013). The seed's and oil composition of Camelia - first romanian cultivar of camelina (*Camelina sativa* L. Crantz), *Romanian Biotechnological Letters*, 18(5), 8594, 8602.

Waraich, E. A., Ahmed, Z., Ahmad, R., Muhammad, Y. A., Saifullah, Naeem, M. S., Rengel, Z. (2013). *Camelina sativa,* a climate proof crop, has high nutritive value and multipleuses: a review, *Australian Journal of Crop Science*.

Waraich, Ejaz & Ahmed, Zeeshan & Ahmad, Rais & Ashraf, Muhammad & Ullah, Saifullah & Naeem, M.S. & Rengel, Zed. (2013). *Camelina sativa,* a climate proof crop, has high nutritive value and multiple-uses: A review. *Australian Journal of Crop Science*, 7. 1551–1559.

Zohary, D., Hopf, M. (2000). *Domestication of Plants in the Old World*, Oxford University Press, New York.

Zubr, J. (1997). Oil-seed crop: *Camelina sativa, Industrial Crops and Products*, Vol. 6, Issue 2, 16, pg. 113–119.

Zubr, J. (2003). Qualitative variation of Camelina sativa seed from different locations, *Industrial Crops and Products*, 17, 161–169.

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FUNGAL BIOTECHNOLOGY OF LIGNOCELLULOSIC WASTE CONVERSION - A REVIEW

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Abstract

Lignocellulosic materials are the constituent elements of all plants and are most renewable feedstock available for most regions of our world. Lignocellulosic waste represents huge amounts of unutilized plant-based bioresurces, difficult to degrade for industry. Very important components of natural lignocellulosic materials are carbohydrate polymers represented by cellulose and hemicellulose, and lignin an aromatic polymer. In its natural lignocellulose state, cellulose is protected from microbial degradation, mainly due to the lignin and hemicellulose polymer components. The biotechnology of lignocellulosic material conversion into bio-products normally requires multistep processes. The focus of this article is a study on the potential cultivation of edible and medicinal mushrooms, using different types of residues as natural substrates: fruit tree wastes, winery and vine wastes, agricultural and agro-industrial wastes. A large number of fungi are capable for selectively degrading lignin. Proper management of lignocellulose biodegradation and utilization can serve to improve the quality of the environment.

Key words*: environment biotechnology, cellulose, hemicellulose, lignin, lignocellulose, biodegradation.*

INTRODUCTION

Lignocellulose is the most abundant source of biomass on earth, originating from fruit tree, forestry, agricultural, and agro-industrial wastes, causing environmental problems.
Lignocellulose is mainly composed from carbohydrate polymers: cellulose and hemicellulose, and they are tightly bound to an aromatic polymer, lignin (Figure 1).

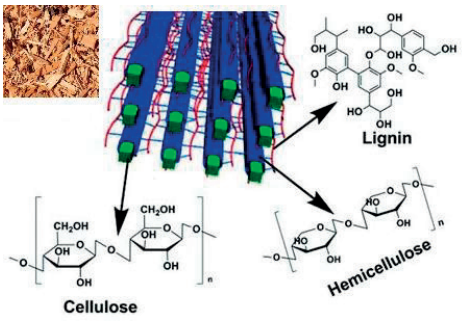

Figure 1. Structure of the lignocellulosic biomass
Source: http://www.iitbmonash.org

Some variety of edible and medicinal fungi can fragment these macromolecules. In natural environments, breakdown of lignin is brought by filamentous fungi belonging to the class of basidiomycetes that secrete an array of enzymes, such as lignin peroxidases (LiP), manganese peroxidases (MnP) and laccases for this purpose (Vicuña Errázuriz, 2000).
Huge amounts of residual plant biomass can potentially be converted into various different value-added products like human nutrients, improved animal feedstuffs, and cheap energy sources for fermentation, chemicals compounds, and high-quality biofuels.
Lignocellulosic enzymes also have significant potential applications in various industries including: chemicals, fuel, food, brewery and wine, animal feed, textile and laundry, pulp and paper, and agriculture (Howard et al., 2003).
Main focus in this review is to highlight significant aspects of fungal biotechnology of lignocellulose waste bioconversion.

MATERIALS AND METHODS

Online research was conducted using PubMed, ScienceDirect, Cochrane, and Embase data.

BIODEGRADATION AND UTILIZATION OF LIGNOCELLULOSIC RESIDUES BY FUNGI

White and brown rot fungi produce extracellular enzymes or exoenzymes, which are synthesised inside the cell and then secreted outside the cell. These enzymes are employed in a number of industries for pollution control. For example, laccases which can be produced by white rot fungi are capable of oxidising several environmental pollutants. Cellulases, represent a mixtures of hydrolytic enzymes produced by basidiomycetes fungi, which catalyses the conversion of carbohydrates and is widely used in pharmaceutical, food and beverage industries. Cellulases include endo-acting (endoglucanases) and exo-acting (cellobiohydrolases) enzymes. The use of laccases as enzymes for biotechnological applications has increased since their discovery in basidiomycetes white-rot fungi. There are many additional technologies that include selective delignification to produce cellulose, the conversion of lignocellulose into feed and biofuels, and also new technologies for treating environmental pollutants and toxic agents generated in vary industrial processes. As a result, laccases enzymes have been extensively studied for various biotechnological applications, including functionalization of lignocellulose materials, modification of wood fibres and remediation of contaminated soil and effluents, also to use in various biosensors. These studies show that laccases are enzymes of interest for utilization in environmental protection applications (Viswanath et al., 2014).

Very often cultivated mushroom in the world are in the following order *Agaricus bisporus* (button mushroom) followed by *Lentinus edodes* (shiitake), *Pleurotu*s spp. (oyster mushrooms), *Auricularia auricula* (wood ear mushroom), *Flammulina velutipes* (winter mushroom), and *Volvariella volvacea* (straw mushroom). Others species of fungi successfully produced on diverse substrates include *Hericium erinaceus*, *Ganoderma* spp., *Agrocybe aegerita*, *Grifola frondosa, Lepista nuda, Hypsizygus marmoreus, Coprinus comatus, Pholiota nameko* and *Stropharia* spp. Even if the mentioned mushroom species, have the capacity to degrade lignocellulosic residues, there are some differences regarding production of enzymes necessary to degrade lignocellulosic substrates. So these fungi present different abilities to grow and fruit on the substrate consisting of bio-residues. White-rot mushroom degrade lignin, leaving decayed wood whitish in colour (bleaching of the wood), and include widespread cultivated species like: *Pleurotus* spp., *Ganoderma* spp. L. *edodes* etc. They are the most efficient lignocellulosic biomass degraders, capable to produces wide variety of hydrolytic enzymes (cellulases and hemicellulases) and a unique oxidative and extracellular ligninolytic system (advanced lignin depolymerisation). Expression of many fungal laccases is influenced by many culture parameters, such as: concentration of carbon and nitrogen sources; media composition; pH varying and temperature; or presence of inducers and lignocellulosic materials (Philippoussis A.N., 2009).

Ram and Kumar (2010) examine morphological parameters of *Agaricus bisporus* fruiting bodies and total cultivation productivity on different agricultural waste. Six nutrient medium formulations were analysed for studies. Maximum weight of fruiting body was obtained with application of casing coconut coir pith + vermin compost + sand.

Pleurotus species (Oyster mushrooms) is the second most commercially cultivated edible mushroom worldwide. Optimal results for *P. ostreatus* growth, yield, biological efficiency and mushroom size have been studied at large-scale, by a big number of authors. Notable results have obtained in Romania from winery + apple wastes (1:1) (cellulose degradation 0.9 g %) (Petre and Petre, 2012) and from apple wastes + 1.5 % barley (cellulose degradation 0.9 g % d.w.) (Petre and Petre, 2013).

The strong enzyme system of *Pleurotus* spp. increase biodegradation of the wide spectrum of substrates, not only sawdust and cereal straw.

Philippoussis et al. (2004), examined the influence of oak-wood sawdust substrates on *Lentinula edodes* mushrooms, and observed colonization rate is much faster on substrates enhanced with wheat straw or corn-cobs in a ratio of 1:2. Higher sporophore yields were observed on oak-wood sawdust substrates and

corn-cobs mixtures, especially in the supplementation ratios 1:1 and 1:2. Substrates with high oak-wood sawdust content (2:1 ratio) appeared to promote mushroom quality and high protein content of the sporophores.

Lentinula edodes cultivation on hard wood saw-dust, rice straw, crushed corn cobs and crushed bagasse supplemented with 20% wheat bran, 1% soy bean flour, 2% gypsum has been investigated by Hassan (2011). Incubation period and early of harvesting yield were estimated. Sawdust produced the highest maximum yield 297 g/kg with wet media while bagasse recorded the lowest values. Sawdust recorded the shortest incubation time and first harvesting day time, while bagasse showed the longest ones.

The effect of pH and temperature variations on the growth of *Volvariella volvacea* cultivated on various agricultural wastes, single and in various combinations has been studied by Akinyele and Adetuyi (2005). A pH range of 5.5 to 8.5 recorded the maximum mycelia yield and the highest mycelia weight was recorded at pH 6.5. High mycelia growth of the mushroom was also observed between 25°C and 30°C. The researcher also evaluated effect of mycelial growth on diverse substrates of *Volvariella* spp. (*V. diplasia* and *V. volvacea*).

Paddy straw, oil palm fibre, sawdust, and a mixture of oil palm fibre and sawdust were screened for the cultivations of *V. volvacea* (Tripathy et al., 2011). Growth and production of fruit bodies on oil palm fibre was similar to that of paddy straw (Onuoha et al., 2009). *V. volvacea* showed that it is an very good agro waste destructor (Barshteyn & Krupodorova, 2016).

Akavia et al. (2009) investigated the cultivation of five *Hypsizygus marmoreus* strains on 24 substrates. Average number of colonized particles per day, number of mushrooms and weight of mushrooms harvested during one month have been studied. The best substrate in terms of 85.6%. biological efficiency, was corn cob with bran and olive press cake. Without olive press cake was only 67.5% biological efficiency.

Researchers are interested not only in *Agaricus* spp., *Pleurotus* spp. and *Volvariella* spp. fruit bodies cultivation on cheap substrates, but also in others edible and medicinal mushrooms, such as:

Ganoderma lucidum cultivated on sawdust and rice bran + 10% of food waste compost, with good biological efficiency;

Hericium erinaceus Good results was obtained from: sawdust (yield = 184 g/kg); wheat straw (protein); sawdust + wheat straw + 20% wheat bran + 1% $CaCO_3$ + 1% sugar (fat*)* investigated cultivation by Hassan (2007);

Auricularia auricula-judae Best results was obtained from dry olive mill residue, by increases peroxidase secretion and produced a sharp decrease in total phenolic content of growing substrate (Reina et al., 2013);

Flammulina velutipes cultivated on rice bran, wheat bran (Peng, 2010) and paddy straw + palm empty fruit bunches (25:75). Biological efficiency was 185.09% (Harith et al., 2014).

Another current and future resource for biotechnology research opportunities is a group of filamentous fungi.

Companies such as AB Enzymes, BASF, Bayer, DuPont, Novozymes, Puratos are global leaders in using filamentous fungi as cell factories in white and red biotechnology. This group of microorganisms is often superior to bacterial and yeast based production systems, in terms of metabolic versatility, robustness and secretory capacity.

Large-scale manufacturing processes have been developed for the production of organic acids, proteins, enzymes and small molecule drugs including antibiotics, statins and steroids. Fungal biotechnology plays a very important role for many industries including: food and feed, pharmaceutical industries, paper and pulp, detergents, textiles and bio-fuels (Meyer et al., 2016).

