University of Agronomic Sciences
and Veterinary Medicine of Bucharest
Faculty of Biotechnology

SCIENTIFIC BULLETIN

SERIES F. BIOTECHNOLOGIES

Volume XXVII, No. 2

2023
Bucharest

SCIENTIFIC BULLETIN
SERIES F. BIOTECHNOLOGIES
VOLUME XXVII, NO. 2, 2023

University of Agronomic Sciences
and Veterinary Medicine of Bucharest
Faculty of Biotechnology

SCIENTIFIC BULLETIN

SERIES F. BIOTECHNOLOGIES

Volume XXVII, No. 2

2023
BUCHARESt

PUBLISHERS:
University of Agronomic Sciences and Veterinary Medicine of Bucharest, Romania – Faculty of Biotechnology
Address: 59 Mărăști Blvd., District 1, Zip code 011464, Bucharest, Romania,
Phone: + 40 21 318 25 64, Fax: +40 21 318 28 88,
E-mail: florentina.matei@biotechnologii.usamv.ro, Webpage: http://biotechnologyjournal.usamv.ro

CERES Publishing House
Address: 29 Oastei Street, District 1, Bucharest, Romania
Phone: + 40 21 317 90 23, E-mail: edituraceres@yahoo.com, Webpage: www.editura-ceres.ro

To be cited: Scientific Bulletin. Series F. Biotechnologies, Volume XXVI, No. 2, 2023

ISSN 2285-1364, CD-ROM ISSN 2285-5521, ISSN Online 2285-1372, ISSN-L 2285-1364

International Database Indexing: COPERNICUS, CABI, GOOGLE SCHOLAR, DOAJ, Scipio, PBN (Polish Scholarly Bibliography), OCLC, Research Bible, Cite Factor (Academic Scientific Journals), Universal Impact Factor

SUMMARY

Scientific Bulletin. Series F. Biotechnologies, Vol. XXVII, No. 2, 2023
ISSN 2285-1364, CD-ROM ISSN 2285-5521, ISSN Online 2285-1372, ISSN-L 2285-1364

ANALYSIS OF NUTRITIONAL CONSTITUENTS IN DIFFERENT FRUIT TISSUES IN FOUR CITRUS SPECIES FROM SOUTH ALBANIA REGION

Ilir LLOHA, Enkeleda BERBERI, Ervin SHISHMANI

Agricultural University of Tirana, Faculty of Biotechnology and Food, Department of Food Science and Biotechnology, "Paisi Vodica" Street, 1050, Tirane, Albania

Corresponding author email: illoha@ubt.edu.al

Abstract

Statistical Office of the European Communities (Eurostat) ranks Albania sixth in Europe for fresh orange production in 2018, and the mandarin production was greater than the global and EU average yields, indicating a high-performing subsector. It is essential to examine the chemical composition of citrus fruits cultivated in Albania to fully benefit from their advantages, which was the motivation for this research. This study comprised four different groupings of citrus species (C. sinensis, C. limon, C. reticulata and C. paradisi) harvested in south Albania region. Among others, total polyphenol content (TPC), total flavonoid content (TFC), vitamin C content (VCC), and antioxidant activity were the focus of our analyses. SPSS and Sigma Plot statistical programs were used to analyse the data. The percentages of different biochemical components varied not only across species, but also between different citrus fruit tissues (Sigma Plot, ANOVA- Tukey test, $p<0.05$). Citrus paradise shows clearly a significantly high TPC compared to other citrus species. Our findings indicate the presence of a significant amount of bioactive compounds in citrus peel and pulp, making them ideal candidates for use in antioxidant-rich diets and the pharmaceutical industry.

Key words*: Citrus species, bioactive compounds, Sigma Plot, SPSS.*

INTRODUCTION

Citrus family is one of the most significant fruit families for human consumption and are among the most studies due to hight antioxidant ability associated with phytochemical and nutrients composition (Liu et al., 2022).

Albania, as part of the Mediterranean region, has a strong tradition in citrus fruits production due to its favourable climate condition, especially in south costal region. In the genus *Citrus* L. of the family Rutaceae, subfamily Aurantioideae are included *Citrus sinensis*, *Citrus paradisi*, *Citrus limon* and *Citrus reticulata*, commonly known as orange, grapefruit, lemon, and mandarin (Wu et al., 2018). According to Eurostat, Albania in 2018 is ranked seventh in Europe to produce fresh oranges with 10,800 tons (Eurostat, 2018). Furthermore. Moreover, according to FAOSTAT, Albania's yield of mandarin production (36 tons/ha) was higher when compared to the world average as well as EU average yield, demonstrating a highly performing subsector (FAOSTAT, 2018). The region of Vlora produces over 59 percent of all citrus fruits in Albania (FAOSTAT, 2018). Also, according to the Institute of Statistics in Albania the highest level of citrus production was achieved in the prefecture of Vlora with 66.05% (32,497 tons) (INSTAT, 2020). If the proper policies are implemented and this sector receives the appropriate investment, the aforementioned data can be taken into consideration as a significant indicator that Albania will probably become one of the leading countries in Europe for the production and export of citrus. In the case of Albania, most citrus fruits are utilized to produce juice, whereas the waste treatment of citrus is underestimated and not been given the proper attention. This approach is in contradiction with numerous studies which evidence the fact that citrus fruit waste offers a lot of potential for use in the food and pharmaceutical industries, as well as for adding value to processed foods and other diets (Chavan et al., 2018; Casas Cardoso et al., 2022). Furthermore, based on the literature, citrus fruits are rich in numerous bioactive compounds, such as ascorbic acid, tocopherols, carotenoids, dietary fibre, minerals, and a few other compounds, such as flavonoids, phenolic acids, and tannins (Lv et al., 2015; Rafiq et al., 2018; Saini et al., 2022).

Most of these compounds have antioxidant properties. We chose to carry on with this study because, as far as we are aware, few research has been done to ascertain the chemical composition of citrus fruits and their antioxidant capacities that grown in Albania. The first preliminary date, published in 2015 (Lloha et al., 2015), where quite encouraging because they indicate a high presence in active natural metabolites that potentially provide benefits for human health as well as a wide range of possible applications in the food industry. In the frame of human health, different studies suggest that a dietary approach rich in fruits, vegetables, and whole grains, are valuable in preventing various chronic diseases (Medina-Remón et al., 2018; Zou et al., 2016).
Therefore, the main aim of this study was to evaluate the total mineral content (TMC), total phenolic content (TPC), total anthocyanin content (TAC), total vitamin A and C as well the antioxidant activities in different tissues of citrus fruits that grow in south Albania regions.

MATERIALS AND METHODS

Fruits belonging to four species of genus *Citrus*, respectively, *Citrus sinensis*, *Citrus limon*, *Citrus reticulata* and *Citrus paradisi*, judge by colour and flavour, were harvested during autumn 2018, in Vlora and Saranda region (Figure 1).

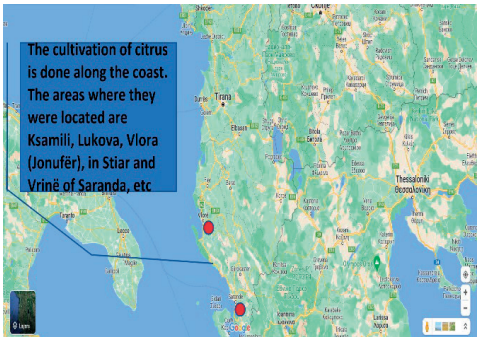


Figure 1: South Albania costal region where the citrus fruits samples were harvested (source google map).

Following collection and during transportation, samples are kept on ice and then were stored at 4^0C at the Laboratory of Food Analyses, Faculty of Biotechnology and Food. Before conducting any analysis in all the fruits was determined the physical characteristics such as dimension, weight, colour etc. About 1 kg sample of each citrus fruit, with fruits having the same dimensions, were analysed. All citrus fruits, of the four citrus species mentioned above, have been peeled and squeezed out and then placed on thermostat (WTB binder) in 80°C for 24 hours. Then the samples were analysed about the moisture content (thermostat) and the mineral content (Muffle furnace) of both fruit pulp and peel samples. After temperature treatment the peels and the dried pulps contacting the seed were grinded. An amount of 10 g of powder samples was extracted with 150 ml distilled water (Heidolph promax) within an interval 60 minutes. After extraction the samples were centrifuged 1500 rpm/min for 10 minutes and then filtered in a vacuum filtering apparatus. For the organic solvent extracts preparation, the powder samples were weighted and an amount of 10 g was extracted with petroleum benzene (50-70°C) in a soxhlet apparatus.
Total phenolic content (TPC) was calculated (Elfalleh et al., 2012) using Folin-Ciocalteu method, with some modification. Briefly, an amount of 150 µl was added with 1ml ethanol and 5 ml distilled water. After vortexes, into the solution was added 0.5 ml Folin-Ciocalteu reagent, and vortexes again for 3 minutes followed by added 1 ml sodium carbonate (Na_2CO_3 5 g/l). The solution was kept in a dark place for 60 minutes and then the absorbance was measured in a photospectrometer at 725 nm length wave. As blank was used distilled water and the results are expressed as gallic acid equivalent.
The total anthocyanin content (TAC) was determinate using the method proposed by Di Stefano et al., 1989. Briefly the samples were diluted (250 µl/5 ml, 500 µl/5 ml, 1000 µl/5ml) in a solution containing ethanol, distilled water and concentrated hydrochlorides acid (70/30/1 v/v/v). The absorbance was measured at a length wave 540 nm. The results are expressed as malvidine-3-glucoside equivalents based on the equation:
TA_{540nm} (mg/ml) = $A_{540\ nm}$ 16.7d (Di Stefano et al., 1989)
Total vitamin A determination was based on spectrophotometric methods (Rutkowski et al., 2007). Briefly 10 ml of each sample was

centrifuged, added with 10 ml KOH and vortexes for 1 minute. Then the flasks were placed on water bath at 60°C for 20 minutes and cooled. After cooling was added 10 ml xylene and vortexes again for 1 minute. Samples were centrifuged (1500 rpm for 10 minutes) and the absorbance (A_1) of the supernatant was measured at 335 nm length wave using xylene as blank. The samples were placed under UV radiation for 30 minutes and measured the absorbance (A_2) at 335 nm length wave using xylene as blank. The vitamin A concentration was determined based on the equation:

$C_x = (A_1\text{-}A_2) \times 22.23$ (Rutkowski et al., 2007)

A rapid and practical method for the total vitamin C content determination is the iodine titration method. Samples water extracts are centrifuged and filtrated and then 5 ml are added with 20 ml distilled water and 2 ml of a water solution containing 1% starch. The mixture is titrated with solution 0.01 N iodine. 1 ml mixture is equivalent of 0.88 mg ascorbic acid.

All samples were tested for the antioxidant activity using the DPPH (2,2-difenil-1-pikrilhidrazil) method (Malacrida et al., 2012) with some modification. Briefly, an amount of 50µ and 100µl of each water extract was placed in a laboratory flask and added with 3 ml DPPH methanol solution (0.04 g/l). After measuring the absorbance A_1 at 517 nm length wave the samples were placed in dark for 60 minutes, after 60 minutes was measured absorbance A_2 at 517 nm with methanol used as blank. The results are calculated using the formula:

$AA = (ABS_{control} - ABS_{sample}/ABS_{control}) \times 100$

All data are expressed as mean and standard deviation of six replicates.

All the data were analysed using statistical program Sigma Plot 12.5 and SPSS (IBM statistic 25.00). A One Way Anova and Tukey post hoc test were performed to observe if there is a significant difference between variables measured in different tissues, pulp and peal, of citrus fruits samples.

RESULTS AND DISCUSSIONS

The following results belong to four citrus species harvested in two regions of Albania, Vlora and Saranda. Total mineral content (TMC), total phenolic content (TPC), and total anthocyanin content (TAC) were determined from each sample extract of citrus fruits, harvested from the Vlora region, using the methods described above, as indicated below (Table 1). There is clearly a difference in value between peal and pulp tissues for all the parameters that are measured, which is in compliance with the literature (Barros et al., 2012). The highest value of TMC was noted in peal mandarin fruits followed by grapefruits and lemon. When compared to other citrus fruits, grapefruit and lemon have higher levels of TPC in peel and the difference is statistically significant ($p<0.01$) which is consistent with the results published from Czech in 2021 and Elkhatim in 2018 (Czech et al., 2021; Elkhatim et al., 2018). Furthermore, TAC value was higher in lemon pulp and peal followed by TAC value observed in orange.

A one-way ANOVA (Statistical programme sigma Plot 12.5) was performed to determine if there is any significance difference between the values of TMC, TPC and TAC extracted in peal and pulp of the four citrus species fruits harvested in the region of Vlora. The data show that there is a significance difference between TMC value in peal and pulp among citrus fruits ($F_{(7,\ 40)} = 7{,}414$; $p < 0.001$) as also there is a significance difference between TPC value in peal and pulp among citrus fruits ($F_{(7,\ 40)} = 4213.4$; $p < 0.001$). Furthermore, the data show that there is a significance difference between TAC value in peal and pulp among citrus fruits ($F_{(7,\ 40)} = 3.417$; $p<0.004$). A Tukey post hoc test was performed to analyse if there is any significant difference between different citrus fruits tissues, pulp, and peal (Table 1). Since both regions are characterized by a similar and very favourable climate for the growth of citrus fruits, quite the same data were extracted from the samples harvested in the region of Saranda. In comparison to the citrus fruits harvested in Vlora, TMC, TPC, and TAC are somewhat higher in peal and pulp for all citrus fruits harvested in Saranda (Table 2). Mandarin fruits harvested in Saranda has a higher level of TMC in peal compared to other citrus fruits. As for TPC value was higher in lemon pulp fruits followed by TPC observed in mandarin pulp fruits. Furthermore, TAC value was higher in

lemon pulp followed by TAC value observed in mandarin pulp fruits.

A one-way ANOVA (Statistical programme Sigma Plot 12.5) was performed to determine if there is any significance difference between the values of TMC, TPC and TAC extracted in peal and pulp of the four citrus species fruits harvested in the region of Saranda. The data show that there is a significance difference between TMC value in peal and pulp among citrus fruits ($F_{(7, 40)} = 5.463$; $p<0.001$) as also there is a significance difference between TPC value in peal and pulp among citrus fruits ($F_{(7, 40)} = 374.073$; $p<0.001$). Furthermore, the data show that there is a significance difference between TCA value in peal and pulp among citrus fruits ($F_{(7, 40)} = 232.910$; $p<0.001$). A Tukey post hoc test was performed to analyse if there is any significant difference between different citrus fruits tissues, pulp, and peal (Table 2).

Table 1. Citrus fruits index from the region of Vlora, Albania. The value of total mineral content (TMC), total phenolic content (TPC) and total anthocyanin content (TAC) in each citrus fruits are express in mean ± Std. Dev. measured as mg/ml samples for TMC, TPC, TAC and in % for the total moisture (TM)

Index	Tissues	*Citrus sinensis* Orange	*Citrus limon* Lemon	*Citrus reticulata* Mandarin	*Citrus paradisi* Grapefruit
TM	Peal	74.2700±0.75551	77.4533±0.87191	67.6517±1.07669	78.3967±0.89021
	Pulp	91.4117±0.86057	92.4000±0.85746	84.1067±0.51640	94.6800±0.69977
TMC	Peal	$0.0822±0.00615^{b}$	$0.1217±0.01169^{b}$	$0.6600±0.43973^{a}$	$0.2033±0.01033^{b}$
	Pulp	0.3217±0.29034	$0.0663±0.00403^{b}$	$0.0462±0.00360^{b}$	$0.0812±0.03508^{b}$
TPC	Peal	$1.08433±0.003011^{bcd}$	$1.38267±0.003077^{bc}$	$0.87400±0.003225^{bcde}$	$1.76267±0.002658^{b}$
	Pulp	$0.95550± 0.004637^{bcde}$	$1.88150±0.014639^{a}$	$1.08700±0.032961^{bcd}$	$0.86050±0.021230^{bcde}$
TAC	Peal	0.15200±0.002757	0.13300±0.005477	0.06283±0.005345	0.19500±0.004382
	Pulp	0.16583±0.237196	$0.23733±0.004502^{a}$	0.08750±0.004324	$0.05883±0.004622^{b}$

*All Pairwise Multiple Comparison Procedures with SigmaPlot 12.5 (Tukey post hoc Test) show a statistical significative difference ($P<0.01$) between parameters analysed in different tissues of citrus fruits.

Table 2. Citrus fruits index from the region of Saranda, Albania. The value of total mineral content (TMC), total phenolic content (TPC) and total anthocyanin content (TAC) in each citrus fruits are express in mean ± Std. Dev. measured as mg/ml samples for TMC, TPC, TAC and in % for the total moisture (TM)

Index	Tissues	*Citrus sinensis* Orange	*Citrus limon* Lemon	*Citrus reticulata* Mandarin	*Citrus paradisi* Grapefruit
TM	Peal	76.437 ± 1.312	82.953 ± 1.950	68.318 ± 1.616	82.397 ± 2.331
	Pulp	92.912 ± 1.511	93.067 ± 0.5166	84.440 ± 0.820	95.347 ± 1.273
TMC	Peal	$0.0983 ± 0.0147^{b}$	0.128 ± 0.0117	$0.515 ± 0.452^{a}$	0.217 ± 0.0186
	Pulp	$0.0628 ± 0.00560^{b}$	$0.0817 ±0. 0117^{b}$	$0.0467 ± 0.0137^{b}$	$0.118 ± 0.0204^{b}$
TPC	Peal	$1.118 ± 0.0189^{bde}$	$1.402 ± 0.0990^{bd}$	$0.973 ± 0.0116^{bde}$	$1.918 ± 0.0797^{c}$
	Pulp	$1.007 ± 0.0451^{bde}$	$1.974 ± 0.0460^{a}$	$1.198 ± 0.00662^{bde}$	$0.929 ± 0.0388^{bde}$
TAC	Peal	$0.171 ± 0.0112^{bc}$	$0.143 ± 0.0116^{bcd}$	$0.0738 ± 0.00725^{bcde}$	$0.212 ± 0.0125^{b}$
	Pulp	$0.0817 ± 0.00763^{bcde}$	$0.252 ± 0.0133^{a}$	$0.0882 ± 0.00479^{bcde}$	$0.0758 ± 0.0159^{bcde}$

*All Pairwise Multiple Comparison Procedures with SigmaPlot 12.5 (Tukey post hoc Test) show a statistical significative difference ($P<0.001$) between parameters analyzed in different tissues of citrus fruits.