Trichoderma reesei are the most widely used strains of filamentous fungi for the production of cellulolytic enzymes and recombinant proteins. Several species of *Aspergillus* as well as other fungi, *Myceliophthora thermophila*, are important for industrial enzyme production. The metabolic diversity of fungi and the broad-range of ecological niches they inhabit, mean that many species, especially the basidiomycetes, have significant potential as sources of novel enzymes for future exploitation. Intensification of research studies on valorisation of hemicellulose and lignin-rich

fractions for high-value applications. These wastes could be used for the growth of filamentous fungi with the capability to convert both hexose and pentose sugars into ethanol, carbon dioxide, and fungal biomass with a relevant nutritional composition (high protein and fat contents, essential amino acids, polyunsaturated fatty acids, cell wall compounds with immunostimulant properties, antioxidants) as an alternative to fishmeal (Karimi et al., 2018).

Another area of research includes filamentous fungi as large-scale producers of pigments and colorants for the food industry with exceptional biological roles (anti-oxidative, free radical killing, anti-carcinogenic, immunostimulation, protection against viruses and bacteria). They produce a wide range of pigments such as carotenoids, citrinin, melanins, flavins, phenazines, quinones, and sometimes monascins, or indigo.

Produced commercially and supplied to the market are: lycopene, β-carotene, astaxanthin, canthaxanthin, lutein, and capxanthin. Red and yellow pigments from *Monascus* sp. are produced in large scales and used as food colorants (Figure 3).

Figure 2. *Monascus purpureus* colony and red pigment Manan M.A. (2017)

The most frequent immunostimulants found in filamentous fungi cell wall are glucans (30-80%), chitin and chitosan (1-15%), mannans and/or galactomannans, and glycoproteins. These compounds enhance the immune system capabilities, stress related responses and resistance to diseases and are present in mycelia, stalks and spore tissues. Filamentous fungi can synthesize water-soluble vitamins such as C (ascorbic acid), B6 (pyridoxine), B2 (riboflavin), nicotinic acid, and nicotinamide. There are reports on the production of pantothenic acid (B5) and β-carotene (pro-vitamin A) by *Fusarium* sp. and *Neurospora* sp. (Karimi et al., 2018).

RESULTS AND DISCUSSIONS

According to data provided by the Food and Agriculture Organization of the United Nations, this statistic illustrates the production in tonnes of mushrooms in the last four years. Asia is in first place, followed by Europe, America, Oceania and Africa. Mondial annual average production is approx. 9 million tons (Figure 3; Table 1).

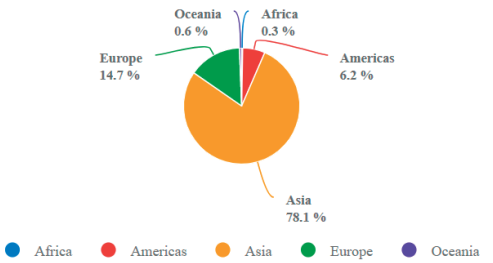

Figure 3. Average production share of edible mushrooms and truffles by region (2015-2018) Source: FAOSTAT (2015-2019)

Table 1. World production of edible mushrooms (tons) Source: FAOSTAT (2015-2019)

World production of edible mushrooms tons	2015	2016	2017	2018	2018/ 2014 (%)
ASIA	7038694	7087944	6956812	7031724	**78.1**
EUROPA	1363833	1307406	1318614	1324198	**14.7**
AMERICA	547269	558782	553764	554462	**6.2**
OCEANIA	45642	53178	47946	54128	**0.6**
AFRICA	25510	26367	27068	28767	**0.3**
Total	**9020948**	**9033677**	**8904204**	**8993279**	

CONCLUSIONS

Lignocellulosic waste being a major pollutant of the environment, containing the complex composition of cellulose, hemicellulose, and lignin along with plant resins and fatty acids. Due to its complex structure; lignin, resin and plant fatty acid they are not easily degradable by microbial communities.

The natural capabilities of microorganisms to degrade lignocellulosic waste efficiently due to highly effective enzymatic systems are attractive as new strategies for the development of industrial processes. Many factors may be

involved in the difference of nutritional composition of mushrooms cultivated in different substrates.

REFERENCES

Akavia, E., Beharav, A., Wasser, S. P. & Nevo, E. (2009). Disposal of agro- industrial by-products by organic cultivation of the culinary and medicinal mushroom *Hypsizygus marmoreus*. *Waste Management*, 29, 1622–1627. http://dx.doi.org/ 10.1016/ j.wasman.2008.10.024

Akinyele, B. J. & Adetuyi, F. C. (2005). Effect of agrowastes, pH and temperature variation on the growth of *Volvariella volvacea*. *African Journal of Biotechnology*, 4, 1390–1395.

Barshteyn, V., & Krupodorova, T. (2016). Utilization of agro-industrial waste by higher mushrooms: modern view and trends. *Journal of Microbiology, Biotechnology and Food Sciences*, *05*(06), 563–577. https://doi.org/10.15414/jmbfs.2016.5.6.563-577

Food and Agriculture Organization of the United Nations. (2015-2019). FAOSTAT statistical database. [Rome] :FA

Howard, R. L., Abotsi, E., Van Rensburg, E. L. J., & Howard, S. (2003). Lignocellulose biotechnology: Issues of bioconversion and enzyme production. *African Journal of Biotechnology*, *2*(12), 702–733. https://doi.org/10.5897/ajb2003.000-1115.

Harith, N., Abdullah, N., Sabaratnam, V. (2014). Cultivation of Flammulina velutipes mushroom using various agro-residues as a fruiting substrate. *Pesquisa Agropecuária Brasileira*, 49(3), 181–188. http://dx.doi.org/10.1590/S0100-204X2014000300004

Hassan, F. R. H. (2007). Cultivation of the Monkey Head Mushroom (*Hericium erinaceus*) in Egypt. *Journal of Applied Sciences Research*, 3(10), 1229–1233.

Hassan, F. R. H. (2011). Utilization of agro and agro-industrial wastes for cultivation of Shiitake (Lentinus *edodes*) an edible and medicinal mushroom and their drying aspects in Egypt. *Research Journal of Agriculture & Biological Sciences*, 7(6), 491–497.

Karimi, S., Soofiani, N. M., Mahboubi, A., & Taherzadeh, M. J. (2018). Use of organicwastes and industrial by-products to produce filamentous fungi with potential as aqua-feed ingredients. *Sustainability (Switzerland)*, *10*(9). https://doi.org/10.3390/ su10093296.

Manan M. A., (2017) The Morphology and Structure of Red Pigment Producing Fungus: *Monascus purpureus*. *J Microbiol Exp* 5(1): 00138. https://doi.org/10.15406/jmen.2017.05.00138

Meyer, V., Andersen, M. R., Brakhage, A. A., Braus, G. H., Caddick, M. X., Cairns, T. C., de Vries, R. P., Haarmann, T., Hansen, K., Hertz-Fowler, C., Krappmann, S., Mortensen, U. H., Peñalva, M. A., Ram, A. F. J., & Head, R. M. (2016). Current challenges of research on filamentous fungi in relation to human welfare and a sustainable bio-economy: a white paper. *Fungal Biology and Biotechnology*, *3*(1), 1–17. https://doi.org/10.1186/s40694-016-0024-8

Onuoha, C. I., Oyibo, G. & Ebibila, J. (2009). Cultivation of straw mushroom (*Volvariella volvacea*) using some agro-waste material. *Journal of American Science*, 5(5), 135–138.

Peng, J. T. (2010). Agro-waste for cultivation of edible mushrooms in Taiwan. Food and Fertilizer Technology Center Publication Database. http://www.agnet.org/library.php?func=view&id=20110725155730&type_id =4. Accessed

Petre, M. & Petre, V. (2012). The semi-solid state cultivation of edible mushrooms on agricultural organic wastes. *Scientific Bulletin, Series F, Biotechnologies*, XVI, 36–39.

Petre, M. & Petre, V. (2013). Biotechnology for controlled cultivation of edible mushrooms through *submerged* fermentation of fruit wastes. *AgroLife Scientific Journal*, J 2(1), 117–120.

Philippoussis, A., Diamantopoulou, P., Arapoglou, D., Bocari, M. & Israilides, C. (2004). Agricultural waste utilisation for the production of the medicinal mushroom *Lentinula edodes*. Protection and Restoration of the Environment VII (Proceedings of the International Conference) Mykonos, http://www.srcosmos.gr/srcosmos/showpub.aspx?aa=7221. Accessed 5 May 2015.

Philippoussis A. N. (2009). Production of Mushrooms Using Agro-Industrial Residues as Substrates. In: Singh nee' Nigam P., Pandey A. (eds) *Biotechnology for Agro-Industrial Residues Utilisation*. Springer, Dordrecht.

Ram, R. C., Kumar, D. S. (2010). Agricultural wastes used as casing mixtures for production of button mushroom. *Indian Journal of Scientific Research*, 1(1), 21– 25.

Reina, R., Liers, C., Ocampo, J. A., García-Romera, I. & Aranda, E. (2013). Solid state fermentation of olive mill residues by wood- and dung-dwelling Agaricomycetes: effects on peroxidase production, biomass development and phenol phytotoxicity. *Chemosphere*, 93(7), 1406–1412. http://dx.doi.org/ 10.1016/j.chemosphere.2013.07.006

Tripathy, A., Sahoo, T. K. & Begera, S. R. (2011). Yield evaluation of paddy straw mushrooms (*Volvariella* spp.) on various lignocellulosic wastes. *Botany Research International*, 4(2), 19–24.

Viswanath, B., Rajesh, B., Janardhan, A., Kumar, A. P., & Narasimha, G. (2014). Fungal laccases and their applications in bioremediation. *Enzyme Research*, *2014*. https://doi.org/10.1155/2014/163242

Vicuña Errázuriz, R. (2000). *Ligninolysis*. *14*.

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PLANTS FROM THE SPONTANEOUS FLORA OF ROMANIA WITH TOPIC SKIN PROTECTION ACTION AGAINST EXTERNAL FACTORS THAT INDUCE OXIDATIVE STRESS - A MINI REVIEW

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Abstract

For skin care, the organic ingredients have been used by folk products. Today, they are becoming more used because of the toxic action of chemical products. Thus, there is a great demand that natural products should be used in cosmetic products. The main benefits that are determined by the plant extracts, used for skin care, contains antioxidant and anti-germ activities or the effect of halting of tyrosinase. Phenol compounds are the main group of natural antioxidants popular on the market and express anti-germ actions, anti-inflammatory or anti-aging, that can get pass the skin barrier. The purpose of the paper was to present a revised and detailed list of the main factors that have effects on the skin, a detailed list of benefits, from a scientific point of view, of topic anti-oxidants, to understand the way of formulating and administering of topic anti-oxidants protection study.

Key words*: plant extracts, anti-oxidant activity, phenols, skin.*

INTRODUCTION

The spontaneous flora of Romania represents a high source of plants and fungi with therapeutic potential (Vasile et al., 2017). Many of these bioactive elements are underrated, and studies that show the effects of a topic administering were not brought up to date according with the new way of things of the biopharmaceutical field.

The active compounds from plants are becoming more popular as cosmetic ingredients, because, when they act, the skin products, the ones made with plants have a lower toxic rate and are biodegradable (Ribeiro et al., 2015). In addition, these compounds have different biological activities and are therapeutic (Jucá et al., 2018).

The use of cosmetic products is rising in the last years, being used for beauty treatments, for improving skin appearance and for treating many other skin problems (Rinaldi, 2008).

Products that protect against the sun have been evolving, from the first tanning oils and have elements that offer protection from UV radiation within (Korac & Khambholja, 2011). Today, sun block products have sophisticated formulas that promise and offer much more than a simple UV block and sun burn protection (Young et al., 2019).

Sunscreens are cosmetic products, with a functional role (Shanbhag et al., 2019). The classification criteria differ from skin care and make-up products. The companies are trying to add functional compounds to main sun screen creams through the incorporation of some bioactive ingredients (Ngoc et al., 2019. Through these formulas, extra benefits are obtained, such as anti-aging effects, hydration, anti-wrinkling etc. While some of these extra ingredients have a more advanced protection role for the skin (Dopcea and Matei, 2018), others do not express their advantages only after a long and regular application of the product, thus the majority of effects are limited because of temporary and limited usage time (Ganceviciene et al., 2012).

Thus, in this mini review the main factors that work on the skin, a detailing of benefits, from a scientific point of view, of topic antioxidants, to understand the formulating and way of administering, of topic antioxidants and identifying new ways for antioxidant protection study.

1. Ingredients of natural origins in cosmetic products

The skin has an extremely vast surface, the biggest organ that gives a diverse sensitivity; it protects the body from external factors of the environment and from germs (Abdallah et al., 2017). It offers a certain protection from sun waves that inevitably affects us, and destroys the „horny layer" of the skin, inducing loss of transepidermal water and reduced elasticity (Hillebrand et al., 2010).

According to EU regulation 1223/2009, cosmetics are substances or products destined to be put in contact with different external parts of the body, such as skin, hair, nails and lips, with the purpose to hydrate, change aspect, clean, perfume, and also to confer a sense of wellbeing.

In the formulation of cosmetic products, you can find natural active ingredients and chemical ones that promote benefits similar to medical products, such as beneficial topic actions, and protection against degenerative skin conditions (Ribeiro et al., 2015). Cosmetic products provide nutrients necessary to skin care and the improvement of its appearance, conferring glow to the skin and diminishing wrinkles. Cosmetics are the sort of products with a rapid growth of the industry of personal care natural products (Mukul et al., 2011; Figure 1).

Figure 1. The aspect of cosmetic products based on functional extracts obtained in Faculty of Biotechnology lab

The extractions with HCl, HNO_3 and acetic acid at 60ºC for 2 hours lead to lower pectin content in comparison with citric acid extraction (Table 4), which recorded the highest content of 23.06% of fresh matter.

By conducting the experiment at 75ºC for 2 hours, the pectin content was also lower for the first 3 acids but higher for citric acid with a value of 14.38% (Table 4).

The use of plants for medical purposes is ensue the appearance of new products on the market that contain natural oils. Plants were the only source of all cosmetic products before the use of industrial substances with similar actions (Ribeiro et al., 2015). Molecules from organic vegetables represents one of the main objectives of the researchers in this field of expertise. With all this, the utilization of extracts needs special attention on the methods of extraction, interaction between plant and solvent and the contents of active ingredients (Zhang et al., 2018). The use of plant extracts in skin care products is pointed out by the demand of consumers that are more preoccupied with the procuring of ecological products that contain ingredients of natural origins (Bruno et al., 2015; Ribeiro et al., 2015).

2. The advantages of extracts of natural origins

The natural products present a rich source of vitamins, protein, anti-oxidant, oils and essential oils, enzymes and other bioactive compounds (Figure 2). Depending on their structure, these extracts can give different elements to cosmetic products (Altemimi et al., 2017).

Figure 2. The aspect of functional extracts obtained in Faculty of Biotechnology lab

2.1. Antimicrobial activity

Pharmaceutical and cosmetic industries have an increasing interest to substitute the synthetic antimicrobial compounds with products that have a working role, without toxicity (Ivanov et al., 2015). Besides the

growing interest of clients for natural agents, the microbial resistance to ordinary antimicrobials is on the rise (Ribeiro et al., 2015) and health problems are appearing.
Phenolic compounds are synthesized by plants and mushrooms (Figure 3) for different purposes. Those can interact with the cellular wall of the microorganism, that leads to cellular destruction. Phenols can get into bacterial cells and can determine the coagulation of its contents (Ribeiro et al., 2015).

Figure 3. Mushroom from the Faculty of Biotechnology garden

2.2. Antioxidant activity

The importance of antioxidants of phenolic origin has raised considerably due to their high capacity to inhibit free radicals (Rahman, 2007). Plants rich in phenols can be used to prevent the toxic effects on the skin by UV radiations (Kurutas, 2016).
Phenolic compounds can be assimilated by the organism from the plant extracts, in the form of medicine, food supplements and cosmetic products (Ribeiro et al., 2015). The pattern of phenolic compounds from an extract is highly influenced by the way of extraction, and also by the solvent that was used (Jiménez-Moreno et al., 2019).
In the last years, there has been a rise in the number of scientific studies regarding plants used in traditional medicine on par with their chemical composition and possible benefits for the health of humans (Ekor, 2014). The species *Centaurea cyanus*, which is known as cornflower, originates from Europe and Asia. In Romania, this regnum is present at the mountain side, and is not yet harnessed. In traditional European medicine, the watery extract from *Centaurea cyanus* is used to treat eye diseases, mental, dermatological and gastric. It also has, a diuretic role, stimulant and tonic (Escher et al., 2018).

2.3. Inhibition effect on tyrosinase

Melanin is a human pigment that gives the color to the eye, hair and skin (Małgorzata and Grzybowski, 2016). It is made and secreted, through a physiological process called melanogenesis in the lowest layer of the skin (Ali & Naaz, 2018). There are two types of melanic pigments produced by melanocytes (Solano, 2014), eumelanin, for black or brown and pheomelanin, for red or yellow. Each person from the same race has, in general, the same number of melanocytes; thus, the type of melanin produced depends on their function, meaning that people who have a dark skin are genetically programmed to constantly produce a higher level of melanin (Ribeiro et al., 2015). When the skin is exposed to solar radiation, the melanogenesis is enhanced by the tyrosinase, a key enzyme of melanogenesis (Zahiu et al., 2010).

3. The benefits of anti-aging creams with solar protection

Exposure to UV radiations is the main source of sun burns on the skin (D'Orazio et al., 2013); harmful quantities of free radicals are produced, oxygen reactive species (ROS), that leads to premature aging (Amaro-Ortiz et al., 2014). The human skin can be protected from the damaging effects of the oxygen reactive species using typical products, such as solar protection cream (Jadoon et al., 2015).
The use of vitamins in sunscreens is widespread (Norval and Wulf, 2009), based on numerous studies, even though the majority of them are on animals or *in vivo*, their "anti-aging" activity is not far off vitamins C and E have a more "preventive" role (Pullat, 2017; antioxidant, that need to be used for sun exposure), while vitamin A and its derivatives are more of a "repairing" type, cancelling some of the damage caused by UV radiations (Lintner, 2017). The main disadvantage is the difficulty to formulate a functional product, thus the right quantity of vitamins can be optimal for a limited amount of time (Lintner, 2017).

CONCLUSIONS

Sun damage prevention can be improved by some of the antioxidant and photoprotector agents; and also, it can be justified that treating sun damage during or immediately after sun exposure with bio-compounds with repairing effect (Lintner, 2017);
Teaching the consumer how to manage sun light, has become a marketing obligation. Prevention supersedes the use of sun screen and includes wearing proper clothes and avoiding to go out during the day at some hours etc;
Many plant extracts, after being properly studied, can be a certain alternative, efficient and cost friendly in cosmetic products;
Today, consumers have a rising interest in natural products, mainly in the case of cosmetic products. In other ideas, many studies refer to the advantages of plant extracts, such as antioxidant capacity, inhibiting tyrosinase and antimicrobial activity, that can be good for reducing and preventing different problems of the skin.

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AUTHOR CONTRIBUTIONS

DCM & EV analyzed the data and wrote the paper. The authors discussed and made comments on the paper.

REFERENCES

Abdallah, F., Mijouin, L., & Pichon, C. (2017). Skin Immune Landscape: Inside and Outside the Organism. *Mediators of inflammation*, 5095293.

Ali, S.A., Naaz, I. (2018). Biochemical aspects of mammalian melanocytes and the emerging role of melanocyte stem cells in dermatological therapies. *International Journal of Health Sciences*, 12(1), 69-76.

Altemimi, A., Lakhssassi, N., Baharlouei, A., Watson, D.G., Lightfoot, D.A. (2017). Phytochemicals: Extraction, Isolation, and Identification of Bioactive Compounds from Plant Extracts. *Plants*, 6(4), 42.

Amaro-Ortiz, A., Yan, B., D'Orazio, J.A. (2014). Ultraviolet radiation, aging and the skin: prevention of damage by topical cAMP manipulation. *Molecules*, 19(5), 6202-6219.

Bruno, F.S., Antonio, C. M., Marlus, C. (2015). Sustainability, natural and organic cosmetics: consumer, products, efficacy, toxicological and regulatory considerations. *Brazilian Journal of Pharmaceutical Sciences*, 51(1), 17-26.

Dopcea G., Matei F. 2018, Review on some current skin antiseptics. *Scientific Bulletin. Series F. Biotechnologies*, XXII, 147-158.

D'Orazio J., Jarrett S., Amaro-Ortiz A., Scott, T. (2013). UV radiation and the skin. *International Journal of Molecular Sciences*, 14, 12222-12248.

Ekor M. (2014). The growing use of herbal medicines: issues relating to adverse reactions and challenges in monitoring safety. *Frontiers in Pharmacology*, 4, 177.

Escher, G.B., Santos, Jâ. Sousa., Rosso, N.D., Marques, M.B., Azevedo, L., do Carmo, Mariana. Araú. Vieira., Daguer, H., Molognoni, L., Prado-Silva, L.D., Sant'Ana, A.S., da Silva, M.C., Granato, D. (2018). Chemical study, antioxidant, anti-hypertensive, and cytotoxic/cytoprotective activities of *Centaurea cyanus* L. petals aqueous extract. *Food and Chemical Toxicology,* 118, 439-453.

Fowler, J. F., Woolery-Lloyd, H., Waldorf, H., Saini, R. (2010). Innovations in natural ingredients and their use in skin care. *Journal of Drugs in Dermatology*, 9(6 Suppl), S72-81.

Ganceviciene, R., Liakou, A.I., Theodoridis, A., Makrantonaki, E., & Zouboulis, C.C. (2012). Skin anti-aging strategies. *Dermato-endocrinology*, 4(3), 308-319.

Hillebrand, G.G., Liang, Z., Yan, X., Yoshii, T. (2010). New wrinkles on wrinkling: an 8-year longitudinal study on the progression of expression lines into persistent wrinkles. *British Journal of Dermatology*, 162(6), 1233-41.

Ivanov I., Petkova N., Denev P., Pavlov A. (2015). Polyphenols content and antioxidant activities in infusion and decoction extracts obtained from *Fragaria vesca* l. Leaves. *Scientific bulletin. Series F. Biotechnologies*, XIX, 145-148.

Jadoon, S., Karim, S., Asad, M.H.H.B., Akram, M.R., Khan, A.K., Malik, A., Chen, C., Murtaza, G. (2015). Anti-Aging Potential of Phytoextract Loaded-Pharmaceutical Creams for Human Skin Cell Longetivity, *Oxidative Medicine and Cellular Longevity*, 2015, 709628.

Jiménez-Moreno, N., Volpe, F., Moler, J.A., Esparza, I., Ancín-Azpilicueta, C. (2019). Impact of Extraction Conditions on the Phenolic Composition and Antioxidant Capacity of Grape Stem Extracts. *Antioxidants*, 8(12), 597.