The total vitamin A and C in both peal and pulp was quantified based on the method described above. The data show that the value for total vitamin A and C are higher in peal than pulp for all the citrus species and for both regions where the citrus fruits are harvested. According to our findings, the peel in general contains more polyphenols and vitamin C than other fruit sections, resulting in increased antioxidant activity. The results are comparable with the finding published for citrus fruits that grow in Cyprus, Greece (Goulas & Manganaris, 2012) with one exception, despite the high levels of TPC in the peal of grapefruits, our findings show that even the pulp of the *C. limon* and *C. reticulata* has the highest content of total polyphenols and the strongest antioxidant activity. We assume, based also on the

literature, that the presence of the presence of seed in dried pulp in samples had a significant impact in increasing the value of TPC in this tissue (Elkhatim et al 2018; Costanzo et al., 2020). The highest cumulative capacity to scavenge free radicals was measured in the peal of *C. paradisi* (87%) followed by the value of the pulp in *C. limon* (86%) and in the pulp of *C. reticulata* (75%). The lowest cumulative capacity to scavenge free radicals was measured in the peal of *C. reticulata* (62%). Statistical analyses of the data with SPSS show a clear correlation (Pearson 2-tailed) between TPC and antioxidant activities. Correlation is significant at the 0.05 level (2-tailed) between total phenolic content in peal of *C. paradisi* and antioxidant activities ($R^2 = 0.859$, $p = 0.028$) as it is a significant correlation at 0.01 level between total phenolic content in peal of *C. limon* and antioxidant activities ($R^2 = 0.929$, $p = 0.007$).

Table 3. Citrus fruits index from the region of of Vlora and Saranda, Albania. The value of total Vitamin A nd Vitamin C content, in each citrus fruits are express in mean ± Std. Dev. measured as mg/ml samples

	Fruits index	Tissues	***Citrus sinensis*** Orange	***Citrus limon*** Lemon	***Citrus reticulata*** Mandarin	***Citrus paradisi*** Grapefruit
Vlora	Total Vit. A	Peal	3.512 ± 0.134^{bc}	5.067 ± 0.134^{b}	5.500 ± 0.0754^{a}	2.460 ± 0.180^{bc}
		Pulp	0.565 ± 0.0579^{bc}	1.507 ± 0.0147^{bc}	1.628 ± 0.0155^{bc}	0.491 ± 0.0454^{bc}
	Total Vit. C	Peal	0.384 ± 0.0141^{b}	0.313 ± 0.0235^{bc}	0.341 ± 0.0102^{bc}	0.419 ± 0.0124^{a}
		Pulp	0.255 ± 0.00792^{bcd}	0.287 ± 0.00775^{bcd}	0.261 ± 0.00987^{bcd}	0.279 ± 0.00770^{bcd}
Saranda	Total Vit. A	Peal	3.930 ± 0.228^{b}	5.882 ± 0.506^{a}	5.985 ± 0.468^{a}	2.890 ± 0.157^{bc}
		Pulp	0.525 ± 0.0959^{bcd}	1.475 ± 0.0426^{bcd}	1.672 ± 0.0387^{bcd}	0.508 ± 0.0655^{bcd}
	Total Vit. C	Peal	0.398±0.0255b	0.338 ± 0.0412^{b}	0.341 ± 0.0486^{b}	0.484 ± 0.0407^{a}
		pulp	0.263 ± 0.0128^{bcd}	0.308 ± 0.0273^{bc}	0.282 ± 0.0250^{bc}	$0.283 \pm 0.0104b^{c}$

*All Pairwise Multiple Comparison Procedures with Sigma Plot 12.5 (Tukey post hoc Test) show a statistical significative difference ($P<0.001$) between parameters analysed in different tissues of citrus fruits.

To the best of our knowledge, this is the first report indicating the bioactive compound profile of four citrus fruits species that grown in south region of Albania along with antioxidant activities in different tissues of these fruits. The data included in this report indicate a high presence of TMC, TPC, TAC, vitamin C and A in both types of tissues analyzed. In general, from the two regions of southern Albania where the samples were collected, the highest values of the aforementioned parameters were identified in grapefruit samples, followed by lemon fruit samples.

Regardless of the data presented in this manuscript, further studies are warranted in the near future to have a full understanding of the bioactive compounds founds in citrus fruits that grow in south Albania region.

CONCLUSIONS

This study was carried out in four citrus fruits species that grow in south Albania region. The data showed a significant difference in the distribution of bioactive compounds between peal and pulp. When compared to other citrus fruits, grapefruit has a higher total phenolic and total anthocyanin content in its peel with excellent scavenging properties. Lemon and mandarin fruits have higher total phenolic content in their pulp. Total vitamin A and total vitamin C was significantly higher in peal in all citrus fruit's species. The value for the bioactive compounds, total mineral content and total vitamin A and C extracted from peal and pulp of citrus species that grow in south Albania region are comparable to findings from previous citrus research studies in other countries. Another result of this study is that we demonstrated the presence of the bioactive compounds in the waste products of the citrus juice producing industry. This indicates that these by-products can be used in various ways in order to exploit the presence of the nutrients in it.

The data presented in this research are very encouraging, nevertheless, the exploitation of citrus by-products in food industry is yet premature. Therefore, further research and

studies in citrus fruits that grow in Albania are needed before conducting these natural resources from waste to the food industries.

ACKNOWLEDGMENTS

The authors wish to thank the Laboratory of Food Science in the Faculty of Biotechnology and Food, Agricultural University of Tirana, Albania for technical support and for providing the materials, reagents and equipment's used for the experiments.

REFERENCES

Barros, H. R. M.; Ferreira, T. A., & Genovese, M. I. (2012). Antioxidant capacity and mineral content of pulp and peel from commercial cultivars of citrus from Brazil, *Food Chemistry,* 134 (4), 1892–1898, https://doi.org/10.1016/j.foodchem.2012.03.090

Casas Cardoso, L., Cejudo Bastante, C., Mantell Serrano, C., & Martínez de la Ossa, E. J. (2022). Application of Citrus By-Products in the Production of Active Food Packaging. *Antioxidants* (Basel, Switzerland), 11(4),738. https://doi.org/10.3390/antiox11040738.

Chavan, P., Singh, A.K., & Kaur, G. (2018). Recent progress in the utilization of industrial waste and by-products of citrus fruits: A review. *Journal of Food Process Engineering,* 41 (2): e12895. https://doi.org/10.1111/jfpe.12895

Costanzo, G.; Iesce, M.R.; Naviglio, D.; Ciaravolo, M.; Vitale, E., & Arena, C. (2020). Comparative Studies on Different Citrus Cultivars: A Revaluation of Waste Mandarin Components. *Antioxidants*, 9 (6): 517. https://doi.org/10.3390/antiox9060517

Czech, A.; Malik, A.; Sosnowska, B., & Domaradzki, P. (2021). Bioactive Substances, Heavy Metals, and Antioxidant Activity in Whole Fruit, Peel, and Pulp of Citrus Fruits, *International Journal of Food Science*, 2021, 6662259. https://doi.org/10.1155/2021/6662259

Di Stefano, R.; Cravero, M.C., & Gentilini, N. (1989). Metodi per lo studio dei polifenoli dei vini. *L'enotecnico*, 5, 83-90.

Elfalleh, W.; Hannachi, H.; Tlili, N.; Yahia, Y.; Nasri, N., & Ferchichi, A. (2012). Total phenolic contents and antioxidant activities of pomegranate peel, seed, leaf and flower. *Journal of Medicinal Plant Research*, 6: 4724e30.

Elkhatim, K.A.S., Elagib, R.A.A., & Hassan, A.B. Content of phenolic compounds and vitamin C and antioxidant activity in wasted parts of Sudanese citrus fruits, *Food Science & Nutrition*, 6, 1214–1219. https://doi.org/10.1002/fsn3.660

Eurostat (2018). Crop production in EU standard humidity.https://appsso.eurostat.ec.europa.eu/nui/submitViewTableAction.do

FAOSTAT (2018) Crop and livestock production, 2018. https://www.fao.org/faostat/en/#data/QCL

Goulas, V., & Manganaris, G. A. (2012). Exploring the phytochemical content and the antioxidant potential of Citrus fruits grown in Cyprus. *Food Chemistry*, 131, 39-47. https://doi.org/10.1016/j.foodchem.2011.08.007

INSTAT (2020). Agriculture Statistics report, 2020, http://www.instat.gov.al/en/themes/agriculture-and-fishery/agriculture/publication/2021/agriculture-statistics-2020/

Liu, Sh., Lou, Y., Li, Y., Zhang, J., Li, P., Yang, B., & Gu, Q. (2022). Review of phytochemical and nutritional characteristics and food applications of Citrus L. fruits, Frontiers in Nutrition, 9 (2022), https://doi.org/10.3389/fnut.2022.968604

Lloha, I.; Peçuli, A.; Hoxha, M., & Mamoçi. E. (2015). Evaluation of the dried pulp and peel composition of several citrus family fruits growing in Albania, *The 3rd International Conference on Research and Educatıon - "Challenges toward the Future",* Shkoder, Albania, October 23-24, 2015.

Lv, X.; Zhao, S.; Ning, Z.; Zeng, H.; Shu, Y.; Tao, O.; Xiao, C.; Lu, C., & Liu, Y. (2015). Citrus fruits as a treasure trove of active natural metabolites that potentially provide benefits for human health. *Chemistry Central Journal*, 9: 68, https://doi.org/10.1186/s13065-015-0145-9

Malacrida, C. R.; Kimura, M., & Jorge, N. (2012) Phytochemicals and Antioxidant Activity of Citrus Seed Oils, *Food Science and Technology Research*, 18 (3), 399–404. https://doi.org/10.3136/fstr.18.399

Medina-Remón, A., Kirwan, R., Lamuela-Raventós, R. M., & Estruch, R. (2018). Dietary patterns and the risk of obesity, type 2 diabetes mellitus, cardiovascular diseases, asthma, and neurodegenerative diseases. Critical reviews in food science and nutrition, 58(2), 262–296. https://doi.org/10.1080/10408398.2016.1158690

Rafiq, S., Kaul, R., Sofi, S.A., Bashir, N.,Nazir, F., & Nayik, G.A. (2018). Citrus peel as a source of functional ingredient: a review. *Journal of the Saudi Society of Agricultural Sciences,* 17, 351–358,

Rutkowski, M., & Grzegorczyk, K. (2007). Modifications of spectrophotometric methods for antioxidative vitamins determination convenient in analytic practice, *Acta Scientiarum Polonorum, Technologia Alimentaria.* 6(3), 17-28.

Saini, R. K., Ranjit, A., Sharma, K., Prasad, P., Shang, X., Gowda, K. G. M., & Keum, Y. S. (2022). Bioactive Compounds of Citrus Fruits: A Review of Composition and Health Benefits of Carotenoids, Flavonoids, Limonoids, and Terpenes. *Antioxidants* (Basel, Switzerland), 11(2), 239. https://doi.org/10.3390/antiox11020239

Wu, G.A., Terol, J., Ibanez, V., Lopez-Garcia, A., Perez-Roman, E., Borreda, C., Domingo, C., Tadeo, F.R., Carbonell-Caballero, J., Alonso, R., et al. (2018). Genomics of the origin and evolution of Citrus. *Nature,* 554, 311–316. doi: 10.1038/nature25447

Zou, Z., Xi, W., Hu, Y., Nie, C., & Zhou, Z. (2016). Antioxidant activity of Citrus fruits. Food chemistry, 196,885–896. https://doi.org/10.1016/j.foodchem.2015.09.072

Scientific Bulletin. Series F. Biotechnologies, Vol. XXVII, No. 2, 2023
ISSN 2285-1364, CD-ROM ISSN 2285-5521, ISSN Online 2285-1372, ISSN-L 2285-1364

BIOPOLYMERS: TYPES AND THEIR POTENTIAL FOR USE IN VARIOUS FIELDS OF BIOMEDICAL AND COSMETIC ENGINEERING

Nicoleta ENE[1, 2], Irina LUPESCU[3], Emanuel VAMANU[1]

[1]Faculty of Biotechnologies, University of Agronomic Sciences and Veterinary Medicine of Bucharest, 59 Marasti Blvd, District 1, Bucharest, Romania,
[2]National Institute for Chemical Pharmaceutical Research and Development-ICCF, Vitan Avenue 112, 031299, Bucharest, Romania
[3]Department of Veterinary Medicine, Spiru Haret University, 256 Basarabia Blvd, 030352, Bucharest, Romania

Corresponding author email: email@emanuelvamanu.ro

Abstract

In recent years, biopolymers have attracted the attention of specialists and researchers in various fields, including biotechnology, materials science, engineering, and medicine. The main driver is the possibility of combining scientific and technological progress with sustainability. This is a vast research topic because there are several classes and varieties of biopolymers. Polyhydroxyalkanoates (PHAs), a specific type of polyesters, are biodegradable polymers with various thermoplastic characteristics formed by microorganisms under adverse growth conditions. In recent years, PHAs have been increasingly employed in biomedical applications owing to their adaptive mechanical features, cytocompatibility, capacity for cell adhesion, and controlled biodegradability. The increasing potential is also indicated by the benefits of 3D-printing technology for fabricating intricate structures, fast prototyping, and personalization. This study aims to detail the types of biopolymers, as well as their areas of use, after a thorough description of the synthesis and manufacture of PHAs. The most recent and significant medical applications of PHAs in tissue engineering, medication delivery, and vascular stenting are listed below.

Key words*: Polyhydroxyalkanoates, biomedical uses, medical devices, biodegradability.*

INTRODUCTION

Polyhydroxyalkanoates (PHAs), a specific class of polyesters, are biodegradable polymers with various thermoplastic characteristics formed by microorganisms under adverse growth conditions (Rodriguez-Perez et al., 2018; Angra et al., 2023). In recent years, PHAs have been increasingly employed in biomedical applications owing to their adaptive mechanical characteristics, cytocompatibility, capacity for cell adhesion, and controlled biodegradability. The benefits of 3D-printing technology also show increasing potential for fabricating intricate structures, fast prototyping, and personalization (Kovalcik, 2021; Cecen, 2023).
In this study, we list the most recent and important medical applications of PHAs in tissue engineering, drug delivery, and vascular stenting. Recently, biopolymers have attracted the attention of specialists and researchers in various fields, such as biotechnology, material science, engineering, and medicine (Rehakova et al., 2023; Kourmentza et al., 2017). The main reason is the possibility of combining scientific and technological progress with sustainability (Koller et al., 2017). Biopolymers can be divided into many classes and variations, making them a broad field of research.
Biofuels are recommended, special mainly because they are environmentally beneficial, renewable, easy to use, and less dependent on petroleum (Dilkes-Hoffman et al., 2018). However, the production costs of biofuels are higher compared to fossil fuels. For this reason it is important to find alternative fuels with lower production costs (Angra et al., 2023). Therefore, the development of suitable production processes is necessary.

1. BIOPOLYMERS

Biopolymers can be obtained in different ways: through bacterial biosynthesis, or through different processes, such as syntheses chemicals from renewable natural materials (polyesters

from lactic acid - obtained by fermentation starting from starch).
They can be obtained from microbial systems, extracted from plants (e.g., corn, soybeans, and various trees), or chemically synthesized from basic biological systems. They have attracted particular interest from the scientific community owing to their wide range of properties and potential uses (Subash et al., 2023). Biopolymers are chain molecules composed of repetitive monomer-building units. They are being materials with specific properties, such as biocompatibility, biodegradability, low antigenicity, high bioactivity, processability to complicated forms with adequate porosity, capacity to support cell growth and proliferation, and adequate mechanical properties (Chai & Isa, 2013). Biopolymers can be used for biomedical applications such as wound healing acceleration or drug carrier.
These characteristics have stimulated research on their potential applications in the production of biosensors, absorbents, packaging, cosmetics, food, electronics, medical devices, and biofuels (Bugnicourt et al., 2014; Keskin et al., 2017; Koller & Mukherjee, 2022). They might be polynucleotides, polypeptides (short-chain monomer polymers), or polysaccharides (linear polymeric carbohydrate structures), depending on the monomer unit in the biopolymer structure.
Biopolymers (Table 1) are abundant in nature and come from various sources.

Table 1. Types of biopolymers and areas of use.

Biopolymers	Resources	Uses	References
Polylactide (PLA)	Renewable resources	Implant devices, stents, vascular prostheses	Da Silva et al., 2018; Amnieh et al., 2021; Blume et al., 2022
Poly-lactic-co-glycolic acid (PLGA / PLG)	Fossil resources	Regenerate bone tissues Tissue/organ deficiencies	Alsaab et al., 2022; Yoo & Won, 2020; Huo et al., 2019
Polycaprolactone (PCL)	Fossil resources	Drug-controlled applications Implants	Dash & Konkimalla, 2022; Baghersad et al., 2022
Poly(3-hydroxybutyrate-co-3-hydroxyvalerate) – PHBV	Renewable resources (microbial polymer)	Packaging Agriculture Medical industries Medical implants	Ponjavic et al., 2023; Kaniuk & Stachewicz, 2021

For example, there are biopolymers obtained from natural sources that are classified in: 1) polysaccharides (starch, cellulose, pectin, chitosan) and 2) proteins and lipids (gluten, soy, casein, gelatin, collagen). Another type of biopolymers are obtained from renewable sources: 1) microbial polymers (polyesters, polyhydroxyalkanoates, pullulan, curdulan) and 2) natural polymers (polylactic acid). The third type of biopolymers are obtained from fossil resources, such as polyvinyl alcohol or aliphatic and aromatic polymers (PGA, PCL, PVA etc.).

1.1. Biodegradable synthetic polymers

- ***Polylactide (PLA)***

Polylactic acid (PLA) is obtained by converting carbohydrates into lactic acid. This process is often used in the food industry (e.g., milk industry, wine production, meat industry, obtaining fermented plant products) (Da Silva et al., 2018). Due to the increased interest the need to replace conventional plastic materials, the development of PLA production bioprocesses has increased significantly. In 2020, approximately 1 million tons of PLA were produced $2 / kg (Rajendran & Han, 2023). Lactic bacteria (LAB) are bacteria that ferment, and are used to produce PLA. *LAB* are gram-positive *Bifidobacterium*, *Enterococcus*, *Lactobacillus*, *Aerococcus*, *Streptococcus*, etc. (Wang et al., 2021).
Polylactic acid (PLA) is an aliphatic polyester with thermoplastic properties. To obtain it you can use various natural raw materials such as rice, corn starch, potatoes, sugar cane, etc. Compared to other biopolymers, PLA has the benefit of relatively low manufacturing costs and is biodegradable (Amnieh et al., 2021). It also exhibits mechanical qualities equivalent to synthetic polypropylene polymers (DeStefano et al., 2020). Furthermore, PLA is a bioabsorbent that may provide advantages over implanted devices (Da Silva et al., 2018).
For instance, in the case of stents, the biopolymer can biodegrade into body fluids after being utilized as an intravascular dilator, preventing the need for a second surgery to remove the stent. Studies show encouraging results after testing biodegradable stents in human models (Soares et al., 2010). Positive findings were also obtained in a study that examined the biodegradation of PLA stents in

the rabbit aorta (Yang et al., 2023). Polyglycolic acid-based PLA-PGA copolymer-based devices have been used in orthopedic applications. Copolymers compressed into plates or screws have been used to repair fractures and fill in bone deformities (Castañeda-Rodríguez et al., 2023). A technique for making woven wavy vascular prostheses made of PLA and polyethylene terephthalate (PET) that restores blood flow in damaged blood artery segments was patented by Rebelo et al. (2017). These prostheses are particularly useful for vascular surgery. To create a system in which the human body may absorb PLA while the other offers mechanical support, these two biocompatible wires have been employed.

PLA has been employed extensively in tissue engineering applications, including bone support (Zhang et al., 2017), cartilage, tendons, and neurons (Schedin-Weiss et al., 2017), and vascular regeneration. Because PLA resorbs (in roughly 4-6 years) and resembles the bone structure in appearance, it has significant potential for bioactivity in mending bone fractures (Zhang & King, 2020).

- ***Poly-lactic-co-glycolic acid (PLGA / PLG)***

Polylactic-co-glycolic acid polyester consists of poly-lactic acid (PLA) and poly-glycolic acid (PGA). Owing to its biocompatibility, biodegradation rate, and capacity to alter surface characteristics to ensure better interaction with biological materials, polylactic-co-glycolic acid (PLGA) is frequently utilized as a primary material for biomedical applications. An examination of the state of the art in this area indicates the existence of novel techniques for fabricating PLGA-based biomimetic supports that can alter cell interactions for better replacement, repair, or enhancement of bone tissue function (Alsaab et al., 2022). PLGA is primarily utilized to regenerate bone tissues and exists in several forms, including microspheres, hydrogels, and porous supports. However, poor osteoconductivity prevents the therapeutic use of pure PLGA in bone repair.

To make PLGA more biomimetic, it is frequently combined with other materials, such as ceramics or bioactive glass, or suitably changed. From a structural standpoint, lactic acid and glycolic acid, the two monomers constituting PLGA, can be combined in various ratios to create a linear copolymer. PLGA supports are now employed to heal tissue/organ deficiencies in the skin (Pathan & Shende, 2021), blood vessels (Han et al., 2011), liver (Ayhan & Ayhan, 2017), and bones (Sharma et al., 2016). Over time, the biopolymer structure was also altered. To considerably enhance tissue regeneration, medications or proteins, in particular growth factors or genes that express growth factors, have been incorporated into porous PLGA supports (Wang et al., 2023).

- ***Polycaprolactone (PCL)***

Polycaprolactone (PCL), an aliphatic polyester prepared using petrochemical processes, is a biocompatible synthetic polymer. It is appropriate as an implantable biomaterial despite its poor mechanical stiffness because its breakdown products do not induce inflammation in the human body (Nejati-Koshki et al., 2017). PCL is frequently employed in drug-controlled applications and long-term implants (which scarcely deteriorate) (Irani et al., 2017). The Food and Drug Administration (FDA) has provided a green light for using PCL-based media in human applications, which are primarily employed in cell therapy (Kamath et al., 2014). The PCL biopolymer increases the production of collagen, making it possible to treat early signs of aging, such as loss of skin elasticity (Dash & Konkimalla, 2022).

Polyacrolactone is used as a composite material in dental medicine to fill root canals and PCL and PLA stents for the treatment of cardiovascular diseases (Guerra et al., 2018). PCL ensures the proliferation of endothelial vessel cells in the outer wall of the stent, while the internal PLA wall prevents the accumulation of cells that cause restenosis.

1.2. Biodegradable natural polymers

- ***Polyhydroxyalkanoates (PHAs)***

Biopolymers of bacterial origin are an exciting area of research with numerous potential applications. These biopolymers are produced by bacteria and are composed of natural macromolecules such as polysaccharides, proteins, and polyesters. One of the most well-known biopolymers of bacterial origin is polyhydroxyalkanoates (PHAs), which are biodegradable and biocompatible polyesters that are synthesized by bacteria as a form of carbon and energy storage (Silva et al., 2012; Krumnow et al., 2009; Kaur & Sharma, 2023).

Polyhydroxyalkanoates (PHA) are a group of biodegradable polymers that have gained considerable attention recently due to their potential use as an alternative to conventional, petroleum-based plastics. A wide range of microorganisms produces PHA polymers and are fully biodegradable under various environmental conditions (Kourmentza et al., 2017; Sabapathy et al., 2020). One of the main advantages of PHA is their ability to biodegrade in water and soil (Koller & Mukherjee, 2022). Microorganisms biodegrade PHA that break down the polymer chains into simpler compounds that can be used as a food source. In water, PHA is degraded more slowly than in soil due to lower microbial activity and other organic matter, such as dissolved organic carbon (Koller, 2018).

However, the biodegradability of PHA in water and soil can be affected by various factors, such as temperature, pH, nutrient availability, and oxygen levels. For example, PHA biodegrades more rapidly in warm, moist environments with high microbial activity and nutrient availability, whereas colder and drier conditions may slow the process (Roy & Visakh, 2014).

PHA biopolymers occur naturally and are produced from renewable carbon sources. They are made as intracellular granules that serve as energy and carbon reserves absorb electrons, protect against stress, and provide cells with a means of survival under stressful conditions (Somleva et al., 2013). A variety of microbial species produces them. PHAs are made as a precaution against nutrient limitation and extreme conditions) (Koller, 2018). PHA accumulates when the culture medium contains too much carbon, but cell development is limited by nitrogen, phosphorus, oxygen, or magnesium.

About 150 different hydroxyalkanoic acids have been identified as PHA constituents, leading to the production of many polyesters of bacterial origin. These polymers can be used in pure form or as additives in petrochemical plastics, such as polyethylene, and have a wide range of applications (packaging materials, including films, boxes, coatings, fibers, and foams); however, they are handy for medical applications because they are fully biodegradable thermoplastic biomaterials that are environmentally friendly and biocompatible (Morgan-Sagastume, 2016). PHA can be used as a material for implanted devices because it degrades slowly and does not cause an immune response in humans (Vicente et al., 2023).

- ***Poly(3-hydroxybutyrate-co-3-hydroxyvalerate) – PHBV***

Polyhydroxybutyrate-valerate (PHBV) is a biopolymer that has attracted attention due to its potential use as a biodegradable plastic. PHBV comprises two monomers, 3-hydroxybutyrate (3HB) and 3-hydroxyvalerate (3HV). It is biodegradable, compostable, and has similar mechanical properties to conventional plastics. PHBV has potential applications in packaging, agriculture, and medical industries, which can be used in medical implants (Ponjavic et al., 2023). The creation of medical devices implanted for dental, orthopedic, thermoplastic, and skin surgeries is one area where PHBV has various uses (Bolbasov et al., 2017). This biopolymer has created numerous medical devices, including PHBV-coated surgical meshes for thermoplastic surgery and wound coatings. Other potential medical devices include bioresorbable surgical sutures, screws, plates for fixing cartilage and bone, biodegradable membranes for periodontal treatment (Chen et al., 2020), and biodegradable membranes.

1.2.1. PHA producing microorganisms

The standard strain described as producing PHA is Bacillus sp. It is the first strain in which Maurice Lemoigne observed PHA accumulation (Gilmore et al., 1990). The carbon source used for microbial growth influences the formation of homopolymers and copolymers of acids [R] -hydroxyalkanoic.

To date, Gram-positive and Gram-negative bacteria from approximately 300 species have been found to produce polyhydroxyalkanoates (Awasthi et al., 2022; Westlie et al., 2022). The most common are Alcaligenes latus, Bacillus megaterium, Cupriavidus necator, and Pseudomonas oleovorans, which can use a variety of carbon sources, such as plant oils or wastes, to do so (Meereboer et al., 2020). PHAs are thus produced from a wide range of substrates, including organic acids, methane, cellulose, and by-products such as molasses and whey, as well as renewable resources such as starch and glycerol (Kiselev et al., 2020; Hafis et al., 2023; Koller & Obruča, 2022).

Many PHA-producing strains are easy to isolate because they come from natural sources, e.g., soil, sea, plants, estuaries, salt rock from mountains, etc. The bioprocess development must occur under certain optimal conditions that meet the needs of the bacterial strain used (Madkour et al., 2013). These conditions include temperature, pH, salinity, carbon substrates, and concentrations of organic or inorganic matter. Recent studies have reported that mesophilic and extremophilic microorganisms can accumulate PHA (Koller, 2017; Kourilova et al., 2021). Halomonas sp. is a strain isolated from marine waters that produce PHA, as mentioned in the Abd El-malek et al. (2020) study. A novelty is the isolation of PHA-producing strains from polluted environments such as oil fields - Aneurinibacillus thermoaerophilus (Musilova et al., 2022; Xiao et al., 2015).

For the industrial production of PHA, species have been sought out that can grow to a final high cell density from basic and inexpensive substrates in a relatively short amount of time and with a high PHA content. As a result, the research focused on Pseudomonas species that fit the requirements above (Khanna & Srivastava, 2005). PHAs develop up as inclusions or granules that are typically above 100 monomers in number and range in size from 0.2 to 0.5 m. These granules are produced and preserved without endangering the bacteria that produce them (Arumugam et al., 2020). Because this form of polymer slows down the autolysis of cells and, ultimately their mortality, it has been demonstrated that bacteria using PHA as spare materials may survive longer during food-limited availability than bacteria without PHA. Additionally, PHA-producing bacteria have shown enhanced resistance to brief environmental stresses such as ultraviolet (UV) light (Slaninova et al., 2018), heat (Sun et al., 2018), and osmotic shock (Sedlacek et al., 2019).

2. BIOMEDICAL ENGINEERING

Bioengineering is an essential field in which biopolymers are preferred. Applying engineering and design concepts and techniques to problems with medical implications is the focus of interdisciplinary scientific disciplines known as biomedical engineering (Huang et al., 2021). To prevent, diagnose, and treat a wide range of diseases and improve human care's quality of life and safety, biomedical engineering is concerned with tools and devices that can significantly improve the delivery of medical interventions from diagnosis to monitoring or treatment (Egbo, 2021).

Biomedical engineering combines the knowledge of engineering with biomedical sciences (such as biomedical electronics, biomaterials, computational biology, cell, tissue, and genetic engineering, medical imaging, orthopedic bioengineering, and bionanotechnology) and clinical practice to develop breakthrough concepts for surgical robots (Rosen et al., 2011), biocompatible prostheses (Shepherd, 2016), new therapeutic drug systems (Ghadi et al., 2014), various medical diagnostic and therapeutic devices, stem cell engineering (Kwon et al., 2018), and printing of biological organs in three dimensions (Agarwal et al., 2023). Tissue and check cell experts aim to duplicate human organs (Wu et al., 2020) and improve the lives of millions of people by perfecting transplants. New implanted devices, including pacemakers, coronary stents, orthopedic implants, prostheses, and dentures, are being developed by experts (Ward et al., 2013; Liu et al., 2023). The use of medical devices in clinical settings requires both reliability and safety. Finally, developments and progress in chemistry, materials science, and biology are linked to the future of biomedical engineering.

Several areas of specialization in biomedical engineering are presented below:

(a) Bioinformatics is the field of understanding biological data using digital technology. It combines disciplines such as engineering, mathematics, statistics, and computer science. With the help of bioinformatics, it is possible to study genes, nucleotide polymorphisms, and single nucleotide polymorphisms (SNP) and determine how genetics and genetic adaptations affect different populations (especially in agriculture). Bioinformatics can provide databases to analyze biodegradation (Bionemo). Therefore, some databases (MetaCyc and BioCyc) help researchers obtain information about the biochemistry and genetics of microbial degradation

(Arora & Bae, 2014). This branch can also be used to predict the toxicity of some chemical substances. Toxicity can negatively influence the bioprocess of polymer production (Buchholz et al., 2022).

(b) Tissue engineering is a rapidly evolving discipline that uses laboratory-assisted manufacturing of organs and tissues to shorten the waiting time for patients needing organ or tissue transplantation. To be used in tissue engineering, biopolymers must be biodegradable, allow cell proliferation, and not cause an immune response in the host organism (Liu et al., 2023). Also, only polymers with mechanical properties and a suitable surface for the place where they will be inserted are used. Some studies show the wound healing effect using PHAs (Kaniuk & Stachewicz, 2021). Researchers have developed bioartificial organs compatible with host organisms by combining synthetic and biological components (Pulingam et al., 2022; Pryadko et al., 2021).

(c) Genetic engineering is a term that characterizes the newly developed field of recombinant DNA technology. Recombinant DNA technology began with the successful cloning of tiny DNA fragments. It evolved into a broad area in which whole-genome cloning, the genome transfer from one cell to another, was possible. This was achieved using various genetic techniques, such as molecular cloning or eyelash splitting. Gene manipulation using biotechnology is led by genetic engineering, often called genetic modification or gene manipulation. The production of human insulin using genetically modified bacteria or the production of erythropoietin in hamster ovaries both have a role in research thanks to techniques and knowledge in the field (Kaparapu, 2018; Saratale et al., 2021). The PHA biosynthesis process itself is controlled by genetic engineering. Many producing microorganisms are genetically modified to use different carbon substrates or increase their PHA production. Another genetic modification that can be made is the addition or increase of the effect of some enzymes with a role in biosynthesis: β-keto thiolase (phaA), acetoacetyl-CoA reductase (phaB), and PHB-polymerase (phaC). The genome of one bacterium can be transferred to another to optimize the PHA production process (Kaparapu, 2018; Wand et al., 2023).

(d) Neural engineering is a discipline in which scientists, doctors, engineers, and neurologists work to comprehend, interfere with, and modify the neurological system. To understand the complexity of the nervous system, neural engineering has been used to study the communication of neurons while employing various quantitative approaches for recording synapses. Treatments for people with different neurological conditions, such as stroke or epilepsy, can be developed using neural engineering (Kaniuk & Stachewicz, 2021). By adding neural stem cells (NSCs) to biopolymers, neural engineering is possible (Huang & Wang, 2017). NSCs can develop into neurons, astrocytes, or oligodendrocytes and can self-renew. The ability of these cells (neural stem cells) to replace or repair damaged neural cells is crucial. Therefore, several neurological illnesses like Parkinson's, Alzheimer's, or focal ischemia can be controlled by combining some biopolymers with NSCs (Zarrintaj et al., 2023; Irioda et al., 2021; Grochowski et al., 2018).

(e) Pharmaceutical engineering. This field focuses on the planning, building, and maintaining drug-manufacturing facilities. Drug variations are created using carefully managed synthetic chemical processes, standardized labor procedures, and the proper safety gear (Koller, 2018).

2.1. Medical devices

In medical devices, PHA can be used in the production of implants and sutures and in coatings for various medical instruments. PHA's biocompatibility, biodegradability, and mechanical properties make it an ideal material for these applications (Table 2). Furthermore, depending on the desired application, PHA can be tailored to have specific properties, such as flexibility or strength (Dwivedi et al., 2020; Elmowafy et al., 2019). Instruments, equipment, implants, machines, tools, reagents, or other similar objects are considered medical devices used to identify, treat, facilitate, or cure a disease or condition. Medical devices, such as

pacemakers, infusion pumps, implants, lenses, and ocular or facial prostheses, achieve their aims by physical, structural, or mechanical activity instead of chemical or metabolic action within or on the body. The Food and Drug Administration (FDA)* assesses medical technology based on patient risk, with higher-risk products and lower-risk things.

Table 2. The medical field and possible uses of biopolymers

Medical field	Uses	References
Bioinformatics	Biochemistry and genetics of microbial degradation	Boyandin et al., 2013
Tissue engineering	Bioartificial organs	Jose et al., 2022
Genetic engineering	Enzymatic modification Genome modification (genomic recombination)	Syed Mohamed et al., 2022
Neural engineering	Improvement of neurological diseases Parkinson's, Alzheimer's or focal ischemia.	Bhatia et al., 2021
Medical devices	Pacemaker, infusion pump, implants, lenses, ocular, facial prosthetics	Ward et al., 2013; Liu et al., 2023
Cosmetic industry	PHA microplastic Mild cleaning products	Gupta et al., 2022; Acharjee et al., 2023

According to the level of risk with which they are connected, the EU or US clearance authorities have regulated and divided medical devices into three classes: devices classified as Class I are low risk and do not need to send data or information to the FDA. Elastic bandages, examination gloves, hand surgical tools, and other commonly used items in this category are typically utilized as mechanical barriers. Wheelchairs, ophthalmic lasers, and hemodialysis catheters are examples of class II devices, which pose a moderate risk and often come into contact with the skin or mucous membranes. Class III devices are cutting-edge, high-risk goods. They can affect health by releasing chemicals. These gadgets were subjected to a series of highly demanding tests to ensure their efficacy and safety. Heart valves, hip or knee implants, implanted cerebellar stimulants, and endoscopic implants (intra-bone) are among the most popular class III devices (Muehlematter et al., 2021; Yaqoob et al., 2019; World Health Organization, 2017).

The fundamental goal of implanted devices is to closely resemble a portion of tissue so that they can be utilized to replace an organ or other damaged component to keep the body operating normally (Gordon & Stern, 2019). They were constructed using conventional materials, including metals, ceramics, and synthetic polymers, but there are several drawbacks associated with their use, including immunological rejection. In addition, the biodegradation products of synthetic polymers may trigger an unintended immunogenic reaction in the body (Bao et al., 2022). Hydrolysis creates Carbon dioxide during the breakdown process, which lowers the local pH and results in cellular and tissue necrosis. In this approach, biopolymers play a significant role as implantable medicinal materials (Yean et al., 2017).

With applications ranging from regenerative medicine to robotics, projected tissues must reproduce the inherent structure of the tissues (Acharjee et al., 2023). Under a microscope, native tissues can be examined to reveal how they are structured and how well their inherent traits can be used to build effective biomimetic constructs. The ability of tissue engineering to solve issues ranging from vascularization to the control/determination of cellular function is becoming more widely recognized. This is because analogous structures may be included at an incredibly ordered microscopic level (Liu et al., 2023; Dhania et al, 2022).

2.2. PHAs microplastic

Conventional plastics negatively affect the environment by polluting soil and water with microplastic and nanoplastic (Ali et al., 2021). Plastic reaches a microplastic of a size of 1 nm and 5000 µm through abrasion and erosion processes. These polluting materials reach the soil and water, then the food chain from the ingested plankton. Then, from plankton to fish and birds and at the end, they reach the human food (Koelmans et al., 2022; Rodrigues et al., 2021).

Plastic recycling is done mainly using biopolymers that can replace it (polymers and biopolymers) (Cunha et al., 2020). The great advantage is that PHA particles are biodegradable and do not pollute (Koller & Mukherjee, 2022). Therefore, in 2021, the production of PHA will reach 43.6 thousand tons, compared to 25,000 tons in 2019 and is expected to increase more than ten times in the next five years. In 2022, biopolymers produced

4.5 million tons, representing only 1% of the total polymers created (Nygaard et al., 2023; Vu et al., 2022).
PHA biopolymers are used in various fields for at least partial if not total, replacement of conventional plastic and to reduce massive pollution (Koller & Mukherjee, 2022; Akinwumi et al., 2022; Elmowafy et al., 2019).