Jucá, M.M., Cysne Filho, F.M.S., De Almeida, J.C., Mesquita, D.D.S., Barriga, J.R.M., Dias, K.C.F., Barbosa, T.M., Vasconcelos, L.C., Leal, L.K.A.M., Ribeiro, J.E., Vasconcelos, S.M.M. (2018). Flavonoids: biological activities and therapeutic potential. *Natural Product Research*, 16, 1-14.

Korać, R.R., Khambholja, K.M. (2011). Potential of herbs in skin protection from ultraviolet radiation. *Pharmacognosy Reviews*, 5(10), 164-173.

Kurutas E.B. (2016). The importance of antioxidants which play the role in cellular response against oxidative/nitrosative stress: current state. *Nutrition journal*, 15(1), 71.

Lintner, K. (2017). Benefits of Anti-Aging Actives in Sunscreens. *Cosmetics*, 4, 7, 1-8.

Małgorzata, N., Grzybowski, A. The Role of the Reactive Oxygen Species and Oxidative Stress in the Pathomechanism of the Age-Related Ocular Diseases and Other Pathologies of the Anterior and Posterior Eye Segments in Adults. *Oxidative Medicine and Cellular Longevity*, 2016, 3164734.

Mukul, S., Surabhi, K., Atul, N. (2011). Cosmeceuticals for the skin: an overview. *Asian Journal of Pharmaceutical and Clinical Research*, 4(2), 1-6.

Ngoc, L.T.N., Tran, V.V., Moon, J.Y., Chae, M., Park, D., Lee, Y.C. (2019). Recent Trends of Sunscreen Cosmetic: An Update Review. *Cosmetics, 6*, 64.

Norval M., Wulf H.C. (2009). Does chronic sunscreen use reduce vitamin D production to insufficient levels? *British Journal of Dermatology*, 161, 4, 732-736.

Pullar, J.M., Carr, A.C., Vissers, M. (2017). The Roles of Vitamin C in Skin Health. Nutrients, 9, 866.

Rahman K. (2007). Studies on free radicals, antioxidants, and co-factors. *Clinical Interventions in Aging*, 2, 219-236.

Ribeiro, A.S. Estanqueiro, M., Oliveira, M.B. Sousa Lobo, J.M. (2015). Main Benefits and Applicability of Plant Extracts in Skin Care Products. *Cosmetics*, 2, 48-65.

Rinaldi, A. (2008). Healing beauty? More biotechnology cosmetic products that claim drug-like properties reach the market. *EMBO reports*, 9(11), 1073-1077.

Shanbhag, S., Nayak, A., Narayan, R., Nayak, U. Y. (2019). Anti-aging and Sunscreens: Paradigm Shift in Cosmetics. *Advanced Pharmaceutical Bulletin*, 9, 348-359.

Schagen, S.K., Zampeli, V.A., Makrantonaki, E., Zouboulis, C.C. (2012). Discovering the link between nutrition and skin aging. *Dermato-endocrinology*, 4(3), 298-307.

Solano, F. (2014). Melanins: Skin Pigments and Much More-Types, Structural Models, Biological Functions, and Formation Routes. *New Journal of Science*, 2014, 498276.

Videira, I.F., Moura, D.F., Magina, S. (2013). Mechanisms regulating melanogenesis. *Anais Brasileiros de Dermatologia*, 88(1), 76-83.

Vasile, D., Dincă, L., Enescu, C. M. (2017). Impact of collecting mushrooms from the spontaneous flora on forest ecosystems in Romania. *AgroLife Scientific Journal*, 6(1), 268-275.

Vișan L.V., Dobrinoiu R.V., Gropoșilă-Constantinescu D., Dănăilă-Guidea S., Tamba-Berehoiu R. (2017). Identification of main volatile compounds from the must of hybrid grapes grown in Romania. *Scientific Bulletin. Series F. Biotechnologies*, XXI, 2285-1364, 99-103.

Young, A.R., Narbutt, J., Harrison, G.I., Lawrence, K.P., Bell, M., O'Connor, C., Olsen, P., Grys, K., Baczynska, K.A., Rogowski-Tylman, M., Wulf, H.C., Lesiak, A., Philipsen, P.A. (2019). Optimal sunscreen use, during a sun holiday with a very high ultraviolet index, allows vitamin d synthesis without sunburn. *British Journal of Dermatology*, 181(5), 1052-1062.

Zhang, Q.W., Lin, L.G., Ye, W.C. (2018). Techniques for extraction and isolation of natural products: a comprehensive review. *Chinese medicine*, 13, 20.

https://s3.amazonaws.com/tonic4quickfix/uv-light-for-skin-conditions-list.html, acces in 18.02.2020

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EBIODEX: A USEFUL MOBILE TOOL IN THE FIELD OF BIOTECHNOLOGY E-LEARNING ECOSYSTEM

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Abstract

As technology evolves and we evolve with it, this means using optimal equipment. Thus, certain segments of the education area in the field of biotechnology could be improved. For example, the teaching support could be managed much better by teachers and students through mobile applications. The aim of the paper is to describe the making of an electronic mobile application suitable in the field of e-learning education. ***eBIODEX*** *is an application for smartphones and tablets, integrated in the e-learning system of the Faculty of Biotechnologies, which will contain theoretical courses and working protocols for the various laboratories that are carried out within the faculty. It is hoped that the* ***eBIODEX*** *application will be a modern and friendly alternative to having the study materials accessible to the students of the Faculty of Biotechnologies.*

Key words*: biotechnology, e-learning, education, internet, mobile application.*

INTRODUCTION

Biotechnology is a technology that uses biological systems, living organisms or parts of them to develop or create different products. According to the European Federation of Biotechnology: *"The integrated use of biochemistry, microbiology and engineering sciences in order to achieve technological application of the capabilities of microorganisms, cultured tissue, cells*" is the definition of Biotechnology (European Federation of Biotechnology - EFB, 1981).

Nowadays, biotechnology covers many different disciplines (for example, genetics, biochemistry, molecular biology, etc.). New technologies and products are developed every year in the fields of eg. Biomedicine (development of new drugs and therapies), agriculture (development of genetically modified plants, biofuels, biological treatment), industrial biotechnology (production of chemicals, paper, textiles and food) or bioinformatics.

"Bioinformatics is the application of information technology and information technology in the field of molecular biology. The term bioinformatics was developed by Paulien Hogeweg in 1979 for the study of computer processes in biotic systems." (Toma, 2016)

"The main purpose of bioinformatics is to increase the understanding of biological processes.

Its primary use since at least the late 1980s has been in genomics and genetics, especially in those areas of genomics that involve large-scale DNA sequencing. Bioinformatics now involves the creation and development of databases, algorithms, computational and statistical techniques and theory for solving formal and practical problems resulting from the management and analysis of biological data." (Gobalan, 2016).

Biotechnology combines disciplines such as genetics, molecular biology, biochemistry, embryology and cell biology, which in turn are related to practical disciplines such as chemical engineering, information technology and robotics (Toma, 2016).

The aim of the paper is to describe the making of an electronic mobile application suitable in the field of e-learning education.

Every one of us has forgotten at least once in our books/books, but the phone has already reached our extension, so having an application that does not require internet connection and which will have all the necessary information for the user will no longer be available. There were excuses like: "I didn't get xerox with the

lab work", "nobody told me we needed them today", etc.
Thus, came up with the idea of **eBIODEX. eBIODEX** is an application for smartphones and tablets, integrated in the e-learning system of the Faculty of Biotechnologies, which will contain theoretical courses and working protocols for the various laboratories that are carried out within the faculty.
Although it is not the first application developed within the Faculty of Biotechnologies (Toma, 2018), but also because these learning elements were very well received by the students of the faculty (Margarit, 2016), this application is intended as an additional tool available to students who come to supplement the benefits offered to them by the e-learning platform of the Faculty of Biotechnologies (Toma, 2013).
It is hoped that the eBIODEX application will be a modern and friendly alternative to having the study materials accessible to the students of the Faculty of Biotechnologies.

MATERIALS AND METHODS

The platform used to develop the application is Unity.
Unity is an engine for creating 3D games. Unity can be used by anyone who wants to create mobile applications for phone, desktop, web and 3D games.
Unity 3d initially started from the collaboration between programmers Nicholas Francis from Denmark and Joachim Ante from Germany. Working on his own game engine, Francis had problems implementing a shader system (a shader is a program that does shading on 3D objects). He asked for assistance on the Mac OpenGL message board. Ante answered a few hours later. As it turned out, Ante was also working on his own game engine. The two developers decided to change their projects and start with the creation of a single engine: Unity (Unity official WebPage).
This solution was chosen over solutions like AndroidStudio because it offers several benefits such as:
- It is excellent for multi-platform development. Cross-development platforms are currently being developed. They are really time consuming and require a lot of effort to develop native applications. Platforms, such as Unity, have made this process quite simple and easy to use compared to previous versions. Using cross-platform development, a single script can be compiled and used for many platforms.
- It is also easier to use compared to many other technologies. There are so many other complicated technologies that become tougher as we use them. Unity is an exception here.
- Unity's technical support is very efficient.
Another reason for choosing Unity was that if Android Studio was used the developed application would become strictly restricted to phones and tablets using the Android Operating System (Android Studio webpage).
To create the application was used Visual Studio 2017, an application that belongs to Unity (i.e. the programming side) for eBIODEX. The program interface looks like this (Figure 1):

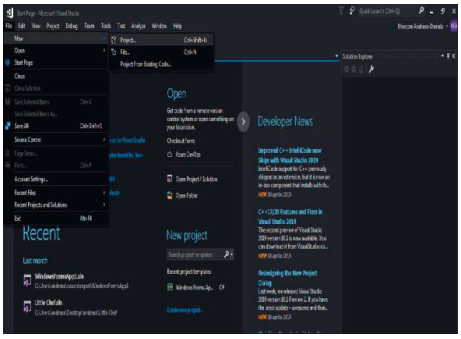

Figure 1. Interface for Visual Studio 2017

The menu interface appears in the following form (Figure 2):

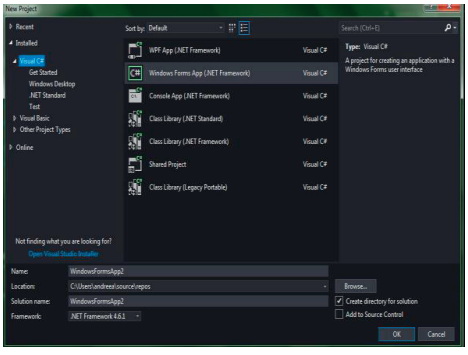

Figure 2. Interface for Menu in Visual Studio 2017

Once selected, the program builds a skeleton / base represented below in the figure where, with the help of the tools provided by the program, they can insert various pieces depending on the final purpose of the application. One of the first screen capture of the base of application is look like this (Figure 3):

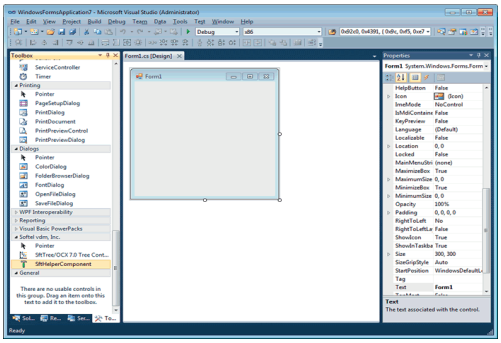

Figure 3. Screen capture

RESULTS AND DISCUSSIONS

Initially it was intended that the program interface would allow us to login with username and password. To do this, it was necessary to download another program. Be it MySQL, Microsoft Access or FireBird. But because it is desired that this application be a free tool, it was decided that this should not be done for time being. But in order to keep this as an open option for the future the next thing was to use one of the containers offered by the program so that the menu is only visible when certain requirements are fulfilled in the code. This process is much more efficient in terms of the memory used and the code. So when you want to log in the application with your username and password, it is not necessary to change all the functions performed by the menu items but only those of the container (which will be transmitted automatically to the component parts). But for now, for this version of the mobile application, on the home page there is only the logo and a button that sends to the main menu of the application. The main menu of the application is depicted in Figure 4.