2.3. Cosmetic industry

PHA biopolymers are also used in cosmetics. The first use is biodegradable packaging (Bugnicourt et al., 2014). A second use is an insertion into creams with UV protection (solar screen), skin cleansing and moisturizing products, shower gels or washing agents. PHA microparticles can reduce the microplastic in wastewater treatment plants, lakes, rivers and the marine environment. By biodegradation, PHA releases chemicals with an increased nitrogen content (nitrates, nitrites) and nitrogen gas through the denitrification process, which helps to improve wastewater treatment (Vicente et al., 2023; Kovalcik et al., 2019; Guleria et al., 2022).

CONCLUSIONS

In conclusion, biopolymers can be obtained in different ways: through bacterial biosynthesis or through different processes, such as the synthesis of chemicals from renewable natural materials (polyesters from lactic acid, obtained by fermentation starting from starch). Biopolymers constitute an intensive subject studied by researchers because they can be used in many fields, including medical implants, stents (polylactide), regenerated bone tissues (poly lactic-co-glycolic acid), drug-controlled applications, implants (polycaprolactone), packaging, medical industries, and medical implants (polyhydrokyalkanoates).
Polyhydroxyalkanoates (PHAs) are naturally biodegradable polymers. To date, approximately 300 species of Gram-positive and Gram-negative bacteria have been found to produce polyhydroxyalkanoates. As we saw previously, this class of biopolymers is important for biomedical engineering. PHAs can be used in various fields such as tissue engineering (for the creation of artificial organs), genetic engineering (by studying enzyme changes or genome changes), and neural engineering (improvement of neurological diseases such as Parkinson's, Alzheimer's, or focal ischemia), or they can be used in medical devices such as pacemakers, infusion pumps, implants, lenses, and ocular and facial prostheses. An adjacent field is cosmetic engineering, where PHA can be used as a mild cleaning product or for the formation of microplastic PHA, which completely degrade in nature and are thus biodegradable.
Therefore, polyhydroxyalkanoates are biomaterials with high potential for use in biomedical engineering and related fields.

REFERENCES

Abd El-malek, F., Farag, A., Omar, S., & Khairy, H. (2020). Polyhydroxyalkanoates (PHA) from Halomonas pacifica ASL10 and Halomonas salifodiane ASL11 isolated from Mariout salt lakes. *International Journal of Biological Macromolecules*, *161*, 1318-1328.

Acharjee, S. A., Bharali, P., Gogoi, B., Sorhie, V., Walling, B., & Alemtoshi. (2023). PHA-based bioplastic: A potential alternative to address microplastic pollution. *Water, Air, & Soil Pollution*, *234*(1), 21.

Acharjee, S. A., Bharali, P., Gogoi, B., Sorhie, V., Walling, B., & Alemtoshi. (2023). PHA-based bioplastic: A potential alternative to address microplastic pollution. *Water, Air, & Soil Pollution*, *234*(1), 21.

Agarwal, P., Arora, G., Panwar, A., Mathur, V., Srinivasan, V., Pandita, D., & Vasanthan, K. S. (2023). Diverse Applications of Three-Dimensional Printing in Biomedical Engineering: A Review. *3D Printing and Additive Manufacturing*.

Akinwumi, A. R., Nwinyi, O. C., Ayeni, A. O., Ahuekwe, E. F., & Chukwu, M. N. (2022). An overview of the production and prospect of polyhydroxyalkanote (PHA)-based biofuels: Opportunities and limitations. *Scientific African*, *16*, e01233.

Ali, S. S., Elsamahy, T., Koutra, E., Kornaros, M., El-Sheekh, M., Abdelkarim, E. A., & Sun, J. (2021). Degradation of conventional plastic wastes in the environment: A review on current status of knowledge and future perspectives of disposal. *Science of The Total Environment*, *771*, 144719.

Alsaab, H. O., Alharbi, F. D., Alhibs, A. S., Alanazi, N. B., Alshehri, B. Y., Saleh, M. A., & Alzhrani, R. M. (2022). PLGA-Based Nanomedicine: History of Advancement and Development in Clinical Applications of Multiple Diseases. *Pharmaceutics*, *14*(12), 2728.

Amelia, T. S. M., Govindasamy, S., Tamothran, A. M., Vigneswari, S., & Bhubalan, K. (2019). Applications of PHA in agriculture. *Biotechnological applications of polyhydroxyalkanoates*, 347-361.

Amnieh, S. K., Mashayekhi, M., Shahnooshi, E., Tavafoghi, M., & Mosaddegh, P. (2021). Biodegradable performance of pla stents affected by geometrical parameters: The risk of fracture and fragment separation. *Journal of Biomechanics*, *122*, 110489.

Angra, V., Sehgal, R., & Gupta, R. (2023). Trends in PHA production by microbially diverse and functionally distinct communities. *Microbial ecology*, 85(2), 572-585.

Arora, P. K., & Bae, H. (2014). Integration of bioinformatics to biodegradation. *Biological Procedures Online*, 16, 1-10.

Arumugam, A., Anudakshaini, T. S., Shruthi, R., Jeyavishnu, K., Sundarra Harini, S., & Sharad, J. S. (2020). Low-cost production of PHA using cashew apple (Anacardium occidentale L.) juice as potential substrate: Optimization and characterization. *Biomass Conversion and Biorefinery*, *10*, 1167-1178.

Awasthi, S. K., Kumar, M., Kumar, V., Sarsaiya, S., Anerao, P., Ghosh, P., & Awasthi, M. K. (2022). A comprehensive review on recent advancements in biodegradation and sustainable management of biopolymers. *Environmental Pollution*, *307*, 119600.

Ayhan, F., & Ayhan, H. (2017). Functional PEG Macromers for Biomedical Applications. *Journal of Composites and Biodegradable Polymers*, 5, 48-61.

Baghersad, S., Hivechi, A., Bahrami, S. H., Milan, P. B., Siegel, R. A., & Amoupour, M. (2022). Optimal Aloe vera encapsulated PCL/Gel nanofiber design for skin substitute application and the evaluation of its in vivo implantation. *Journal of Drug Delivery Science and Technology*, *74*, 103536.

Bao, Y., Paunović, N., & Leroux, J. C. (2022). Challenges and opportunities in 3D printing of biodegradable medical devices by emerging photopolymerization techniques. *Advanced Functional Materials*, *32*(15), 2109864.

Bhatia, D., Grozdanov, V., Ruf, W. P., Kassubek, J., Ludolph, A. C., Weishaupt, J. H., & Danzer, K. M. (2021). T-cell dysregulation is associated with disease severity in Parkinson's Disease. *Journal of Neuroinflammation*, *18*(1), 1-10.

Blume, C., Kraus, X., Heene, S., Loewner, S., Stanislawski, N., Cholewa, F., & Blume, H. (2022). Vascular implants–new aspects for in situ tissue engineering. *Engineering in life sciences*, *22*(3-4), 344-360.

Bolbasov, E. N., Antonova, L. V., Stankevich, K. S., Ashrafov, A., Matveeva, V. G., Velikanova, E. A., & Barbarash, L. S. (2017). The use of magnetron sputtering for the deposition of thin titanium coatings on the surface of bioresorbable electrospun fibrous scaffolds for vascular tissue engineering: A pilot study. *Applied Surface Science*, *398*, 63-72.

Boyandin, A. N., Prudnikova, S. V., Karpov, V. A., Ivonin, V. N., Đỗ, N. L., Nguyễn, T. H., & Gitelson, I. I. (2013). Microbial degradation of polyhydroxyalkanoates in tropical soils. *International Biodeterioration & Biodegradation*, *83*, 77-84.

Buchholz, P. C., Feuerriegel, G., Zhang, H., Perez-Garcia, P., Nover, L. L., Chow, J., & Pleiss, J. (2022). Plastics degradation by hydrolytic enzymes: The plastics-active enzymes database - PAZy. *Proteins: Structure, Function, and Bioinformatics*, 90(7), 1443-1456.

Bugnicourt, E., Cinelli, P., Lazzeri, A., & Alvarez, V. A. (2014). Polyhydroxyalkanoate (PHA): Review of synthesis, characteristics, processing and potential applications in packaging.

Castañeda-Rodríguez, S., González-Torres, M., Ribas-Aparicio, R. M., Del Prado-Audelo, M. L., Leyva-Gómez, G., Gürer, E. S., & Sharifi-Rad, J. (2023). Recent advances in modified poly (lactic acid) as tissue engineering materials. *Journal of Biological Engineering*, *17*(1), 21.

Cecen, B. (2023). FDM-based 3D printing of PLA/PHA composite polymers. *Chemical Papers*, 1-8.

Chai, M. N., & Isa, M. I. N. (2013). The oleic acid composition effect on the carboxymethyl cellulose based biopolymer electrolyte.

Chen, Y., Abdalkarim, S. Y. H., Yu, H. Y., Li, Y., Xu, J., Marek, J., & Tam, K. C. (2020). Double stimuli-responsive cellulose nanocrystals reinforced electrospun PHBV composites membrane for intelligent drug release. *International journal of biological macromolecules*, *155*, 330-339.

Cunha, C., Silva, L., Paulo, J., Faria, M., Nogueira, N., & Cordeiro, N. (2020). Microalgal-based biopolymer for nano-and microplastic removal: a possible biosolution for wastewater treatment. *Environmental Pollution*, *263*, 114385.

Da Silva, D., Kaduri, M., Poley, M., Adir, O., Krinsky, N., Shainsky-Roitman, J., & Schroeder, A. (2018). Biocompatibility, biodegradation and excretion of polylactic acid (PLA) in medical implants and theranostic systems. *Chemical Engineering Journal*, *340*, 9-14.

Dalton, B., Bhagabati, P., De Micco, J., Padamati, R. B., & O'Connor, K. (2022). A review on biological synthesis of the biodegradable polymers polyhydroxyalkanoates and the development of multiple applications. *Catalysts*, *12*(3), 319.

Dash, T. K., & Konkimalla, V. B. (2012). Polymeric modification and its implication in drug delivery: poly-ε-caprolactone (PCL) as a model polymer. *Molecular pharmaceutics*, 9(9), 2365-2379.

DeStefano, V., Khan, S., & Tabada, A. (2020). Applications of PLA in modern medicine. *Engineered Regeneration*, *1*, 76-87.

Dhania, S., Bernela, M., Rani, R., Parsad, M., Grewal, S., Kumari, S., & Thakur, R. (2022). Scaffolds the backbone of tissue engineering: Advancements in use of polyhydroxyalkanoates (PHA). *International Journal of Biological Macromolecules*, *208*, 243-259.

Dilkes-Hoffman, L. S., Lane, J. L., Grant, T., Pratt, S., Lant, P. A., & Laycock, B. (2018). Environmental impact of biodegradable food packaging when considering food waste. *Journal of Cleaner Production*, 180, 325-334.

Dwivedi, R., Pandey, R., Kumar, S., & Mehrotra, D. (2020). Poly hydroxyalkanoates (PHA): Role in bone scaffolds. *Journal of oral biology and craniofacial research*, *10*(1), 389-392.

Egbo, M. K. (2021). A fundamental review on composite materials and some of their applications in biomedical

engineering. *Journal of King Saud University-Engineering Sciences*, *33*(8), 557-568.

Elmowafy, E., Abdal-Hay, A., Skouras, A., Tiboni, M., Casettari, L., & Guarino, V. (2019). Polyhydroxyalkanoate (PHA): Applications in drug delivery and tissue engineering. *Expert review of medical devices*, 16(6), 467-482.

Ghadi, A., Mahjoub, S., Tabandeh, F., & Talebnia, F. (2014). Synthesis and optimization of chitosan nanoparticles: Potential applications in nanomedicine and biomedical engineering. *Caspian journal of internal medicine*, *5*(3), 156.

Gilmore, D. F., Fuller, R. C., & Lenz, R. (1990). Biodegradation of poly (beta-hydroxyalkanoates). *CRC Press*, Boca Raton, Florida, USA.

Gordon, W. J., & Stern, A. D. (2019). Challenges and opportunities in software-driven medical devices. *Nature biomedical engineering*, *3*(7), 493-497.

Gregory, D. A., Taylor, C. S., Fricker, A. T., Asare, E., Tetali, S. S., Haycock, J. W., & Roy, I. (2022). Polyhydroxyalkanoates and their advances for biomedical applications. *Trends in Molecular Medicine*.

Grochowski, C., Radzikowska, E., & Maciejewski, R. (2018). Neural stem cell therapy—Brief review. *Clinical neurology and neurosurgery*, *173*, 8-14.

Guerra, A. J., Cano, P., Rabionet, M., Puig, T., & Ciurana, J. (2018). 3D-printed PCL/PLA composite stents: Towards a new solution to cardiovascular problems. *Materials*, *11*(9), 1679.

Guleria, S., Singh, H., Sharma, V., Bhardwaj, N., Arya, S. K., Puri, S., & Khatri, M. (2022). Polyhydroxyalkanoates production from domestic waste feedstock: A sustainable approach towards bio-economy. *Journal of Cleaner Production*, *340*, 130661.

Gupta, S., Sharma, S., Nadda, A. K., Husain, M. S. B., & Gupta, A. (2022). Biopolymers from waste biomass and its applications in the cosmetic industry: A review. *Materials Today: Proceedings*, *68*, 873-879.

Hafis, H., Putri, R. U. S. A., Ahmad, L. O., & Susilowati, P. E. (2023, May). Potential of waste starch substrate as a bioplastics production substrate by microbes. In *AIP Conference Proceedings* (Vol. 2704, No. 1). AIP Publishing.

Han, J., Lazarovici, P., Pomerantz, C., Chen, X., Wei, Y., & Lelkes, P. I. (2011). Co-electrospun blends of PLGA, gelatin, and elastin as potential nonthrombogenic scaffolds for vascular tissue engineering. *Biomacromolecules*, *12*(2), 399-408.

Huang, H., Feng, W., & Chen, Y. (2021). Two-dimensional biomaterials: material science, biological effect and biomedical engineering applications. *Chemical Society Reviews*, *50*(20), 11381-11485.

Huang, L., & Wang, G. (2017). The effects of different factors on the behavior of neural stem cells. *Stem cells international*, *2017*.

Huo, X., Zhang, Y., Jin, X., Li, Y., & Zhang, L. (2019). A novel synthesis of selenium nanoparticles encapsulated PLGA nanospheres with curcumin molecules for the inhibition of amyloid β aggregation in Alzheimer's disease. *Journal of Photochemistry and Photobiology B: Biology*, *190*, 98-102.

Irani, M., Sadeghi, G. M. M., & Haririan, I. (2017). A novel biocompatible drug delivery system of chitosan/temozolomide nanoparticles loaded PCL-PU nanofibers for sustained delivery of temozolomide. *International journal of biological macromolecules*, *97*, 744-751.

Irioda, A. C., Mogharbel, B. F., Stricker, P. E. F., Oliverira, N. B., da Rosa, N. N., Perussolo, M. C., & de Carvalho, K. A. T. (2021). The natural functional biopolymer matrix demonstrates to be a promise for safe cell therapy in Parkinson's disease. *European Journal of Public Health*, 31(Supplement_2), ckab120-104.

Jose, A. A., Hazeena, S. H., Lakshmi, N. M., Madhavan, A., Sirohi, R., Tarafdar, A., & Binod, P. (2022). Bacterial biopolymers: From production to applications in biomedicine. *Sustainable Chemistry and Pharmacy*, *25*, 100582.

Kamath, M. S., Ahmed, S. S., Dhanasekaran, M., & Santosh, S. W. (2014). Polycaprolactone scaffold engineered for sustained release of resveratrol: therapeutic enhancement in bone tissue engineering. *International journal of nanomedicine*, 183-195.

Kaniuk, Ł., & Stachewicz, U. (2021). Development and advantages of biodegradable PHA polymers based on electrospun PHBV fibers for tissue engineering and other biomedical applications. *ACS Biomaterials Science & Engineering*, 7(12), 5339-5362.

Kaniuk, Ł., & Stachewicz, U. (2021). Development and advantages of biodegradable PHA polymers based on electrospun PHBV fibers for tissue engineering and other biomedical applications. *ACS Biomaterials Science & Engineering*, *7*(12), 5339-5362.

Kaparapu, J. (2018). Polyhydroxyalkanoate (PHA) production by genetically engineered microalgae: a review. *J. New Biol. Rep*, 7, 68-73.

Kaur, P., & Sharma, R. (2023, February). Emerging role of natural polymers in drug delivery systems. In *AIP Conference Proceedings* (Vol. 2558, No. 1). AIP Publishing.

Keskin, G., Kızıl, G., Bechelany, M., Pochat-Bohatier, C., & Öner, M. (2017). Potential of polyhydroxyalkanoate (PHA) polymers family as substitutes of petroleum based polymers for packaging applications and solutions brought by their composites to form barrier materials. *Pure and Applied Chemistry*, *89*(12), 1841-1848.

Khanna, S., & Srivastava, A. K. (2005). Recent advances in microbial polyhydroxyalkanoates. *Process biochemistry*, *40*(2), 607-619.

Kiselev, E. G., Demidenko, A. V., Zhila, N. O., Shishatskaya, E. I., & Volova, T. G. (2022). Sugar beet molasses as a potential C-substrate for PHA production by Cupriavidus necator. *Bioengineering*, *9*(4), 154.

Koelmans, A. A., Redondo-Hasselerharm, P. E., Nor, N. H. M., de Ruijter, V. N., Mintenig, S. M., & Kooi, M. (2022). Risk assessment of microplastic particles. *Nature Reviews Materials*, *7*(2), 138-152.

Koller, M. (2017). Production of polyhydroxyalkanoate (PHA) biopolyesters by extremophiles. *MOJ Polymer Science*, *1*(2), 1-19.

Koller, M. (2018). Biodegradable and biocompatible polyhydroxy-alkanoates (PHA): auspicious microbial macromolecules for pharmaceutical and therapeutic applications. *Molecules*, *23*(2), 362.

Koller, M., & Mukherjee, A. (2022). A new wave of industrialization of PHA biopolyesters. *Bioengineering*, *9*(2), 74.

Koller, M., & Obruča, S. (2022). Biotechnological production of polyhydroxyalkanoates from glycerol: A review. *Biocatalysis and Agricultural Biotechnology*, *42*, 102333.

Koller, M., Maršálek, L., de Sousa Dias, M. M., & Braunegg, G. (2017). Producing microbial polyhydroxyalkanoate (PHA) biopolyesters in a sustainable manner. *New biotechnology*, 37, 24-38.

Kourilova, X., Novackova, I., Koller, M., & Obruca, S. (2021). Evaluation of mesophilic Burkholderia sacchari, thermophilic Schlegelella thermodepolymerans and halophilic Halomonas halophila for polyhydroxyalkanoates production on model media mimicking lignocellulose hydrolysates. *Bioresource Technology*, *325*, 124704.