Figure 4. The main menu of eBIODEX

In the first phase of the menu design, a discipline selector, a PDF reader and several buttons were added, plus a display box that together formed a computer.

Because the program is meant to display protocols for certain engineering disciplines that are based on multiple calculations, a computer is useful for solving calculation formulas. But I was disappointed again with my own computer. The program code looks like in the figures below (some fragments are shown in Figures 5-8).

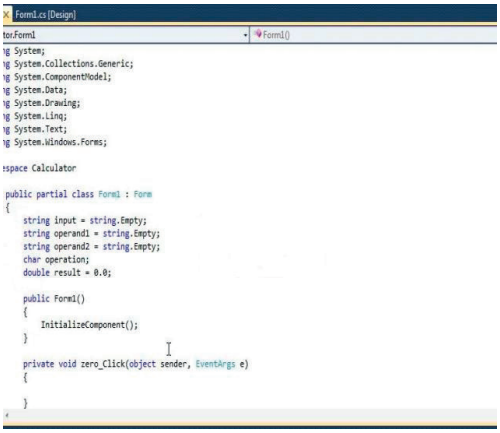

Figure 5. Screen capture of the programming code

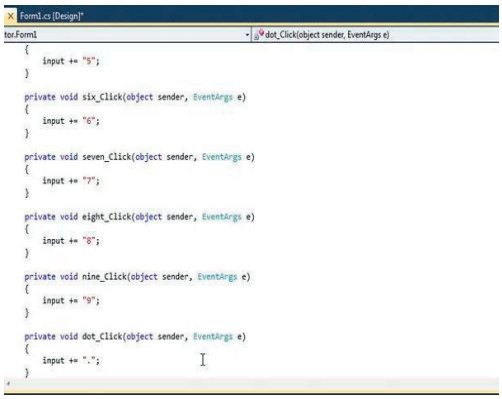

Figure 6. Screen capture of the programming code

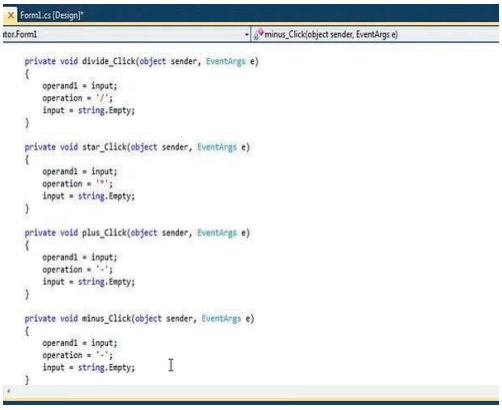

Figure 7. Screen capture of the programming code

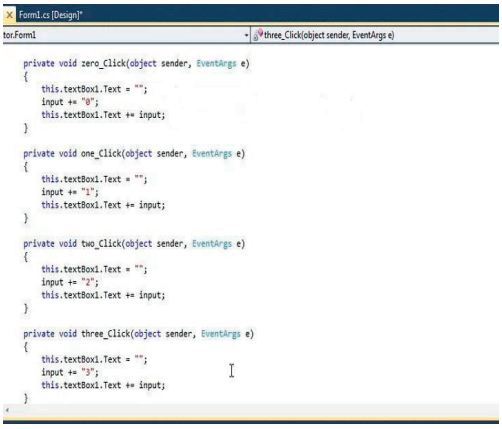

Figure 8. Screen capture of the programming code

The laboratories/seminars displayed in the program are currently only within the subject "Technologies for processing raw materials for plants and animals" of course used with the agreement of Professor Ranga Ionut. These were scanned and saved locally in the resource folder of the program. It follows that in the immediate future, the application will be completed with materials from other disciplines, grouped by years of study, by specialization and by the type of study program.

Below is a partial sequence from the code that is the basis for the pdf display of the theoretical materials presented in pdf format present in the mobile application (Figure 9).

Figure 9. Screen capture of the programming code

The mobile application looks, at this moment, as represented in the following figures (Figures 10-11).

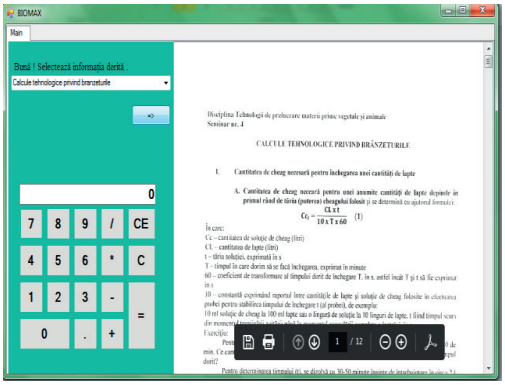

Figure 10. Screen capture of the application interface

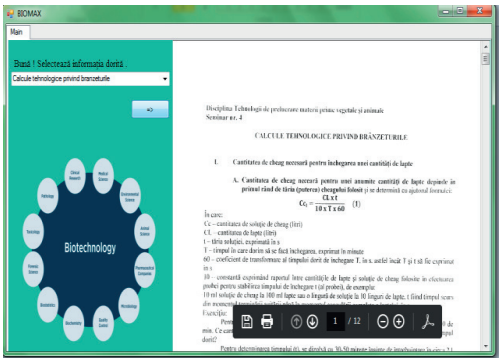

Figure 11. Screen capture of the application interface

The final result is a mobile application for Android and iOS smartphones and tablets suitable in the field of e-learning education which will contain theoretical courses and working protocols for the various laboratories that are carried out within the faculty.

CONCLUSIONS

Developing educational software to make the term biotechnology easier to understand for high school students in order to attract them to this field and potentially transform into new students within this faculty (Faculty of Biotechnologies) is a desiderate important to be achieved.

The described application can be improved by adding updates such as:

- Login with username and password;
- An integrated scientific computer;
- More information available;
- Self-assessment methods;
- A subunit of drawing for drawing and deepening the graphics;
- A database that stores the results obtained by each user in addition to an efficient way in which he can access them.

Also, collaboration with various projects in the field of ecology and environmental protection in order to educate the general public is one of the ideas for future applications.

Obtaining the agreement to use as many laboratories as possible in this project will help me create an increasingly comprehensive library of information.

The interface built on the classic application models makes this application easier to understand and use.

It is hoped that the eBIODEX application will be a modern and friendly alternative to having the study materials accessible to the students of the Faculty of Biotechnologies.

REFERENCES

European Federation of Biotechnology (EFB) (1981). Raport.

Margarit G.L., Toma R.C., Groposila Diana, Barba Dana (2016). Satisfaction level of studying through e-learning system at the students enrolled in the biotechnology education, *Scientific Bulletin, Series F, Biotechnologies*, vol XX, Bucharest, ISSN2285-1364.

Toma R.C., Pomohaci C.M. (2005). Linux şi SST o soluţie fiabilă pentru infrastructura sistemului de educaţie informatizată, *Lucrări Ştiinţifice*, USAMV Bucureşti, Seria F, Vol. IX.

Toma R.C. (2013). *Operarea şi programarea calculatoarelor*, Curs si aplicatii practice, AMC - USAMV Bucureşti.

Toma R.C., Margarit Gabriela Lucica, Groposila Diana, Barba Dana (2016). Benefits of the e-learning platforms and cloud computing in the biotechnology education, *Scientific Bulletin, Series F. Biotechnologies*, vol XX, Bucharest, ISSN2285-1364.

Toma R.C., Margarit Gabriela Lucica, Groposila Diana, Barba Dana, Gabriela Popa (2018). The use of mobile applications in the field of biotechnologies, *Scientific Bulletin, Series F, Biotechnologies*, vol XX, Bucharest, ISSN2285-1364.

Toma R.C. (2016). *Informatica aplicata in biotehnologii.* Ed. Sf. Ierarh Nicolae, Bucuresti, ISSN 978-606-30-0579-4.

Gobalan K, Ahamed John (2016). Applications of bioinformatics in genomics and proteomics, *JOAASR*, Vol. 1, No. 3.

Unity official WebPage https://unity.com/

Android Studio Developer Official WebPage https://developer.android.com/studio

MYSQL Official WebPage https://www.mysql.com/

FireBird Official WebPage https://firebirdsql.org/

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MICROSCOPIC CHARACTERISTICS OF SOME *LAMIACEAE* SPECIES - A SELECTION OF LIGHT MICROSCOPY IMAGES

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Abstract

The Lamiaceae family is well known for its aromatic plants that are important in the food, cosmetics and pharmaceutical industry. Also, there are Lamiaceae species that are important melliferous plants, or are used as ornamental plants. In Romania several Lamiaceae species of pharmaceutical relevance can be found (most are cultivated, but some are also spontaneous plants): Hyssopus officinalis, Lamium album, Lavandula angustifolia, Majorana hortensis, Marrubium vulgare, Melissa officinalis, Mentha piperita, Ocimum basilicum, Origanum vulgare, Rosmarinus officinalis, Salvia officinalis, Satureja hortensis, Thymus serpyllum, Thymus vulgaris.
Lamiaceae species have specific characteristics both at macroscopic level as well as at microscopic level, for example their stem and leaves have numerous secretory hairs that can be analysed using wet mounts of hand-sections, surface preparations or samples of powdered plant material. Thus, the present study illustrates the results of such a microscopic approach, for 10 Lamiaceae species obtained commercially in Bucharest (Romania).

Key words*: Lamiaceae species, light microscopy, microscopic pictures.*

INTRODUCTION

The Lamiaceae (Labiatae) family is one of the major plant families (~4000 species - Sârbu, 1999) in the Lamiales order. Known for the numerous aromatic species that can be easily propagated and are widely cultivated, the "mint family" plants are important in the food, cosmetics or pharmaceutical industry. Also, there are Lamiaceae species that are important melliferous plants, ornamental plants, or have religious significance (for example basil).
The Lamiaceae are commonly herbaceous or shrub plants, rarely trees or vines, with stems that are frequently square in cross section and opposite leaves with no stipules and verticillate inflorescences. Aerial reproductive and vegetative organs are covered with glandular (secretory) trichomes (hairs) that produce volatile oil.
The current study presents a microscopic evaluation of plant material from several Lamiaceae species to record some of the parameters of taxonomic relevance using a simple method.

MATERIALS AND METHODS

Plant material

Fresh plant material and/or dried plant material were analysed, as follows: *Lamium album* (white nettle or white dead-nettle) - herbal tea (*herba*); *Lavandula angustifolia* (lavender or true lavender) - plant in pot and herbal tea (*flores*); *Melissa officinalis* (lemon balm) - herbal tea (*folium*); *Mentha piperita* (peppermint) - cultivated and herbal tea (*folium*); *Ocimum basilicum* (basil) - fresh plant material and herbal tea (*herba*); *Majorana hortensis* sin. *Origanum majorana* (marjoram) - fresh plant material; *Origanum vulgare* (oregano) - fresh plant material; *Rosmarinus officinalis* (rosemary) - fresh plant material; *Salvia officinalis* (sage) - fresh plant material and herbal tea (*folium*); *Thymus serpyllum* - (creeping thyme) - herbal tea (*herba*) (Figures 1-2). Plants in pots were obtained from local shops (Aikaterina flower shop in Bucharest or the Garden Services S.C. situated within the Agronomie Herăstrău University Campus).