Kourmentza, C., Plácido, J., Venetsaneas, N., Burniol-Figols, A., Varrone, C., Gavala, H. N., & Reis, M. A. (2017). Recent advances and challenges towards sustainable polyhydroxyalkanoate (PHA) production. *Bioengineering*, *4*(2), 55.

Kovalcik, A. (2021). Recent advances in 3D printing of polyhydroxyalkanoates: a review. *The EuroBiotech Journal,* 5(1), 48-55.

Kovalcik, A., Obruca, S., Fritz, I., & Marova, I. (2019). Polyhydroxyalkanoates: their importance and future. *BioResources*, *14*(2), 2468-2471.

Krumnow, A. A., Sorokulova, I. B., Olsen, E., Globa, L., Barbaree, J. M., & Vodyanoy, V. J. (2009). Preservation of bacteria in natural polymers. *Journal of Microbiological Methods*, *78*(2), 189-194.

Kwon, S. G., Kwon, Y. W., Lee, T. W., Park, G. T., & Kim, J. H. (2018). Recent advances in stem cell therapeutics and tissue engineering strategies. *Biomaterials Research*, *22*(1), 1-8.

Liu, J., Zhou, Z., Li, H., Yang, X., Wang, Z., Xiao, J., & Wei, D. X. (2023). Current status and challenges in the application of microbial PHA particles. *Particuology*.

Liu, S., Yu, J. M., Gan, Y. C., Qiu, X. Z., Gao, Z. C., Wang, H., ... & Hou, H. H. (2023). Biomimetic natural biomaterials for tissue engineering and regenerative medicine: new biosynthesis methods, recent advances, and emerging applications. *Military Medical Research*, *10*(1), 16.

Liu, Y., Yu, Q., Yang, L., & Cui, Y. (2023). Materials and biomedical applications of implantable electronic devices. *Advanced Materials Technologies*, *8*(4), 2200853.

Madkour, M. H., Heinrich, D., Alghamdi, M. A., Shabbaj, I. I., & Steinbüchel, A. (2013). PHA recovery from biomass. *Biomacromolecules*, *14*(9), 2963-2972.

Meereboer, K. W., Misra, M., & Mohanty, A. K. (2020). Review of recent advances in the biodegradability of polyhydroxyalkanoate (PHA) bioplastics and their composites. *Green Chemistry*, *22*(17), 5519-5558.

Morgan-Sagastume, F. (2016). Characterisation of open, mixed microbial cultures for polyhydroxyalkanoate (PHA) production. *Reviews in Environmental Science and Bio/Technology*, *15*(4), 593-625.

Muehlematter, U. J., Daniore, P., & Vokinger, K. N. (2021). Approval of artificial intelligence and machine learning-based medical devices in the USA and Europe (2015–20): a comparative analysis. *The Lancet Digital Health*, *3*(3), e195-e203.

Musilova, J., Kourilova, X., Pernicova, I., Bezdicek, M., Lengerova, M., Obruca, S., & Sedlar, K. (2022). Novel thermophilic polyhydroxyalkanoates producing strain Aneurinibacillus thermoaerophilus CCM 8960. *Applied Microbiology and Biotechnology*, *106*(12), 4669-4681.

Nejati-Koshki, K., Pilehvar-Soltanahmadi, Y., Alizadeh, E., Ebrahimi-Kalan, A., Mortazavi, Y., & Zarghami, N. (2017). Development of Emu oil-loaded PCL/collagen bioactive nanofibers for proliferation and stemness preservation of human adipose-derived stem cells: possible application in regenerative medicine. *Drug development and industrial pharmacy*, *43*(12), 1978-1988.

Nygaard, D., Yashchuk, O., & Hermida, É. B. (2023). Polyhydroxyalkanoates (PHAs) production from residual glycerol by wild type Cupriavidus necator. *Waste and Biomass Valorization*, *14*(5), 1489-1496.

Pathan, N., & Shende, P. (2021). Strategic conceptualization and potential of self-healing polymers in biomedical field. *Materials Science and Engineering: C*, *125*, 112099.

Ponjavic, M., Malagurski, I., Lazic, J., Jeremic, S., Pavlovic, V., Prlainovic, N., & Nikodinovic-Runic, J. (2023). Advancing PHBV Biomedical Potential with the Incorporation of Bacterial Biopigment Prodigiosin. *International Journal of Molecular Sciences*, *24*(3), 1906.

Prakash, P., Lee, W. H., Loo, C. Y., Wong, H. S. J., & Parumasivam, T. (2022). Advances in Polyhydroxyalkanoate Nanocarriers for Effective Drug Delivery: An Overview and Challenges. *Nanomaterials*, *12*(1), 175.

Pryadko, A., Surmeneva, M. A., & Surmenev, R. A. (2021). Review of hybrid materials based on polyhydroxyalkanoates for tissue engineering applications. *Polymers*, *13*(11), 1738.

Pulingam, T., Appaturi, J. N., Parumasivam, T., Ahmad, A., & Sudesh, K. (2022). Biomedical applications of polyhydroxyalkanoate in tissue engineering. *Polymers*, *14*(11), 2141.

Rajendran, N., & Han, J. (2022). Integrated polylactic acid and biodiesel production from food waste: Process synthesis and economics. *Bioresource Technology*, *343*, 126119.

Rebelo, R., Fernandes, M., & Fangueiro, R. (2017). Biopolymers in medical implants: a brief review. *Procedia engineering*, 200, 236-243.

Rehakova, V., Pernicova, I., Kourilova, X., Sedlacek, P., Musilova, J., Sedlar, K., & Obruca, S. (2023). Biosynthesis of versatile PHA copolymers by

thermophilic members of the genus Aneurinibacillus. *International Journal of Biological Macromolecules*, 225, 1588-1598.

Rodrigues, S. M., Elliott, M., Almeida, C. M. R., & Ramos, S. (2021). Microplastics and plankton: Knowledge from laboratory and field studies to distinguish contamination from pollution. *Journal of Hazardous Materials*, *417*, 126057.

Rodriguez-Perez, S., Serrano, A., Pantión, A. A., & Alonso-Fariñas, B. (2018). Challenges of scaling-up PHA production from waste streams. A review. Journal of environmental management, 205, 215-230.

Rosen, J., Hannaford, B., & Satava, R. M. (Eds.). (2011). Surgical robotics: systems applications and visions. *Springer Science & Business Media*.

Roy, I., & Visakh, P. M. (Eds.). (2014). *Polyhydroxyalkanoate (PHA) based blends, composites and nanocomposites* (Vol. 30). *Royal Society of Chemistry*.

Sabapathy, P. C., Devaraj, S., Meixner, K., Anburajan, P., Kathirvel, P., Ravikumar, Y., & Qi, X. (2020). Recent developments in Polyhydroxyalkanoates (PHAs) production–A review. *Bioresource technology*, *306*, 123132.

Saratale, R. G., Cho, S. K., Saratale, G. D., Kadam, A. A., Ghodake, G. S., Kumar, M., & Shin, H. S. (2021). A comprehensive overview and recent advances on polyhydroxyalkanoates (PHA) production using various organic waste streams. *Bioresource technology*, *325*, 124685.

Schedin-Weiss, S., Inoue, M., Hromadkova, L., Teranishi, Y., Yamamoto, N. G., Wiehager, B., & Tjernberg, L. O. (2017). Monoamine oxidase B is elevated in Alzheimer disease neurons, is associated with γ-secretase and regulates neuronal amyloid β-peptide levels. *Alzheimer's Research & Therapy*, 9(1), 1-19.

Sedlacek, P., Slaninova, E., Koller, M., Nebesarova, J., Marova, I., Krzyzanek, V., & Obruca, S. (2019). PHA granules help bacterial cells to preserve cell integrity when exposed to sudden osmotic imbalances. *New biotechnology*, *49*, 129-136.

Sharma, S., Parmar, A., Kori, S., & Sandhir, R. (2016). PLGA-based nanoparticles: A new paradigm in biomedical applications. *TrAC trends in analytical chemistry*, *80*, 30-40.

Sharma, V., Sehgal, R., & Gupta, R. (2021). Polyhydroxyalkanoate (PHA): Properties and modifications. *Polymer*, 212, 123161.

Shepherd, R. K. (Ed.). (2016). Neurobionics: The biomedical engineering of neural prostheses. *John Wiley & Sons*.

Silva, T. H., Alves, A., Ferreira, B. M., Oliveira, J. M., Reys, L. L., Ferreira, R. J. F., ... & Reis, R. L. (2012). Materials of marine origin: a review on polymers and ceramics of biomedical interest. *International Materials Reviews*, *57*(5), 276-306.

Slaninova, E., Sedlacek, P., Mravec, F., Mullerova, L., Samek, O., Koller, M., & Obruca, S. (2018). Light scattering on PHA granules protects bacterial cells against the harmful effects of UV radiation. *Applied microbiology and biotechnology*, *102*, 1923-1931.

Soares, J. S., Moore Jr, J. E., & Rajagopal, K. R. (2010). Modeling of deformation-accelerated breakdown of polylactic acid biodegradable stents.

Somleva, M. N., Peoples, O. P., & Snell, K. D. (2013). PHA bioplastics, biochemicals, and energy from crops. *Plant biotechnology journal*, *11*(2), 233-252.

Subash, A., Naebe, M., Wang, X., & Kandasubramanian, B. (2023). Biopolymer–a sustainable and efficacious material system for effluent removal. *Journal of Hazardous Materials*, 443, 130168.

Sun, J., Shen, J., Chen, S., Cooper, M. A., Fu, H., Wu, D., & Yang, Z. (2018). Nanofiller reinforced biodegradable PLA/PHA composites: Current status and future trends. *Polymers*, *10*(5), 505.

Syed Mohamed, S. M. D., Ansari, N. F., Md Iqbal, N., & Anis, S. N. S. (2022). Polyhydroxyalkanoates (PHA)-based responsive polymers. *International Journal of Polymeric Materials and Polymeric Biomaterials*, *71*(17), 1283-1302.

Vicente, D., Proença, D. N., & Morais, P. V. (2023). The Role of Bacterial Polyhydroalkanoate (PHA) in a Sustainable Future: A Review on the Biological Diversity. *International Journal of Environmental Research and Public Health*, *20*(4), 2959.

Vu, D. H., Mahboubi, A., Root, A., Heinmaa, I., Taherzadeh, M. J., & Åkesson, D. (2022). Thorough investigation of the effects of cultivation factors on polyhydroalkanoates (PHAs) production by Cupriavidus necator from food waste-derived volatile fatty acids. *Fermentation*, *8*(11), 605.

Wang, J., Liu, S., Huang, J., Cui, R., Xu, Y., & Song, Z. (2023). Genetic engineering strategies for sustainable polyhydroxyalkanoate (PHA) production from carbon-rich wastes. *Environmental Technology & Innovation*, 30, 103069.

Wang, Y., Wu, J., Lv, M., Shao, Z., Hungwe, M., Wang, J., & Geng, W. (2021). Metabolism characteristics of lactic acid bacteria and the expanding applications in food industry. *Frontiers in bioengineering and biotechnology*, *9*, 612285.

Wang, Y., Zhao, L., Zhou, L., Chen, C., & Chen, G. (2023). Sequential release of vascular endothelial growth factor-A and bone morphogenetic protein-2 from osteogenic scaffolds assembled by PLGA microcapsules: A preliminary study in vitro. *International Journal of Biological Macromolecules*, *232*, 123330.

Ward, C., Henderson, S., & Metcalfe, N. H. (2013). A short history on pacemakers. *International journal of cardiology*, *169*(4), 244-248.

Westlie, A. H., Quinn, E. C., Parker, C. R., & Chen, E. Y. X. (2022). Synthetic biodegradable polyhydroxyalkanoates (PHAs): Recent advances and future challenges. *Progress in Polymer Science*, 101608.

World Health Organization. (2017). WHO global model regulatory framework for medical devices including in vitro diagnostic medical devices.

Wu, Q., Liu, J., Wang, X., Feng, L., Wu, J., Zhu, X., & Gong, X. (2020). Organ-on-a-chip: Recent breakthroughs and future prospects. *Biomedical engineering online*, *19*, 1-19.

Xiao, Z., Zhang, Y., Xi, L., Huo, F., Zhao, J. Y., & Li, J. (2015). Thermophilic production of polyhydroxyalkanoates by a novel Aneurinibacillus strain isolated from Gudao oilfield, China. *Journal of Basic Microbiology*, *55*(9), 1125-1133.

Yang, L., Wu, H., Lu, L., He, Q., Xi, B., Yu, H., ... & Zhang, X. (2021). A tailored extracellular matrix (ECM)-Mimetic coating for cardiovascular stents by stepwise assembly of hyaluronic acid and recombinant human type III collagen. *Biomaterials*, *276*, 121055.

Yaqoob, T., Abbas, H., & Atiquzzaman, M. (2019). Security vulnerabilities, attacks, countermeasures, and regulations of networked medical devices—A review. *IEEE Communications Surveys & Tutorials*, *21*(4), 3723-3768.

Yean, O. S., Yee, C. J., & Kumar, S. (2017). Degradation of polyhydroxyalkanoate (PHA): a review. *Журнал Сибирского федерального университета. Биология*, *10*(2), 211-225.

Yoo, J., & Won, Y. Y. (2020). Phenomenology of the initial burst release of drugs from PLGA microparticles. *ACS Biomaterials Science & Engineering*, *6*(11), 6053-6062.

Zarrintaj, P., Seidi, F., Azarfam, M. Y., Yazdi, M. K., Erfani, A., Barani, M., ... & Mozafari, M. (2023). Biopolymer-based composites for tissue engineering applications: A basis for future opportunities. *Composites Part B: Engineering*, *258*, 110701.

Zhang, F., & King, M. W. (2020). Biodegradable polymers as the pivotal player in the design of tissue engineering scaffolds. *Advanced healthcare materials*, *9*(13), 1901358.

Zhang, H., Mao, X., Zhao, D., Jiang, W., Du, Z., Li, Q., & Han, D. (2017). Three dimensional printed polylactic acid-hydroxyapatite composite scaffolds for prefabricating vascularized tissue engineered bone: An in vivo bioreactor model. *Scientific reports,* 7(1), 15255.

Zhang, J., Shishatskaya, E. I., Volova, T. G., da Silva, L. F., & Chen, G. Q. (2018). Polyhydroxyalkanoates (PHA) for therapeutic applications. *Materials Science and Engineering: C*, *86*, 144-150.

Scientific Bulletin. Series F. Biotechnologies, Vol. XXVII, No. 2, 2023
ISSN 2285-1364, CD-ROM ISSN 2285-5521, ISSN Online 2285-1372, ISSN-L 2285-1364

GENETIC RELATIONSHIPS AMONG DIFFERENT BROOMRAPE RACES FROM THE BLACK SEA BASIN

Maria DUCA, Ina BIVOL

Center of Functional Genetics, Moldova State University, 60 Mateevici Street, MD-2009, Chisinau, Republic of Moldova

Corresponding author email: bivolinga@yahoo.com

Abstract

Microsatellite loci represent prevalent molecular markers for assessing genetic diversity, phylogenetic and evolutionary relationships between different in origin broomrape taxon. In the present study, the genetic differences and relationships among 3 broomrape races from Bulgaria, Turkey, Moldova and Romania were identified by ISSR markers. The obtained results on genetic diversity parameters showed that race H is more variable than races G and E. However, the gene diversity within populations was the greatest in race G, followed by race H and E. The limited gene flow for races H and G determined significant genetic differentiation of populations. Conversely, a comparatively moderate gene flow for race E pointed to a little differentiation among populations. These findings were also corroborated by AMOVA, demonstrating high genetic diferences within (89%) and lower (11%) among races. The investigation also revealed the existence evolutionary differences between all races. This situation proves the action of evolutive molecular mechanisms which offered, in time, discrete genetic particularities at broomrape, indicating that the existing races are unstable over time and they are constantly adapting to the new more sunflower resistance hybrids.

Key words*: Orobanche cumana, ISSR markers, genetic relationships, broomrape races.*

INTRODUCTION

The current situation related to progression of broomrape in modern agriculture, and therefore reducing of the world's total production of sunflower seed and oil is worrisome. Moreover, it should be kept in mind that, depending on the agro-ecological conditions, the different factors induce imbalances in the coexistence degrees between the parasite and its host, resulting in significant harvest losses, for example, the small number of intensive autochthonous varieties and hybrids (Strelnikov, 2017), the application of large doses of fertilizers (Pinashkin, 2012), non-compliance with rotation periods (Lukomets & Antonova, 2015), failure to comply of some technological procedures (Pimakhin et al., 2000; Pinashkin, 2012).

One of the most effective and environmentally safe ways to combat this parasitic plant is the development of continuous breeding programs, aimed at creating of sunflower hybrids resistant to new highly virulent broomrape biotypes.

To do this, it is important to know the racial structure of parasite populations, the characteristics of new virulent biotypes and their relationship with sunflower. Finding and creating of resistance sources to the new most virulent broomrape races is becoming increasingly relevant.

For about 200 years, eight broomrape races (A, B, C, D, E, F, G and H) were recorded (Molinero-Ruiz et al., 2015). Over the last 10-12 years, new more aggressive biotypes, like E, F, G and H are considered as the most virulent (Melero-Vara et al., 2000; Kaya et al., 2004), which they are rapidly spreading and taking over new areas across the globe (Fernández-Martínez, 2012). At present, races H+ and I were registered (Duca et al., 2017). The problem of highly virulent broomrape races is very important for countries geographically located in the Black Sea region, including Bulgaria, Turkey, Romania and Republic of Moldova (Kaya et al., 2004; Duca, 2014, 2019).

In Bulgaria, infections of sunflower by races A and B of *O. cumana* were reported for the first time in 1935. Then in 1966, it was found the sort Peredovik has been the most severely affected by the race C (Petrov, 1968). At the beginning of the 1990s, the new physiological races of the parasite D and E massively

attacked the area under crops of sunflower in many regions of the country (Shindrova et al., 1998). In the year 2000, it was found a very aggressive population of broomrape in the northeastern part of Bulgaria identified as race H which was not reported later (Shindrova, 2006; 2012). In the first years of the 21st century race E of *O. cumana* remained predominant in Bulgaria until race F was widely spread (Shindrova, 2006). Nowadays, the prevailing presence of races E (35%), G (80%) and a decline in the presence of race F (16%) was determined in the sunflower growing areas of Bulgaria (Batchvarova, 2014; Encheva, 2018).

The expanded of the parasite across sunflower cultivation areas in the Trakya region of Turkey was first reported in the second half of 1940s (Demirci & Kaya, 2009). The period of immunity until the early 80s ensured by the introduction of sunflower resistant sorts, in the 1990s gave way to explosion of virulence of new race F in the Trakya region (Demirci & Kaya, 2009). Nowadays, race F is widely distributed in Trakya region, which accounts for more than half of the total sunflower areas in the country (Kaya et al., 2004; Semerci et al., 2010). Moreover, the existence of more virulent race G was confirmed in several locations of the Trakya region (Molinero-Ruiz et al., 2013; Kaya, 2014). In the past few years, the infections of sunflower crops by *O. cumana* have also become frequent in other important production areas such as Cukurova, Middle Anatolia and Black Sea regions, but the race/s of the parasite have not been determined yet (Evci et al., 2011; Kaya, 2014).

In Romania, the broomrape has been observed since the 1940s, which began to rapidly forming races with high virulence (Vranceanu et al., 1980; Pacureanu, 2014). In the period of 1960–1963, two broomrape races were identified as A and B, in the years 1975-1980, three other races (C, D and E), after 16-19 years (in 1996-1999) appeared race F, and recently, after a period of only 8-10 years, a new population of the parasite that overcomes the genes of resistance to race F, called race G (Pacureanu, 2014). As occurred in other countries, races A to E in Romania were effectively controlled through genetic resistance for several decades, until race F was identified (Pacureanu, 2014). In recent years, especially in the area of Tulcea and Brăila, the presence even more virulent form of the parasite (race H) was found (Rîşnoveanu et al., 2016).

The close commercial ties of Moldova with many states of the East and West, mild climate, 76% fertile agricultural soils and isolated private farms with numerous difficulties in management contributed to the creation of favourable conditions for the rapid emergence of new local broomrape races. The first-ever report of broomrape (race B) to the sunflower growing areas in Moldova dates back to 1937 (Arhiva Naţională a Republicii Moldova. Fond 3, inv. 2, d. 376). Several decades later (in the 1960s), a new broomrape biotype in different regions of the country, named the Moldovan race or race C, has been identified (Sharova, 1969). The replacing of the old sunflower susceptible cultivars with the new resistant ones allowed to forget about the problem until the 1990's. In the early 2000s, broomrape again manifested itself in its highly aggressive new races E and F, and, recently, more-virulent races G and H have been recorded in sunflower growing areas of Moldova (Gisca et al., 2013; Duca, 2014; 2019).

Identification of broomrape races is useful for breeding studies and analysis about genetic diversity and characterization of *O. cumana* populations by molecular markers. These are effective possible tools of gaining knowledge about the genetic structure of race and their evolution progress.

Although numerous molecular studies have been carried out, regarding the genetic structure and variability of the sunflower broomrape in various countries, the mechanisms of races appearance and their evolution are not yet fully understood.

The main purpose of our study was to investigate the genetic diversity and genetic differentiation of 23 *O. cumana* populations from the Black Sea basin, belonging to 3 races, using ISSR (Inter Simple Sequence Repeats) molecular markers. This technique is based on the amplification of DNA segments between two microsatellite repeated regions by means of unanchored or anchored at 3` or 5` end with 1 to 4 degenerate bases extended into the flanking sequences. For the first time this

method was proposed and implemented on the different animals and plants by Zietkiewicz and Gupta in 1994 (Zietkiewicz et al., 1994; Gupta et al., 1994). Thus, the information from this study allows a better understanding of parasite diversity and may be useful in different breeding programs on the creation of sunflower hybrids resistant to broomrape.

MATERIALS AND METHODS

Plant material. In the frame of this study, 23 sunflower broomrape populations from 4 countries (Bulgaria, Turkey, Republic of Moldova and Romania), located in Black Sea region were used. In order to establish race composition of them, greenhouse experiment was realised. The germination of *O. cumana* seeds was realized in pots, using four Romanian differential lines and hybrids of sunflower for the races E, G and H, as well as susceptible control Performer hybrid, that does not contain specific resistance genes (Or) (Clapco, 2021). A set of sunflower differential lines for broomrape races from A to E, each line carrying a single dominant gene (Or1 through Or5, respectively) were kindly provided by National Agricultural Research and Development Institute (Fundulea, Romania). Two broomrape populations were classified as race E, five identified as race G and the other 16 belonged to race H (Table 1).

Table 1. Origin and race status of 23 studied *O. cumana* populations

Nr.	Population code	Population/ Origin	Number of accessions
Race E			
1.	RM12	Căzănești/Telenești/Moldova	11
2.	RM13	Congaz/Comrat/Moldova	12
Race G			
3.	RM10	Popeasca/Stefan Voda/Moldova	21
4.	RM11	Chisinau/ Moldova	22
5.	B1	Debovo/Pleven/ Nikopol/Bulgaria	12
6.	B2	Selanovtsi/Vratsa/ Oryahovo/Bulgaria	12
7.	T5	Trakia/Turkey	11
Race H			
8.	RM1	Izbiște/Criuleni/Moldova	12
9.	RM2	Svetlii/Comrat/Moldova	12
10.	RM3	Taraclia/Moldova	11
11.	RM4	Soroca/Moldova	12
12.	RM5	Alexander field/Cahul/Moldova	8
13.	RM6	Bălți I/Moldova	12
14.	RM7	Bălți II/Moldova	11
15.	RM8	Prepelița/Sîngerei/Moldova	12
16.	RM9	Grigorievca/Căușeni/Moldova	12
17.	B3	Radnevo/Stara Zagora/Radnevo/Bulgaria	12
18.	B4	Rosenova/Dobrichka/Dobrich/Bulgaria	12
19.	T1	Keşan/Edirne/Thrace/Turkey	9
20.	T2	Adana/Turkey	7
21.	T3	Merkez/Edirne/Thrace/Turkey	7
22.	T4	Lüleburgaz/Kirklareli/Thrace/Turkey	7
23.	R1	Brăila/Romania	12

Fresh tissue samples from each population were collected and stored at –70°C until DNA extraction. In total 269 broomrape accessions representing 23 populations were included in a comparative study on genetic diversity as well as genetic structure analysis of populations.

DNA extraction. Frozen plants were used for total DNA extraction by means of Thermo Scientific GeneJET Plant Genomic DNA Purification Mini Kit #K0791 according to the manufacturer's protocol (Thermo Fisher Scientific, USA). Quality and quantity of isolated DNA were determined by spectrophotometer (model T60 UV-VIS, PG Instruments Limited, England) and, also checked by 0.8% agarose gel electrophoresis in 1xTAE buffer (40 mM Tris-acetat, pH 8.0; 1mM EDTA) at 2.5 V/cm (Sambrook & Russell, 2001).

ISSR analysis. Thirteen most effective ISSR primers were used for PCR amplification, which were selected out of readily available 14 primers (Table 2). This set included those primers reported by Benharat et al. (2002) in analyse of genetic diversity among broomrape populations.

Table 2. Inter simple sequence repeat (ISSR) primers used in this study

Nr.	Primer code	Sequence (5′→ 3′)	NBN	GC, %
1.	BC 807	AGAGAGAGAGAGAGAGT	17	47
2	BC 810	GAGAGAGAGAGAGAGAT	17	47
3.	BC 835	AGAGAGAGAGAGAGAGYC	18	56
4.	BC 841	GAGAGAGAGAGAGAGAYC	18	56
5.	BC 857	ACACACACACACACACYG	18	56
6.	$(CAA)_5$	CAACAACAACAACAA	15	33
7.	$(GACA)_4$	GACAGACAGACAGACA	16	50
8.	$(CA)_8RG$	CACACACACACARG	14	57
9.	$(CTC)_4RC$	CTCCTCCTCCTCRC	14	71
10.	$(CAG)_5$	CAGCAGCAGCAGCAG	15	67
11.	$(CT)_8TC$	CTCTCTCTCTCTCTCTTC	18	50
12.	$(CA)_6AC$	CACACACACACAAC	14	50
13.	$(AG)_8YA$	AGAGAGAGAGAGAGAGYA	18	50

R = (A, G); Y=(C, T); NBN = nitrogenous bases number; GC, % = percentage content of cytosine (C) and guanine (G) nucleotides in primer.

The PCR solution (15 µl) contained: 60 ng ADN; 200 µM dNTP mixture (dATP, dCTP, dGTP, dTTP); 0.4 µM concentration of each primer; 1 U/µL DreamTaq Green DNA polymerase in buffer solution (1x); ultrapure water; 2.5 mM $MgCl_2$ (Thermo Scientific, USA). The amplification was carried out in the thermocycler Genset 9700 (Applied Biosystems, USA) according to the standard steps of polymerase chain reaction (Sambrook & Russell, 2001). PCR reactions were

performed under the following conditions: 5 min. at 95°C for initial denaturation, 35 cycles of 30 s at 95°C (denaturation), 45 s at 45°C (annealing), and 2 min at 72°C (extension), followed by a final extension of 5 min at 72°C. Amplified products were separated by electrophoresis according to their molecular weight on 2% agarose gels submerged in 1xTAE buffer, and then stained with ethidium-bromide solution (final concentration of 0.5 μg/ml) for 20 min. The molecular size of amplicons was estimated by running GeneRuler Express DNA Ladder ready-to-use SM1553 in the gel as standard size marker (Thermo Fisher Scientific, USA). The amplified DNAs visualized on a UV transilluminator (wave length λ = 305 nm) and documented using the Gel documentation system Doc-Print VX2, model SXT-F20.M (Vilber Lourmat, France).

Data analysis. The DNA amplified fragment analysis was carried out with the Photo-Capt V.15.02 program. The data generated by the program were compiled into a binary data matrix, namely (1) as presence and (0) absence of ISSR fragments for each primer.

The polymorphism information content (PIC) of each dominant ISSR marker or its discriminatory ability, that depends on the number of alleles detected and their distribution frequency, was computed according to Roldán-Ruiz (2000) as:

$PIC_i = 2f_i(1-f_i)$, where f_i – is the frequency of the amplified allele (band present), and $(1-f_i)$ is the frequency of the null allele.

Resolving power (*Rp*) is a parameter used to characterize the ability of the primer/s to detect the differences in allele's distribution between a large number of the sampled genotypes and divide it's into a group (Prevost & Wilkinson, 1999):

$Rp = \sum Ibi$, where *Ibi* describes the relative amplicon informativeness *i* and is calculated as $Ibi = 1 - [2 \times |0.5 - pi|]$, *pi* is the proportion of individuals with identified amplicon *i*.

The Simpson's coefficient (h_j) or discrimination potential of each primer was calculated by formula (Lüdtke et al., 2010):

$h_j = \sum \left(\frac{1 - \sum p_i^2}{n} \right)$, where p_i is the frequency of the i-th allele and n is corresponds to the number of loci detected by each primer. A value of 1 indicates that the primer is able to discriminate between all samples, and a value of 0 indicates that all samples are identical.

The marker index (*MI*) is used to evaluate the efficiency of a marker system and calculated as the product of the effective multiplex ratio (*EMR*) and the polymorphism information content (*PIC*) (or average expected heterozygosity (*He*)) (Powell et all, 1996): $MI = PIC \times EMR$, where EMR is defined as the product of the total number of loci per primer (n) and the fraction of polymorphic loci (n_p) by formula: $EMR = n_p \left(\frac{n_p}{n} \right)$.

The observed number of alleles (Na), effective number of alleles (Ne), Nei's gene diversity (H), Shannon's Information index (I), number of polymorphic loci (NPL), percentage of polymorphic loci (PPL), total genetic diversity (Ht), genetic diversity within populations (Hs), coefficient of genetic differentiation (Gst) and gene flow among populations (Nm) were calculated using POPGENE V.1.32 software. XLSTAT 2014 V.2014.5.03 and also POPGENE V.1.32 software packages were used to calculate the Nei's and Euclidean genetic distances as well as to construct dendrograms by UPGMA and Ward's methods. AMOVA (Analysis of molecular variance) test as implemented in GenAlex 6.501 was used to analyse of the genetic diversity and genetic structure of broomrape populations.

RESULTS AND DISCUSSIONS

In the present study, the different origin broomrape populations from the Black Sea basin were selected in order to acquire a deeper insight into their population structure and genetic variability at the race level. Within physiological races, there are exchanges of genes/polygenes (introgression) that deviate from the normal distribution of the rare segregating recessive forms, and also the rare heterozygous forms, which later are eliminated by natural selection (Louarn et al., 2016). These continuous variations can only be maintained under mutation pressure, thus contributing to the appearance of new characters (Pineda-Martos et al., 2013), such as

the level of aggression of the sunflower broomrape races (Rodríguez-Ojeda et al., 2013).

For these reasons, it is of scientific and practical interest to investigate the broomrape races discrimination using molecular markers (i), evaluate their comparative genetic diversity and detect the different levels of variability within race E, G and H (ii) and to ascertain whether the genetic relationships findings based on ISSR markers confirm or conflict the physiological data (iii).

Out of 14 ISSR primers tested, 13 primers have proved a high reproducible and informative in estimating broomrape genepool available. Investigation of the genetic diversity among 23 *O. cumana* populations belonging to 3 races based on molecular ISSR-markers has allowed for revealing both the significant differences and similarities depending on the analysed race or type of primer. The polymorphism level in *O. cumana* races (E-86.64%, G-90.80%, H-94.48%) is high enough (Table 3).

Table 3. The marker polymorphism and amplification profile of 13 ISSR primers used to analyze the genetic diversity of *O. cumana*

Primer	Fragment sizes, bp				Percentage of polymorphism, %			
	O. cumana	race E	race G	race H	*O. cumana*	race E	race G	race H
BC807	300-4358	340-2200	340-4358	300-3000	89.66	75.00	80.77	91.3
BC810	430-5353	430-2500	430-4749	430-5333	92.86	83.33	89.47	92.59
BC835	444-4577	490-2536	490-3899	444-4577	100.00	88.89	95.00	90.48
BC841	280-5000	280-1660	320-2680	280-5000	96.55	81.82	95.00	96.30
BC857	385-3000	420-2340	385-2429	420-3000	95.65	100.00	94.44	100.00
$(CA)_6AC$	395-3759	950-2558	395-3759	744-2558	91.30	100.00	94.12	90.00
$(CA)_6RG$	454-2476	454-1402	454-2476	454-1749	93.75	70.00	91.67	85.71
$(CT)_8TC$	689-5333	900-2520	689-5333	840-4867	96.30	90.91	100.00	95.24
$(AG)_8YA$	230-4699	536-1320	230-4699	230-4699	97.22	100.00	100.00	91.67
(CAA)5	429-4254	660-2200	429-4254	660-3348	87.50	90.00	80.95	95.00
(CAG)5	620-5000	620-2575	620-5000	620-4838	96.43	100.00	95.65	100.00
$(CTC)_4RC$	418-5353	500-3898	418-5133	500-5353	96.43	71.43	86.36	100.00
(GACA)4	388-3902	1117-2134	388-3902	816-3118	86.67	75.00	76.92	100.00
Total/Mean	230-5353	280-3898	230-5333	230-5353	93.87	86.64	90.80	94.48

For race E, 13 ISSR primers generated a total of 129 amplicons in different size ranging from 280 to 3898 bp out of which 113 (87.60%) products were polymorphic, 15 (11.63%) were specific and one (0.78%) amplicon in 700 bp, generated by the primer $(CTC)_4RC$ was monomorphic (Figure 1).

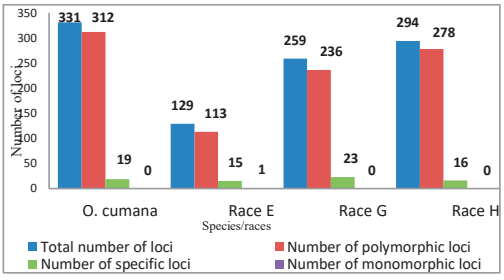


Figure 1. Marker polymorphism of 13 ISSR primers used to analyze the genetic diversity of *O. cumana*

It should be noted that for race G these same primers produced a total of 259 amplified bands, which size ranged from 230 to 5333 bp out of which 236 (91.12%) products were polymorphic, 23 (8.88%) were specific (Figure 1).

However, for race H, the highest number of loci (a total 294) was found, which range from 230 to 5353 bp, out of which 278 (94.56%) products were polymorphic and 16 (5.44%) were specific (Figure 1).

From our data, the specific loci were detected in all races, namely for race E-11.63% or 15 out of the total 129 studied loci, at race G-8.88% or 23 loci out of 259, race H- 5.44% or 16 loci out of 294 (Figure 1). The monomorphic band (700 bp with $(CTC)_4RC$) was observed only in the race E. These data clearly shows that the race E contains the largest percentage of specific loci that distance it evolutionarily from the race G and to a greater extent from the race H.

According to the frequency distribution analysis (331 alleles for 13 loci at 3 races), the number of abundant alleles are with roughly equal in all 3 races. However, it is worth noting that the rare alleles are 15 at race E, but races G and H each has 47 and 48, from which we conclude that the genome of the race E is more stable than the races G and H genomes. Regarding the common alleles in the genome of

race E was revealed only 58, while in the genomes of races G and H - there are 169 and 201, respectively (Figure 2).

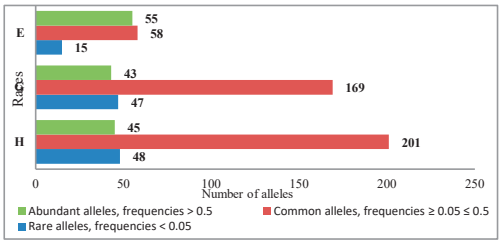


Figure 2. Frequency distribution
of all 331 alleles at 13 loci for 3 races

From these results we can conclude that races G and H are more similar to each other than to race E, which confirms again that race E is more distant of races G and H in evolutionary time. Also the fact should be emphasized that for the first time race E was mentioned in 1980 in Spain (Melero-Vara et al., 1989), rase G – in 2006 in Bulgaria (Shindrova & Penchev, 2012) and race H – in 2014 in Romania (Păcureanu et al., 2014).

However, we received more information about population structure of *O. cumana* races while analysing the ISSR bands distribution on 7 groups of allele frequencies (0.0-0.2, 0.2-0.3, 0.3-0.4, 0.4-0.6, 0.6-0.7, 0.7-0.9, 0.9-1.0) (Figure 3). These results showed the lowest diversity according to different allele frequency at race E in comparison with the races G and H. It just confirms once again the conclusions on the availability of immigration activity or gene passive transfer into populations of race E through gene flow, that over time violates the interpopulation isolation, equalizes gene background and serves as a deterrent regarding speciation. It was found, that the increase genetic diversity for the different allele frequency in races G and H is due to the activating the genetic drift process. Simultaneously, against this background, there was observed the dominant number of rare alleles which could lead to further formative mutations.

The adaptive challenges that matter most in the survival of species in environmental changes are what lead to evolutionary changes in the populations that form this species. Assessment of the level of genetic variation in broomrape populations and also within races to which they relate, it is determined largely by the percentage of polymorphism. Moreover, understanding the mechanisms and selective forces of gene flow between the populations may not only contribute to knowledge of parasitic species evolutionary and phylogeny, but it is also critical to these processes management.

Evaluation of the genetic diversity of broomrape populations by the statistical parameters revealed that the high proportion of diversity among the different broomrape races was accounted for race H (Na=1.89, Ne=1.37, H=0.23, I=0.36, NPL=294, PPL=88.82) and lower values of these index - for races G and E. In addition, the total gene diversity (Ht) and coefficient of gene differentiation among populations (Gst) of race H, were the highest (0.25 and 0.62, respectively), which cannot be said about races G (0.21 and 0.52, respectively) and especially E (0.12 and 0.30, respectively) (Table 4).