Figure 1. Herbal teas used in the present study

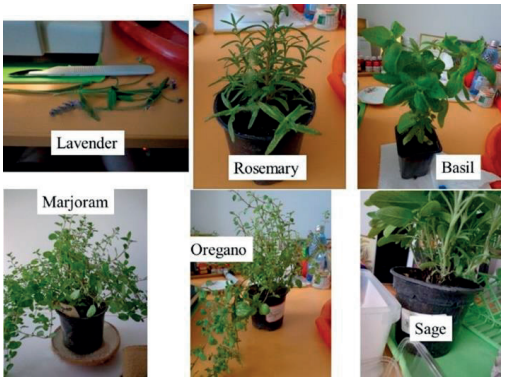

Figure 2. Fresh plant material used in the present study

Microscopic analysis

The analysis was carried out at the Laboratory of Biology, Faculty of Biotechnologies of the University of Agronomic Sciences and Veterinary Medicine of Bucharest.

Wet mounts of hand-sections, surface preparations or samples of powdered plant material were examined using a Novex Holland optical microscope with ocular micrometer (calibration ratio was 1 µm for ob. 100×, 2.5 µm for ob. 40×, 10 µm for ob. 10×). To colour, toluidine blue was added. Microscopic images were photograped with a Sony Cyber-shot® digital camera (Carl Zeiss Vario-Tessar 5× zoom lens) and were later compared to descriptions found in published data. There is a vast literature in this field, for example AHPA (2014), Azzazy (2019), Bosabalidis & Sawidis (2014), Celep et al. (2011), Choi & Kim (2013), Gîrd et al. (2015), Groşan et al. (2019), Jia et al. (2013), Kahraman et al. (2010), Marin et al. (2006), Popescu et al. (2009), Tozin et al. (2016), Turner et al. (2000), however, most of the descriptions used in the present study are based on the work of Gîrd et al. (2010a, b).

RESULTS AND DISCUSSIONS

Microscopically several specific or common anatomical elements were identified, as follows:

Lamii albi herba: conical unicellular non-glandular hairs (Figure 3); multicellular uniseriate, unbranched non-glandular hairs with a thin apex (Figure 4); peltate glandular trichomes and 3-colpate pollen grains. Oil globules were present also.

Figure 3. Conical non-glandular hairs (white nettle)

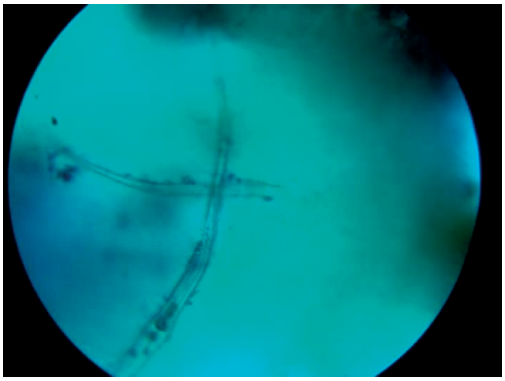

Figure 4. Long, multicellular non-glandular hairs with a thin apex (white nettle)

Lavandula angustifolia **(sin.** ***Lavandula officinalis, Lavandula vera, Lavandula spica*****):** non-glandular multicellular trichomes, uniseriate with warty cuticle and branched in dichotomic manner (star type *hairs*) (Figures 5-6), peltate glandular trichomes and diacytic stomata.

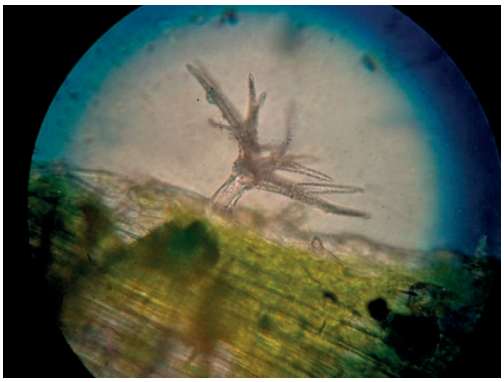

Figure 5. Branched non-glandular multicellular trichome (lavender)

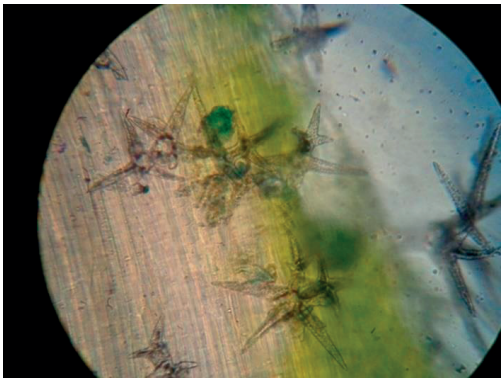

Figure 6. Several branched non-glandular multicellular trichomes (lavender)

In the lavender flower: there were papillae (conical cells) present in the epidermal layer of the corolla (Figure 7); 6-colpate, medium size pollen grains (~ 30 µm) (Figure 8); dense non-glandular trichomes that cover the surface having a protective role (Figure 9). On the ovary there are glandular hairs with elongated stalk and unicellular gland (capitate trichomes) (Figure 10).

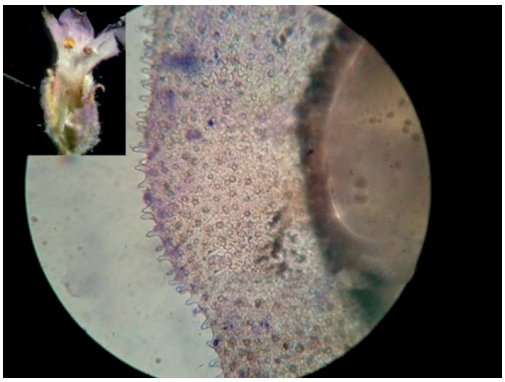

Figure 7. Corolla papillae in the lavender flower

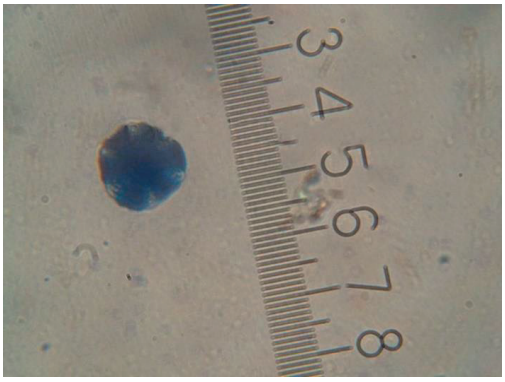

Figure 8. Medium size (~ 30 µm), 6-colpate, pollen grains (lavender)

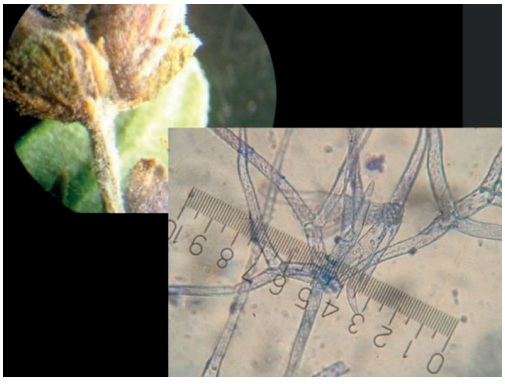

Figure 9. Dense non-glandular trichomes on the lavender flower

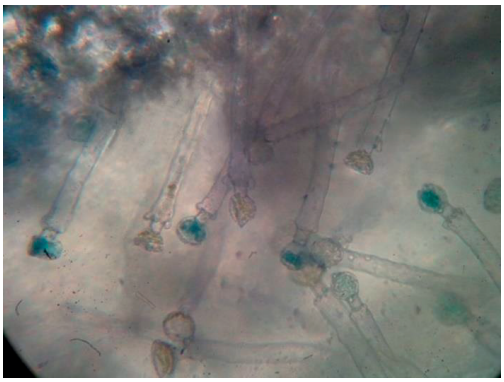

Figure 10. Glandular capitate trichomes in the lavender flower

Lavandulae flores: *numerous non-glandular* multicellular trichomes, uniseriate with warty cuticle and branched in dichotomic manner (star type *hairs*), *that were whole or fragmented* (Figure 11).

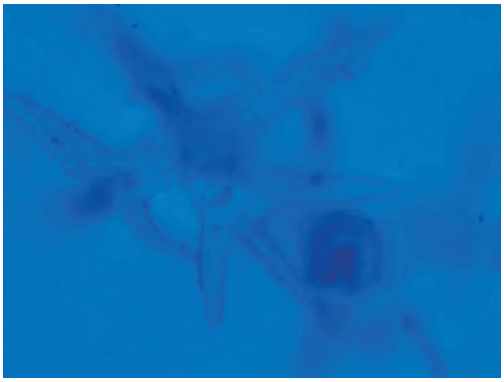

Figure 11. Fragmented star type *hairs* (*Lavandulae flores*)

Melissae folium: epidermal cells and diacytic stomata; non-glandular unicellular trichomes with warty cuticle and a sharp tip, "with the appearance of a canine tooth" (Gîrd et al., 2010), hairs specific to lemon balm (Figure 12); multicellular trichomes with warty cuticle (Figures 13-14); glandular peltate trichomes (Figures 15).

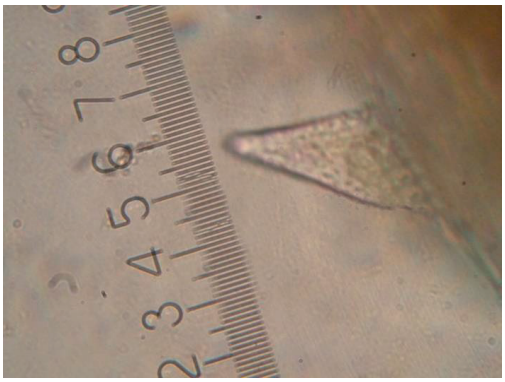

Figure 12. Non-glandular unicellular trichome with warty cuticle and a sharp tip, "with the appearance of a canine tooth" (Gîrd et al., 2010) (*Melissae folium*)

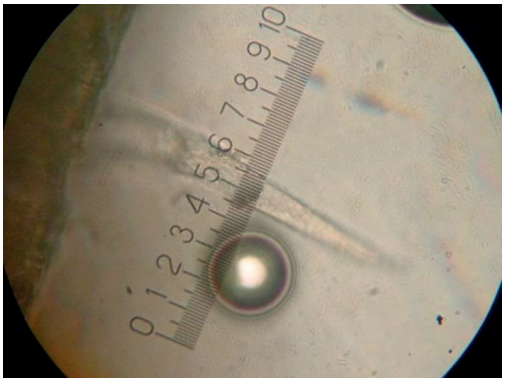

Figure 13. Non-glandular multicellular trichome with warty cuticle (*Melissae folium*)

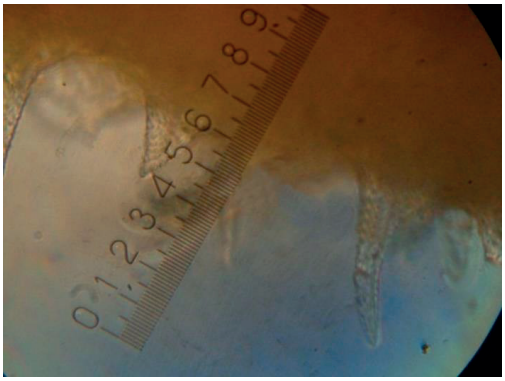

Figure 14. Non-glandular unicellular and multicellular trichomes with warty cuticle (*Melissae folium*)

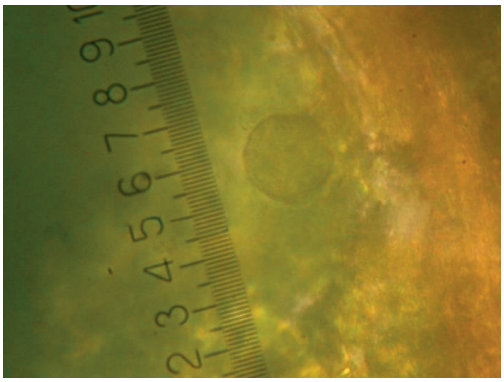

Figure 15. Glandular peltate trichome (*Melissae folium*)

Menthae piperitae folium: non-glandular uniseriate, multicellular, arched hairs with a sharp tip (Figure 16); peltate glandular trichomes.