However, the gene diversity within populations was the greatest in race G (Hs=0.10), followed by race H (Hs=0.09) and E (Hs=0.08). Limited gene flow for races H and G (0.30 and 0.45,

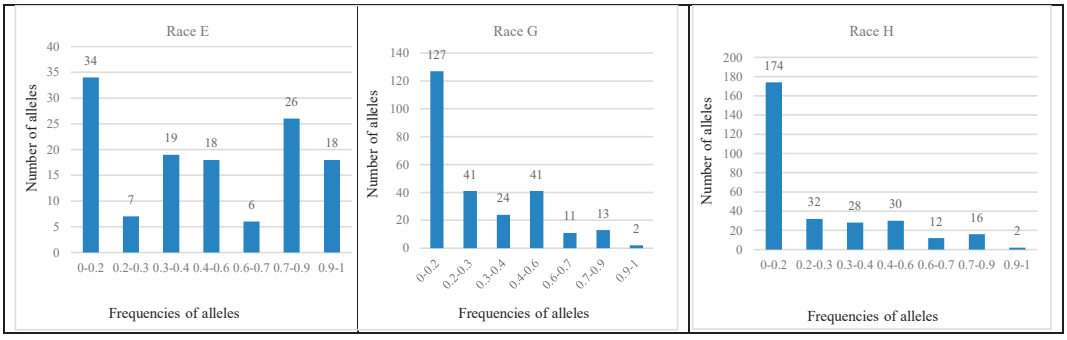


Figure 3. Distribution of the ISSR fragments with different frequencies in 3 *O. cumana* races from the Black Sea Basin

respectively) showed a significant level of genetic differentiation of populations. Conversely, a comparatively moderate level of gene flow was found to be for race E (Nm=1.19), which points to a little differentiation among populations (Table 4). The genetic diversity parameters of the races G (H=0.22, I=0.34, NPL=259, PPL=78.25) and H (H=0.23, I=0.36, NPL=294, PPL=88.82) were higher than at race E (H=0.12, I=0.18, NPL=128, PPL=38.67). This is explained by the moderate level of gene flow for the race E (Nm=1.19), which prevents the differentiation their populations (Gst=0.30) and the limited gene flow for races H and G (Nm=0.30 and Nm=0.45, respectively), that favors the growth of the significant level of genetic differentiation of populations these races (Gst=0.62 and Gst=0.52, respectively).

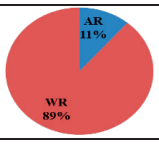
Table 4. Genetic diversity parameters and genetic differentiation of *O. cumana* species/races using 13 ISSR markers

Species/ races		Number of analysed samples	Na	Ne	H	I	Ht	Hs	Gst	Nm (Gst)	NPL	PPL
O. cumana	Mean	269	2.00	1.37	0.23	0.37	0.24	0.18	0.28	1.30	331	100.00
	SD		0.00	0.32	0.17	0.22	0.03	0.02				
E	Mean	23	1.39	1.19	0.12	0.18	0.12	0.08	0.30	1.19	128	38.67
	SD		0.49	0.31	0.17	0.25	0.03	0.02				
G	Mean	78	1.78	1.37	0.22	0.34	0.21	0.10	0.52	0.45	259	78.25
	SD		0.41	0.36	0.19	0.26	0.04	0.01				
H	Mean	168	1.89	1.37	0.23	0.36	0.25	0.09	0.62	0.30	294	88.82
	SD		0.32	0.33	0.17	0.23	0.03	0.01				

Na=observed number of alleles; Ne=effective number of alleles; H=Nei's gene diversity; I=Shannon's Information index; Ht=total gene diversity; Hs=gene diversity within populations; Gst=(Ht-Hs)/Ht, coefficient of gene differentiation among populations; Nm=gene flow among populations from Gst; NPL=number of polymorphic loci; PPL=percentage of polymorphic loci; SD=standard deviation.

AMOVA analysis was carried out on the distance matrix, demonstrating significant genetic differences between and within races. Of the total genetic diversity, 11% was due to differences among races and 89% was contributed to differences within races ($P<0.001$) (Table 5). Out of this analysis, it follows that the highest genetic diversity of broomrape occurred within races (89%).

Table 5. AMOVA analysis of *O. cumana* different origin populations grouped by race status based on ISSR data

Source of variation	Degree of freedom, df	Sum of squares, SS	Mean squares, MS	Estimate of variance component, Est. Var.	Differentiation, (Phi), P<0.001	Molecular dispersion distribution diagram
Among races	2	718.427	359.214	4.627	PhiPT=0.112	
Within races	266	9713.335	36.516	36.516		
TOTAL	268	10431.76	-	41.143	P<0.001	

Our results confirm that the races at the first stage of divergent evolution appear and develop within populations in the geographically grouped regions, being influenced under the same environmental factors. For these reasons, the process of natural selection has a more or less uniform character, so that most races possess a common pool of genes (Duca & Martea, 2020) and common phenotypic characters (Clapco et al., 2020). The pairwise race PhiPT values revealed minimum variation between races H and G (0.096), similar to H and E (0.104). Maximum variation between races G and E (0.231) was established (Figure 4).

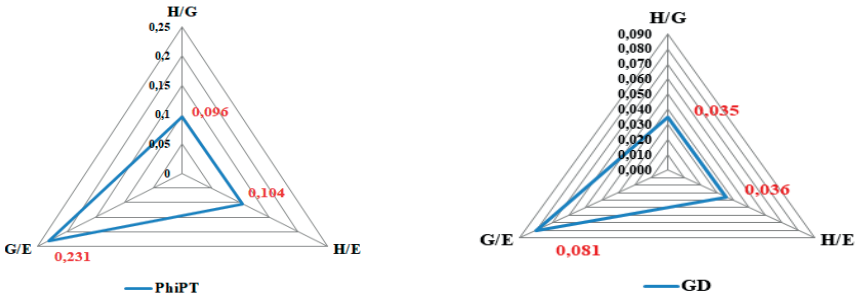


Figure 4. Pairwise comparisons between races on based PhiPT values (on the left) and Nei's unbiased measures of genetic distance (on the right)

Nei's unbiased measures of genetic distance (GD) between broomrape races were calculated with POPGENE (version 1.32) using ISSR data. Based on the results for pairwise comparisons of GD values between 3 races was identified a closer genetic similarity between races G and H (0.035), more genetic distant were races E and H (0.036) and the most genetically distant were found to be races E and G (0.081) (Figure 4).

Relationships between races were further illustrated by dendrograms with the UPGMA method based on Nei's genetic distance and Ward's method by means of Euclidean distance. In both cases, the broomrape populations on race category were classified into 2 groups: the first group combined populations belonging to race G and H, whereas populations of race E fell into separate second group (Figure 5).

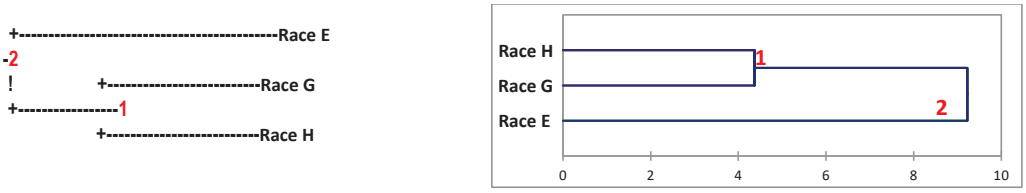


Figure 5. Dendrogram generated using UPGMA method with Nei's genetic distance (on the left) and Ward's method by means of Euclidean distance (on the right), showing relationships between 3 broomrape races, using ISSR data

CONCLUSIONS

The presented data are intended to fill the gaps in knowledge of genetic structure of the broomrape populations at the race level that are characteristic of the Black Sea basin.

1. ISSR analysis of 23 broomrape populations from Black Sea region revealed quite high levels of DNA polymorphism (93.87%). All 13 primers demonstrated a good capacity for race differentiation that is already found at the level of percentage of polymorphism index (races E-86.64%, G-90.80% and H-94.48%). The discrimination power of ISSR markers at race level was observed on most of the molecular-genetic indices and was graphically identified by the cluster analysis.

2. The comparative evaluation of genetic diversity between races E, G and H detected the different levels of variability at them. Monomorphic loci were observed only in the race E, while the specific loci were detected in all races (E-11.63%, G-8.88% and H - 5.44%). The highest percentage of specific loci was found for race E that it is more distant from races G and H in evolutionary time. The frequency distribution analysis confirms this conclusion and reveals that the races G and H are more similar to each other.

3. PhiPT coefficient values and genetic distance data have led to similar conclusions about evolutionary relationships between three broomrape races: the race E has a bigger molecular difference to that of the race G than from H, the last two races being related

genetically most closely. Clustering of populations revealed the pattern of genetic and evolutionary subdivision into 2 groups: the most virulent new races G and H were comprised in the first and less virulent race E in the second, thus being confirmed by the physiological data and genetic relationships detected by using ISSR markers.

ACKNOWLEDGEMENTS

This research was carried out using the broomrape seeds kindly provided by prof. dr. Yalcin Kaya (Trakya University, Edirne, Turkey), dr. Valentina Encheva (Dobrudzha Agricultural Institute, General Toshevo, Bulgaria), dr. Maria Joiţa-Păcureanu (National Agricultural Research and Development Institute, Fundulea, Romania). It was financed from Project of the State Program 20.80009.5107.01 *Genetic, molecular and biotechnological studies of the sunflower in the context of sustainable management of agricultural ecosystems.*

REFERENCES

Arhiva Naţională a Republicii Moldova. Fond 3, inv. 2, d. 376.

Batchvarova, R. (2014). Current situation of sunflower broomrape in Bulgaria. *Proceedings of the Third International Symposium on Broomrape (Orobanche spp.) in Sunflower,* Córdoba, Spain, June 3rd to 6th, 51–54.

Benharrat, H., Veronesi, C., Theodet, C., & Thalouarn, P. (2002). Orobanche species and population discrimination using intersimple sequence repeat (ISSR). *Weed Research, 42*(6), 470–475.

Clapco, S. (2021). Virulence and aggressiveness of some sunflower broomrape populations belonging to different countries. *Scientific Papers. Series A. Agronomy*, *LXIV*(1), 266–272.

Clapco, S., Port, A., Wang, C., & Duca, M. (2020). The study of broomrape diversity in different sunflower cultivating countries based on morphological parameters of parasite seeds. *Lucrări Ştiinţifice. Seria Agronomie*, *1*(63), 135–140.

Demirci, M., & Kaya, Y. (2009). Status of *Orobanche cernua* Loefl. and weeds in sunflower production in Turkey. *Helia, 32*(51), 153–160.

Duca, M. (2014). Current situation of sunflower broomrape in the Republic of Moldova. *Proceedings of the Third International Symposium on Broomrape (Orobanche spp.) in Sunflower,* Córdoba, Spain, 3–6 June, 44–50.

Duca, M., Acciu, A., & Clapco, S. (2017). Distribuţia geografică şi caracteristica unor populaţii de *O. cumana* din Republica Moldova. *Buletinul Academiei de Ştiinţe a Moldovei. Ştiinţele Vieţii, 2*(332), 65-76.

Duca, M., Joiţa-Păcureanu, M., Port, A., Martea, R., Boicu, A., Rîşnoveanu L., & Clapco S. (2019) Genetic diversity analysis of sunflower broomrape populations from Republic of Moldova using ISSR markers. *Romanian Agricultural Research, 37,* 89–97.

Duca, M., & Martea, R. (2020). Using different dataset and different algorithms for genetic diversity analysis in sunflower broomrape. *International Journal of Advanced Research in Biological Sciences*, *7*(8), 99–110.

Encheva, V. (2018). Distribution and race composition of broomrape (*Orobanche cumana* Wallr.) in Bulgaria during 2008–2017. *Proceedings of the 4th International Symposium on Broomrape in Sunflower,* Bucharest, Romania, 2–4 July, 58.

Evci, G., Sezer, N., Pekcan, V., Yılmaz, M.I., & Kaya, Y. (2011). Broomrape control in sunflower production in Turkey. *Buletinul AŞM. Ştiinţele vieţii, 2*(314), 111–117.

Fernández-Martínez, J.M., Velasco, L., & Pérez-Vich, B. (2012). Progress in research on breeding for resistance to sunflower broomrape. *Helia*, *35*(57), 47–56.

Gisca, I., Acciu, A., Glijin, A., & Duca, M. (2013). Highly virulent races of sunflower broomrape in the Republic of Moldova. *Proceedings of European Biotechnology Congress. Current Opinion in Biotechnology, 24*(1), 132.

Gupta, M., Chyi, Y.S., Romero-Severson, J., & Owen, J.L. (1994). Amplification of DNA markers from evolutionarily diverse genomes using single primers of simple-sequence repeats. *Theoretical and Applied Genetics, 89*(7-8), 998–1006.

Kaya, Y. (2014). Current situation of sunflower broomrape in Turkey. *Proceedings of the Third International Symposium on Broomrape (Orobanche spp.) in Sunflower,* Córdoba, Spain, 3–6 June, 55.

Kaya, Y., Evci, G., Pekcan, V., & Gucer, T. (2004). Determining new broomrape infested areas, resistant lines and hybrids in Trakya region of Turkey. *Helia*, *27*(40), 211–218.

Louarn, J., Boniface, M.-C., Pouilly, N., Velasco, L., Pérez-Vich, B., Vincourt, P., & Muños, S. (2016). Sunflower resistance to broomrape (*Orobanche cumana*) is controlled by specific QTLs for different parasitism stages. *Frontiers in Plant Science*, *7*, 14.

Lüdtke, R., Agostini, G., Miotto, S.T.S., & Souza-Chies, T.T. (2010). Characterizing *Polygala* L. (Polygalaceae) species in Southern Brazil using ISSR. *Plant Molecular Biology Reporter*, *28*(2), 317–323.

Lukomets, V.M., & Antonova, T.S. (2015). *Zarazikha (Orobanche cumana Wallr.) na podsolnechnike i mery borby s nei.* Maslichnye kultury. Nauchno-tekhnicheskii byulleten Vserossiiskogo nauchno-issledovatelskogo instituta maslichnykh kultur. *3*(163), 6.

Melero-Vara, J.M., Domínguez, J. & Fernández-Martínez J.M. (1989). Evaluation of differential lines

and a collection of sunflower parental lines for resistance to broomrape (*Orobanche cernua*) in Spain. *Plant Breeding*, *102*(4), 322–326.

Melero-Vara, J.M., Dominguez, J., & Fernandez-Martinez, J.M. (2000). Update on sunflower broomrape situation in Spain: racial status and sunflower breeding for resistance. *Helia*, *23*(33), 45–55.

Molinero-Ruiz, L., Delavault, P., Pérez-Vich, B., Pacureanu-Joita, M., Bulos, M., Emiliano Altieri, M., & Domínguez, J. (2015). History of the race structure of *Orobanche cumana* and the breeding of sunflower for resistance to this parasitic weed: A review. *Spanish Journal of Agricultural Research*, *13*(4), 19.

Molinero-Ruiz, L., García-Carneros, A.B., Collado-Romero, M., Raranciuc, S., Domínguez, J., & Melero-Vara, J.M. (2013). Pathogenic and molecular diversity in highly virulent populations of the parasitic weed *Orobanche cumana* (sunflower broomrape) from Europe. *Weed Research, 54*(1), 87–96.

Pacureanu, M. (2014). Current situation of sunflower broomrape (*Orobanche cumana* Wallr.) in Romania. *Proceedings of the Third International Symposium on Broomrape (Orobanche spp.) in Sunflower,* Córdoba, Spain, 3–6 June, 39–43.

Petrov, D. (1968). A new physiological race of broomrape (*Orobanche cumana* Wallr.) in Bulgaria. *Comptes Rendus de l'Academie Bulgare des Sciences, 1,* 27–30.

Pimakhin, V.F., Lekarev, V.M., & Sokolov N.M. (2000). *Biologicheskie i agrotekhnicheskie osnovy vozdelyvaniya podsolnechnika: Rekomendatsii.* Saratov: Izd-vo NIISKh Yugo-Vostoka.

Pinashkin, N.N. (2012). *Vliyanie agrotekhnicheskikh priemov na urozhainost i maslichnost podsolnechnika v zone yuzhnykh chernozemov Volgogradskoi oblasti.* Dissertatsiya na soiskanie uchènoi stepeni kandidata selskokhozyaistvennykh nauk. Volgograd.

Pineda-Martos, R., Velasco, L., Fernandez-Escobar, J., Fernandez-Martinez, J.M., & Pérez-Vich, B. (2013). Genetic diversity of *Orobanche cumana* populations from Spain assessed using SSR markers. *Weed Research, 53*(4), 279–289.

Powell, W., Morgante, M., Andre, C., Hanafey, M., Vogel, J., Tingey, S., & Rafalski, A. (1996). The comparison of RFLP, RAPD, AFLP and SSR (microsatellite) markers for germplasm analysis. *Molecular Breeding, 2*(3), 225–238.

Prevost, A., & Wilkinson, M.J. (1999). A new system of comparing PCR primers applied to ISSR fingerprinting of potato cultivars. *Theoretical and Applied Genetics, 98*(1), 107–112.

Rîşnoveanu, L., Joiţa-Păcureanu, M., & Anton, F.G. (2016). The virulence of broomrape (*Orobanche cumana* Wallr.) in sunflower crop in Braila Area, in Romania. *Helia*, *39*(65), 189–196.

Rodríguez-Ojeda, M.I., Pineda-Martos, R., Alonso, L.C., Fernández-Escobar, J., Fernández-Martínez, J.M., Pérez-Vich, B., & Velasco, L. (2013). A dominant avirulence gene in *Orobanche cumana* triggers *Or5* resistance in sunflower. *Weed Research*, 53(5), 322–327.

Roldán-Ruiz, I., Dendauw, J., Van Bockstaele, E., Depicker, A., & De Loose, M. (2000). AFLP markers reveal high polymorphic rates in ryegrasses (*Lolium* spp.). *Molecular Breeding, 6*(2), 125–134.

Sambrook, J., & Russell, D. (2001). *Molecular cloning. A laboratory manual.* New York: Cold Spring Harbor Laboratory Press.

Semerci, A., Kaya, Y., Sahin, I., & Citak, N. (2010). Determination of the performances and adoption levels of sunflower cultivars based on resistance to broomrape in farm conditions in Thrace region. *Helia, 33*(53), 69–76.

Sharova, P.G. (1969). Selekcija podsolnechnika na immunitet k agressivnym rasam moldavskoj populjacii zarazih. *Trudy V Vsesojuzn. Soveshhanija po immunitetu rastenij*. Kiev, 28–31.