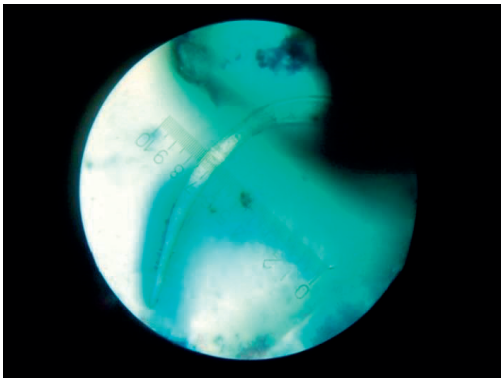

Figure 16. Non-glandular uniseriate multicellular trichome (*Menthae folium*)

Ocimum basilicum: non-glandular trichomes either unicellular or multicellular (Figures 17-18); glandular capitate and peltate trichomes (Figures 19-20); and large size (~ 52.5 µm), 6-colpate pollen grains (Figure 21).

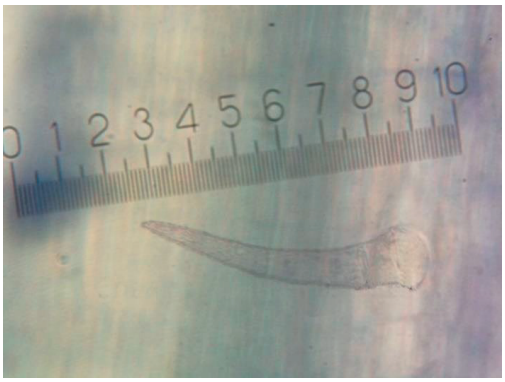

Figure 17. Non-glandular unicellular trichome (basil)

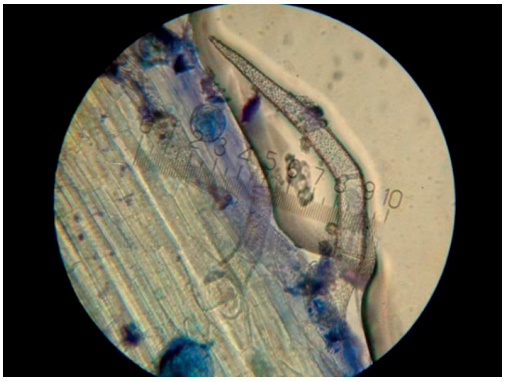

Figure 18. Non-glandular multicellular trichome (basil)

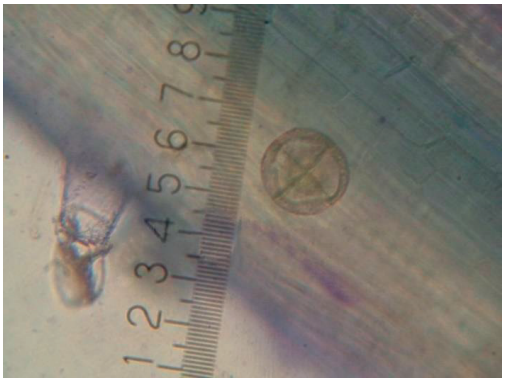

Figure 19. Glandular capitate trichome and glandular peltate trichome with a globular head composed of 4 cells (basil)

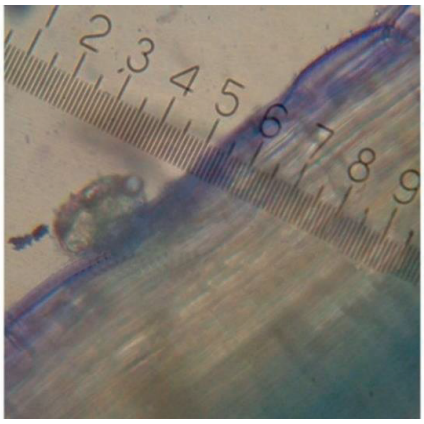

Figure 20. Glandular peltate trichome - side view (basil)

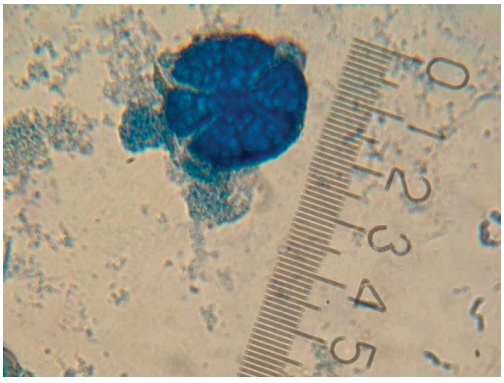

Figure 21. Large size (~ 52.5 µm), 6-colpate pollen grain with reticulate surface pattern (basil)

Basilici herba: non-glandular trichomes (Figure 22); glandular peltate trichomes and epidermal cells with diacytic stomata (Figure 23); oil globules were also present.

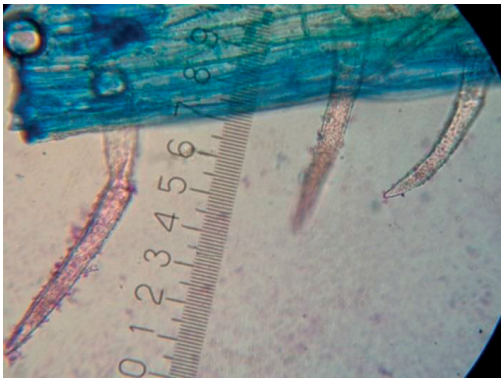

Figure 22. Non-glandular trichome (*Basilici herba*)

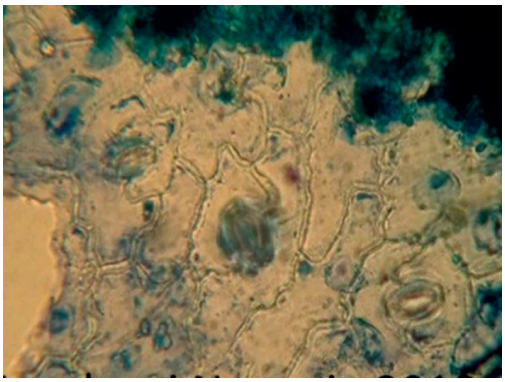

Figure 23. Epidermal cells; diacytic stomata (*Basilici herba*)

Majorana hortensis* sin. *Origanum marjorana*, *Majorana majorana: non-glandular unicellular and multicellular trichomes (Figure 24); numerous peltate glandular trichomes (Figure 25).

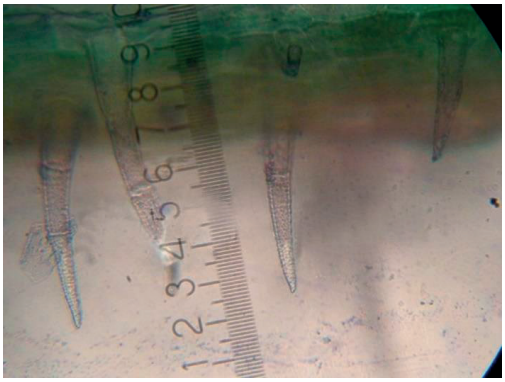

Figure 24. Non-glandular trichomes (marjoram)

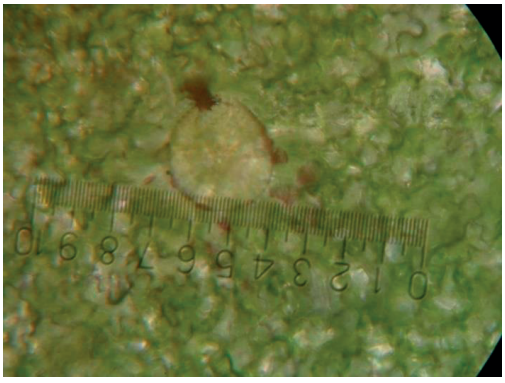

Figure 25. *Peltate glandular trichome* (marjoram)

***Origanum vulgare**:* non-glandular unicellular and multicellular trichomes (Figure 26); peltate glandular trichomes (Figure 27); oil globules were also seen.

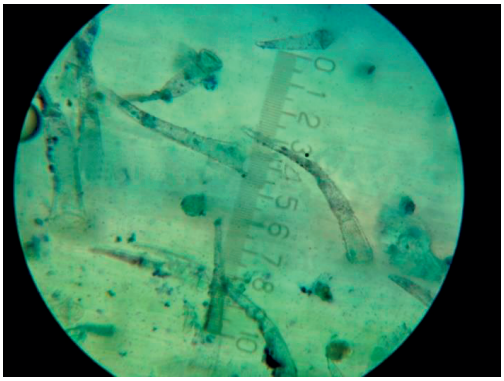

Figure 26. Non-glandular unicellular and multicellular trichomes (oregano)

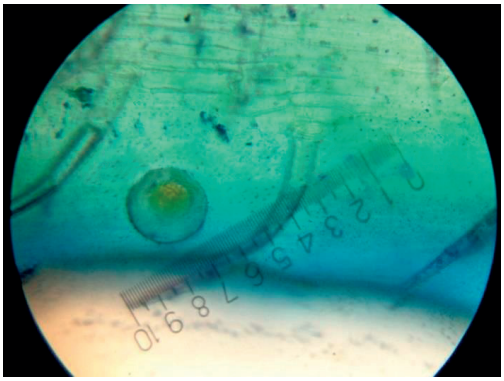

Figure 27. Non-glandular trichomes and peltate trichome (resin sphere visible) (oregano)

Rosmarinus officinalis: numerous non-glandular uniseriate, multicellular, branched trichomes with sharp tips (star type *hairs*) (Figures 28-29); capitate glandular trichomes (Figures 30-31); peltate glandular trichomes (Figure 32); numerous oil globules were also present.

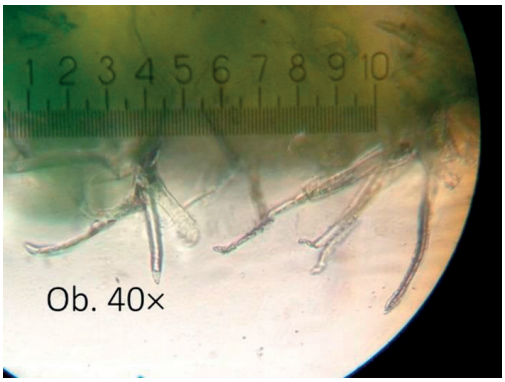

Figure 28. Non-glandular multicellular, branched trichomes with sharp tips (side view) (rosemary)

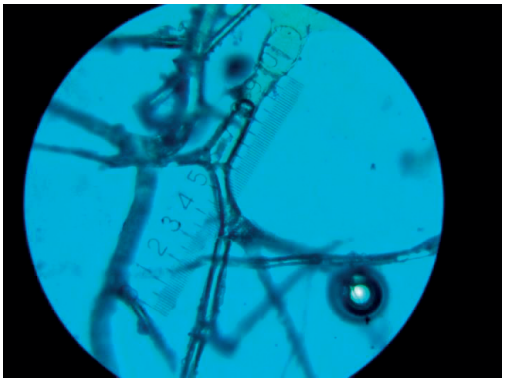

Figure 29. Non-glandular uniseriate, multicellular, branched trichomes with sharp tips (rosemary)

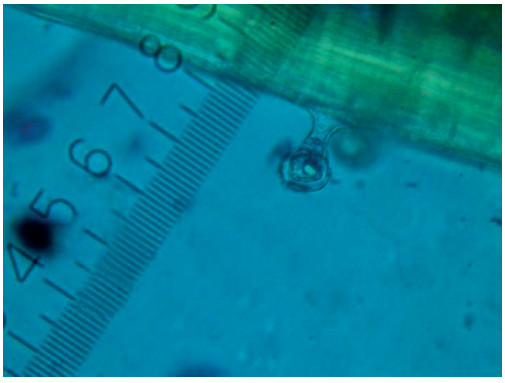

Figure 30. Capitate glandular hair with short stalk (rosemary)

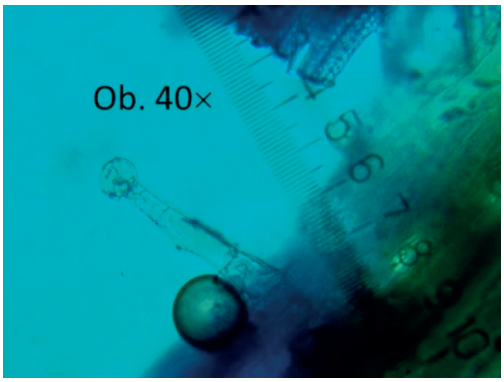

Figure 31. Capitate glandular hair with long stalk (rosemary)

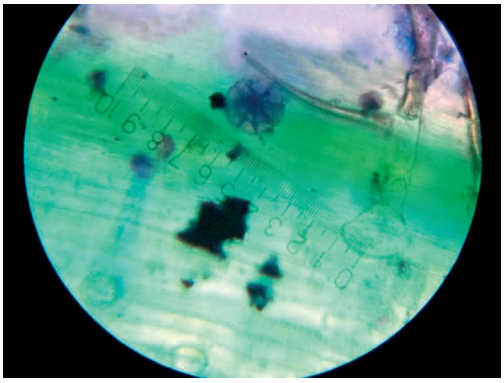

Figure 32. Peltate glandular trichome (rosemary)

Salvia officinalis: numerous non-glandular uniseriate, long and thin, multicellular trichomes with sharp tips (Figures 33-34); capitate glandular trichomes (Figure 35); diacytic stomata.

Figure 33. Numerous non-glandular uniseriate, long and thin, multicellular trichomes with sharp tips (sage)

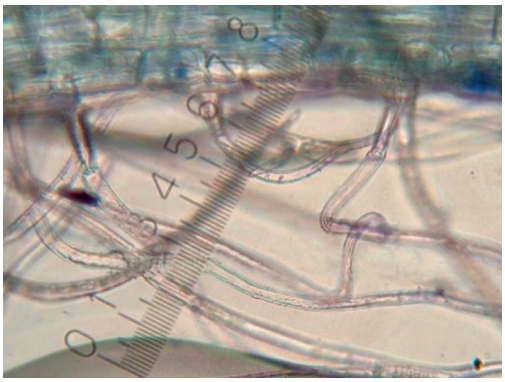

Figure 34. Non-glandular uniseriate, long and thin, multicellular trichomes (sage)

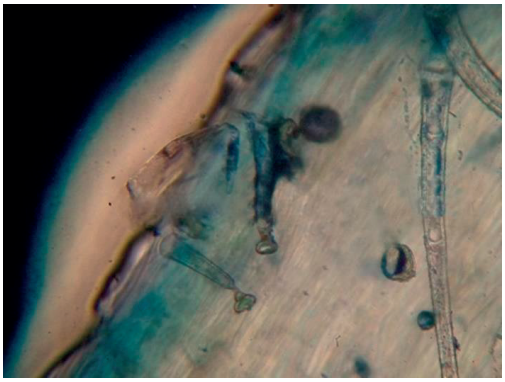

Figure 35. Capitate glandular trichomes (sage)

Salviae folium: non-glandular uniseriate, multicellular trichomes (Figure 36); oil globules (Figure 37).

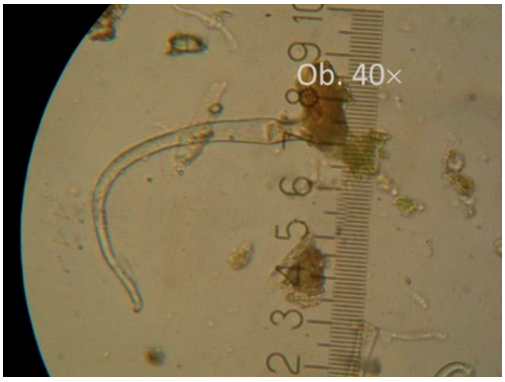

Figure 36. Non-glandular uniseriate trichome (*Salviae folium*)

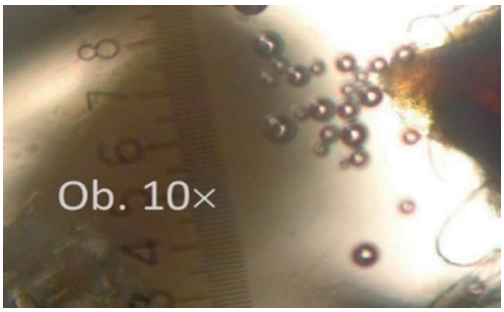

Figure 37. Oil globules (*Salviae folium* - wet mount)

Serpylli herba: 6-colpate pollen grains, oblate in lateral view; non-glandular uniseriate, multicellular trichomes (Figure 38); non-glandular tricellular trichomes with large base (Figure 39); numerous glandular peltate and capitate hairs (Figures 40) and epidermal fragments with diacytic stomata.

Figure 38. Non-glandular uniseriate, multicellular trichome (*Serpylli herba*)

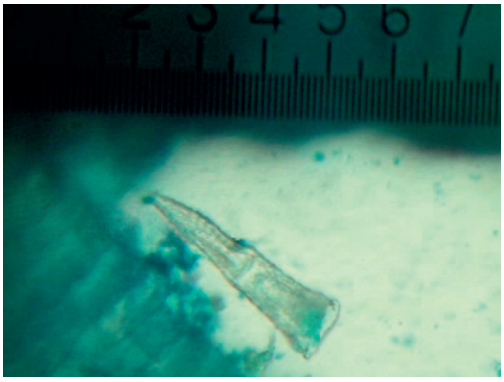

Figure 39. Non-glandular tricellular trichomes with large base (*Serpylli herba*)

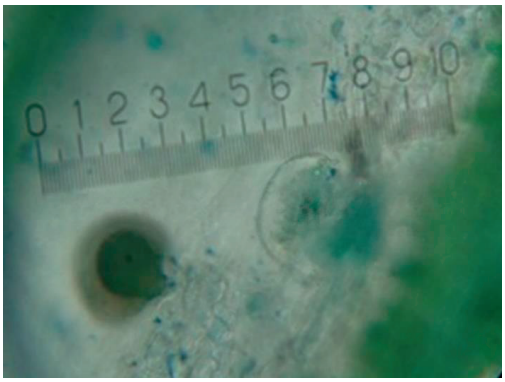

Figure 40. Glandular capitate hair (resin sphere visible) (*Serpylli herba*)

CONCLUSIONS

Three main types of trichomes were observed: glandular peltate trichomes, capitate glandular trichomes and non-glandular trichomes. The two types of glandular trichomes can be determined in light microscopy based on the length of the stalk (which is longer in capitate hairs and short in peltate hairs), while the peltate hairs can also be described further if the number of secretory cells is visible. The non-glandular hairs also showed morphological variety.

The results of the present analysis show some of the structures indicated in the scientific literature for the species that were analysed. Knowledge of the specific tissues and anatomical characteristics of a plant can be applied together with chemical profiling for standardization and quality assurance purposes. Thus, the macroscopic and microscopic analysis of medicinal plants are still used as a preliminary step in the botanical identification of herbal products that is carried out using standard techniques as part of the quality control procedure in pharmaceutical labs and industries.

REFERENCES

AHPA (2014). AHPA Botanical Identity References Compendium. http://www.botanicalauthentication.org/, site accesat în 8 mai 2018.

Azzazy, M. (2019). Micromorphology of Pollen Grains, Trichomes of Sweet Basil, Egypt, 5, 427-433. 10.31031/ACAM.2019.05.000604.

Bosabalidis, A. & Sawidis, T. (2014). Glandular and non-glandular hairs in the seasonally dimorphic *Origanum dictamnus* L. (Lamiaceae) as a means of adaptation to cold stress. *Acta Agrobotanica*, 67: 15-20. 10.5586/aa.2014.010.

Celep, F., Kahraman, A., Atalay, Z. & Dogan, M. (2011). Morphology, anatomy and trichome properties of *Lamium truncatum* Boiss. (Lamiaceae) and their systematic implications. *Australian Journal of Crop Science* 5(2): 147-153.

Choi, J.S. & Kim, E.S. (2013). Structural Features of Glandular and Non-glandular Trichomes in Three Species of Mentha. *Applied Microscopy*, 43(2), 47-53. https://doi.org/10.9729/am.2013.43.2.47.

Gîrd, C.E., Costea, T., Nencu, I., Duțu, L.E., Popescu, M.L. & Balaci, T.D. (2015). Comparative pharmacognostic analysis of Romanian *Ocimum basilicum* L. and *O. basilicum* var. *purpurascens* Benth. aerial parts. *Farmacia,* 63(6): 840-844.

Gîrd, C.E., Duțu, L.E., Popescu, M.L., Iordache, A.T., Tudor, I. & Costea, T. (2010) *Bazele teoretice şi practice ale analizei farmacognostice*, vol. I şi II, Editura Curtea Veche, Bucureşti.

Groșan, A., Ștefănescu, R., Laczkó-Zöld, E., Eșianu, S. & Muntean, D.L. (2019). Identification of the herbal drug Prunellae spica based on macroscopic and microscopic characteristics. *Acta Biologica Marisiensis*, 2(1): 12-20.

Jia, P., Liu, H., Gao, T. & Xin, H. (2013). Glandular trichomes and essential oil of *Thymus quinquecostatus*. *Scientific World Journal*. 2013:387952. doi:10.1155/2013/387952

Kahraman, A., Celep, F. & Dogan, M. (2010). Anatomy, trichome morphology and palynology of *Salvia chrysophylla* Stapf (Lamiaceae). *South African Journal of Botany*, 76(2): 187-195.

Marin, M., Koko, V., Duletić-Laušević, S., Marin, P.D., Rančić, D. & Dajic-Stevanovic, Z. (2006). Glandular trichomes on the leaves of *Rosmarinus officinalis*: Morphology, stereology and histochemistry. *South African Journal of Botany*, 72(3): 378-382.

Popescu, M.L., Dinu, M. & Toth, O. (2009). Contributions to the pharmacognostical and phytobiological study on *Leonurus cardiaca* (L.) (*Lamiaceae*). *Farmacia*, 57(4): 424-431.

Sârbu, A. (1999). *Biologie vegetală. Note de curs*. Editura Universității din București.

Tozin, L.R., de Melo Silva, S.C. & Rodrigues, T.M. (2016). Non-glandular trichomes in Lamiaceae and Verbenaceae species: morphological and histochemical features indicate more than physical protection. *New Zealand Journal of Botany*, 54(4): 446-457. 10.1080/0028825X.2016.1205107.

Turner, G.W., Gershenzon, J. & Croteau, R.B. (2000). Development of peltate glandular trichomes of peppermint. *Plant Physiol*., 124(2): 665-680.

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