Shindrova, P. (2006). Broomrape (*Orobanche cumana* Wallr.) in Bulgaria – distribution and race composition. *Helia*, *29*(44), 111–120.

Shindrova, P., & Penchev, E. (2012). Race composition and distribution of broomrape (*Orobanche cumana* Wallr.) in Bulgaria during 2007–2011. *Helia, 35*(57), 87–94.

Shindrova, P., Ivanov, P., & Nikolova, V. (1998). Effect of broomrape (*Orobanche cumana* Wallr.) intensity of attack on some morphological and biochemical indices of sunflower (*Helianthus annuus* L.). *Helia*, *21*(29), 55–62.

Strelnikov, E.A. (2017). *Analiz rasovoi struktury populyatsii zarazikhi Orobanche cumana Wallr. i sozdanie ustoichivogo iskhodnogo materiala podsolnechnika.* Dissertatsiya na soiskanie uchènoi stepeni kandidata biologicheskikh nauk. Krasnodar.

Vranceanu, A.V., Tudor, V.A., Stoenescu, F.M., & Pirvu, N. (1980). Virulence groups of *Orobanche cumana* Wallr. differential hosts and resistance sources and genes in sunflower. *Proceedings of the International Sunflower Conference*, Torremolinos, Spain, 8–13 July, 74–80.

Zietkiewicz, E., Rafalski, A., Labuda, D. (1994). Genomic fingerprinting by simple sequence repeat (SSR) anchored polymerase chain reaction amplification. *Genomics*, *20*(2), 176–183.

Scientific Bulletin. Series F. Biotechnologies, Vol. XXVII, No. 2, 2023
ISSN 2285-1364, CD-ROM ISSN 2285-5521, ISSN Online 2285-1372, ISSN-L 2285-1364

MECHANISM OF TOXICITY AND MICROSCOPICAL TESTS FOR *Datura* HERB

Monica ENACHE, Radu MORARU

University of Agronomic Sciences and Veterinary Medicine of Bucharest, 59 Marasti Blvd, District 1, Bucharest, Romania

Corresponding author email: monica.enache@biotehnologii.usamv.ro

Abstract

Datura sp. and related plants mandrake (Mandragora officinarum and M. autumnalis), black henbane or stinking nightshade (Hyoscyamus niger), belladonna (Atropa belladonna) and angel's trumpets (Brugmansia sp.) are considered some of the deadliest plant species. Datura stramonium (jimson weed, thorn apple or devil's snare) is a weed that sometimes is cultivated as ornamental for its large, white funnel-shaped flowers, but it is considered dangerous due to accidental or deliberate ingestion of any plant parts. Tropane alkaloids (atropine, hyoscyamine, scopolamine) with strong anticholinergic properties can cause a variety of adverse effects that can lead to death in humans and other animals. They are pharmaceutically active and their mind-altering properties have been known since ancient times. Characteristic toxic symptoms can also occur after skin penetration alone and neither drying nor boiling decrease the plant toxicity. Naturally occuring alkaloids (for example from Stramonii folium and Daturae innoxiae herba) or synthetic and semisynthetic substitutes have found use in modern medicine too. The present paper comprises a review on the mechanism of toxicity and photomicrographs of characteristic microscopic features of Datura leaf.

Key words*: anticholinergic effect; Datura sp.; light microscopy analysis; optical microscope; tropane alkaloids.*

INTRODUCTION

Datura sp., *Mandragora* sp. (mandrake), *Hyoscyamus niger* (black henbane or stinking nightshade), *Atropa belladonna* (belladonna or deadly nightshade), *Brugmansia* sp. (angel's trumpets) and other related species of the Solanaceae family are considered some of the deadliest species in the plant world. They are rich in tropane alkaloids with strong anticholinergic properties. The accidental or deliberate ingestion of fresh or dry plant parts can cause a variety of adverse effects that can lead to death in humans and other animals. Characteristic toxic symptoms can also occur after skin penetration alone and neither drying nor boiling decrease the plant toxicity.

The genus *Datura* has a confused taxonomy because of the variations that appear in different environmental conditions (Mahr, 2023). There are only 8 or 9 species, for example *D. stramonium, D. metel, D. inoxia*. According to Preda (1989), *Datura stramonium* is a herbaceous annual plant, 0.30-1.20 m tall, with unpleasant smell, that sometimes is cultivated as ornamental for its large, white funnel-shaped flowers, that appear throughout summer and autumn. The fruit of *Datura inoxia* and *D. stramonium* is round and spiky and contains black seeds (Figure 1). *Datura* plants tend to self-seed, are fast-growing and can become invasive (Mahr, 2023) (Figure 2).

It is believed the genus *Datura* is native to Central America and Mexico and to East India (Preda, 1989), but it has become naturalised in temperate and tropical regions world-wide (Mahr, 2023).

The genus *Datura* used to also include the semi-woody small trees or shrubs (~3-6 m height) such as *Datura arborea* and *Datura suaveolens* (angel's trumpets), which are now classified in the separate genus *Brugmansia* (Lockwood, 1973). *Brugmansia* are popular ornamental plants, highly appreciated for their large (20-30 cm long), pendulous, trumpet-shaped flowers. They are grown as ornamental in their native tropical regions of South and Central America, and also as container plants worldwide (Figure 3).

Figure 1. (a) *Datura* plant growing in Bucharest (summer 2018); (b) *Datura* fruit

Figure 2. Self-sown dense group of *Datura* plants in Bucharest (summer 2021)

Figure 3. (a)-(b) *Brugmansia* sp. growing in Mexico City (Photos used with permission from Ref. Mrs A.T.E., 2018)

Both *Datura* and *Brugmansia* species contain the same major toxic tropane alkaloids - scopolamine, hyoscyamine and atropine (Evans & Lampard, 1972) that are also pharmaceutically active and have mind-altering properties, known since ancient times in some cultures.

The naturally occuring alkaloids obtained from

dried *Datura stramonium* leaves, or from dried leaves and flowering tops of *D. metel* or *D. innoxia* (the latter two are still being cultivated for scopolamine extraction) or synthetic and semisynthetic substitutes have found use in modern medicine too (Gîrd, 2010; Gîrd et al., 2010; Wink, 2015).
The present paper presents a review on the mechanism of toxicity and photomicrographs of a light microscopy analysis of *Datura* sp. leaf.

MICROSCOPIC ANALYSIS

Materials and methods

The present study was carried out at the Laboratory of Biology of the Faculty of Biotechnologies, the University of Agronomic Sciences and Veterinary Medicine of Bucharest, using dry plant material. Wet mounts of hand-sections or surface preparations of plant material were examined whole or after clarification, using a Novex Holland optical microscope. To colour, toluidine blue was sometimes added (Figure 4).
Microscopic images have been photographed with a Sony Cyber-shot® digital camera (Carl Zeiss Vario-Tessar 5× zoom lens) or with an S-Eye 2.0 microscope digital camera, and were later compared to descriptions found in the literature (for example Pharmacognosy, 2023; for the pollen: Bombosi & Heigl, 2020 and Stebler, 2023).

Figure 4. *Datura* sp.: (a) dry plant material and (b) wet mount of clarified leaf used in the present study

Results and discussions

Several microscopic features cited for *Datura* sp. were seen in the leaves:

- epidermis in surface view showing anisocytic stomata (Figures 5-7);
- cluster crystals of calcium oxalate, sometimes prismatic calcium oxalate crystals are also present in cells of the crystal layer (Figures 8-9);
- covering multicellular, uniseriate, warty trichomes with a large base (Figure 10);
- vessels with spiral thickenings (Figure 11);
- pollen grains were also found caught on the leaf (Figures 12-13).

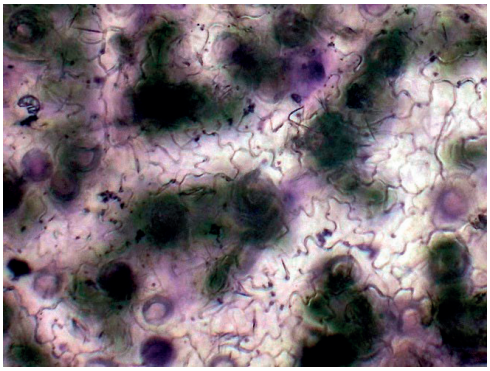


Figure 5. Lower epidermis in surface view, cells have wavy walls

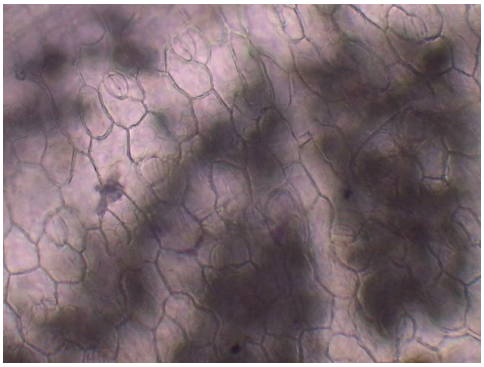


Figure 6. Upper epidermis in surface view

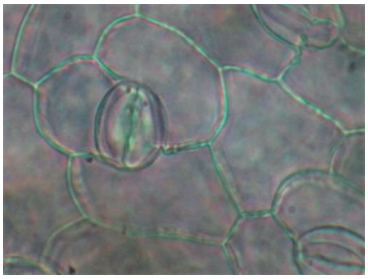


Figure 7. Anisocytic stomata in upper epidermis

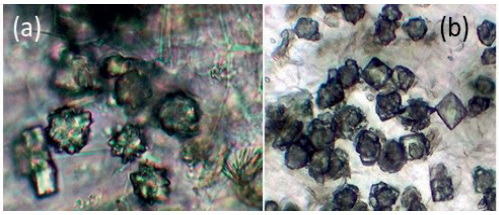


Figure 8. (a) Cluster crystals of calcium oxalate, (b) sometimes also calcium oxalate prisms are present in *Datura stramonium* leaves

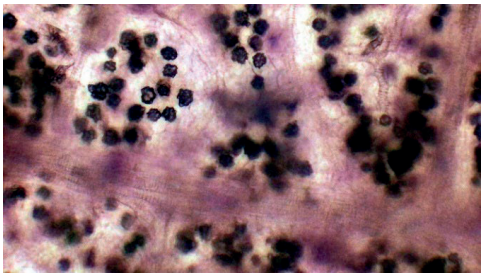


Figure 9. Surface view of the crystal layer in *Datura* leaf

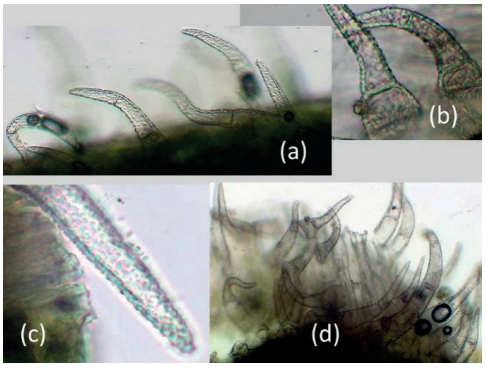


Figure 10. (a)-(d) Covering trichomes in *Datura* leaves

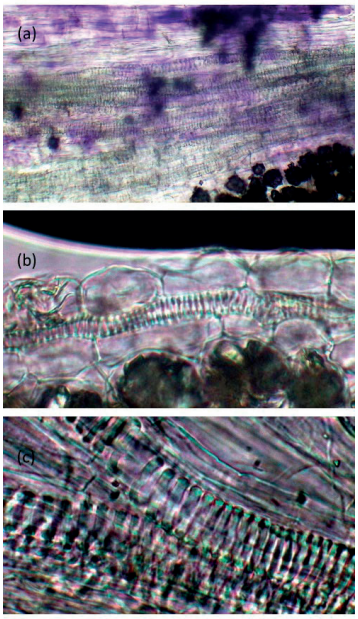


Figure 11. (a)-(c) Vessels with spiral thickenings in *Datura* leaf

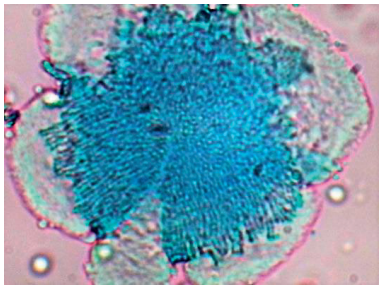


Figure 13. *Datura* sp. pollen grain rupture – striate ornamentation

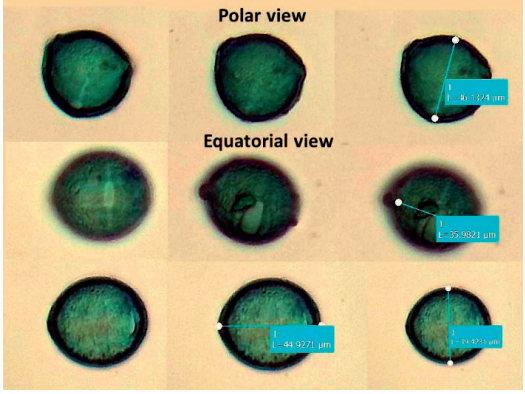


Figure 12. Medium size, tricolporate *Datura* sp. pollen grains

MECHANISM OF TOXICITY

The tropane alkaloids present in *Datura* species have strong anticholinergic properties and are present in all plant parts, but highest concentration is found in seeds and roots (Wink, 2010).

The cholinergic synapses are using acetylcholine (ACh) as a neurotransmitter. Acetylcholine was the first neurotransmitter discovered and it is found in the brain, neuromuscular junctions and the autonomic ganglia (Table 1).

There is a vast literature on the cell biology of the cholinergic synapse (a review can be found in Whittaker, 1990, for example), but relevant information is presented below from Waymire (2023) and Auf Der Heide (2007):

Firstly, acetylcholine is synthesized in the presynaptic (cholinergic) neuron and transported to the end of the axon where it is taken up into secretory vesicles by active transport. Ca^{2+} signaling controls the fusion of the secretory vesicle membrane with the neuron membrane and the acetylcholine secretion in the synaptic space. Then, acetylcholine binds to the cholinergic receptors of the postsynaptic cells, namely the nicotinic (nAChR) and muscarinic receptors (mAChR). ACh binds only briefly to the receptors. After dissociation from the receptor, ACh is rapidly hydrolyzed by the enzyme acetylcholinesterase (AChE). AChE is synthesized in the cell body of cholinergic and non-cholinergic neurons and distributed throughout the neuron. AChE is present in the extracellular matrix.

Drugs that inhibit ACh breakdown are effective in altering cholinergic neurotransmission. This inhibition is produced because ACh molecules accumulate in the synaptic space and keep the receptors occupied. Cholinesterase inhibitors also include certain insecticides and chemical warfare nerve agents.

Datura toxic tropane alkaloids are naturally occurring cholinergic blocking agents that act directly by blocking ACh from binding to muscarinic receptors, because they are competitive antagonists to acetylcholine at muscarinic receptors.

Since these substances bind to the muscarinic cholinergic receptors postsynaptically without activating them, they are muscarinic antagonist or antimuscarinic agents.

The muscarinic receptors are G-protein-coupled receptors that activates a heterotrimeric G-protein when bound to extracellular ACh. Muscarinic receptors do not influence skeletal muscles, but they influence the activity of smooth muscle, exocrine glands and the cardiac conduction system (Table 1).

In the case of plant ingestion the symptoms of toxicity appear after 1-4 hours and include tachycardia, disturbances of vision, dilated pupils due to mydriatic effect, dry skin, dry mouth, inhibition of motility of the gastrointestinal tract, urinary retention, restlessness, delirium, disorientation, hallucinations, convulsions and could lead to coma and death (Dodd-Butera & Broderick, 2005; Serrano, 2018; Wink, 2015).

Table 1. Location of cholinergic synapses in the central and peripheral nervous system (Auf Der Heide, 2007; Waymire, 2023)

CNS	Between neurons in the brain and spinal cord (nAChR, mAChR)		
PNS	Somatic nervous systems	At the neuromuscular junction between the motor nerve and skeletal muscle (nAChR)	
	Autonomic nervous systems	Sympathetic nervous systems	In the preganglionic neurons (nAChR)
			In the postganglionic neurons that inervate the sweat glands (mAChR)
		Parasympathetic nervous systems	In the preganglionic neurons (nAChR)
			In the postganglionic neurons that inervate the heart, exocrine glands and smooth muscle (mAChR)

CONCLUSIONS

Datura species are plants associated with poisoning, but they have applications in the pharmaceutical industry. Recent reports of intoxications from *Datura* use as a recreational drug show the need for a more increased awareness of its harmful and deadly consequences.

REFERENCES

A.T.E. Mrs., (2018), Personal photos collection from Mexico City own garden,.presented in Figure 3. (a)-(b); Unpublished work.

Auf Der Heide, E. (Agency for Toxic Substances and Disease Registry) (2007). Cholinesterase Inhibitors: Including Insecticides and Chemical Warfare Nerve Agents. Part 4: The Cholinergic Toxidrome. Retrieved June 4th, 2023, from https://www.atsdr.cdc.gov/csem/cholinesterase-inhibitors/toxidrome.html.

Bombosi P., Heigl H. 2020. Datura stramonium. In: PalDat - A palynological database. https://www.paldat.org/pub/Datura_stramonium/304468; accessed 2023-06-03.

Evans, W.C. & Lampard, J.F. (1972). Alkaloids of *Datura suaveolens. Phytochemistry*, *11*(11): 3293–3298.

Dodd-Butera, T. & Broderick, M. (2005). Plants, Poisonous. *Encyclopedia of Toxicology* (Second Edition). Elsevier Inc., pp. 443-448.

Gîrd, C.E. (2010). Curs de Farmacognozie, Fitochimie, Fitoterapie. Vol. II. Editura Curtea Veche, Bucureşti.

Gîrd, C.E., Duţu, L.E., Popescu, M.L., Iordache, A.T., Tudor, I., Costea, T. (2010). Bazele teoretice şi practice ale analizei farmacognostice, vol. II, Editura Curtea Veche, Bucureşti. https://doi.org/10.1016/S0031-9422(00)86392-X.

Lockwood, T.E. (1973). Generic recognition of *Brugmansia*. *Botanical Museum Leaflets, 23*(6), 273-283.*https://ia902805.us.archive.org/27/items/biostor-160868/biostor-160868.pdf.*

Mahr, S. (2023). Datura. University of Wisconsin – Madison. Retrieved June 2nd, 2023, from *https://hort.extension.wisc.edu/articles/datura/.*

Pharmacognosy (2023). Herbs – *Datura stramonium*. Retrieved June 2nd, 2023, from https://thepharmacognosy.com/datura-stramonium/.

Preda, M. (1989). *Dicţionar dendrofloricol*. Editura Ştiinţifică şi Enciclopedică, Bucureşti.

Serrano, R. (2018). Toxic Plants: Knowledge, Medicinal Uses and Potential Human Health Risks. *Environment and Ecology Research 6,* pp. 487-492. DOI:10.13189/eer.2018.060509.

Stebler Th. (2023). *Datura inoxia*, Pollen-Wiki. Retrieved June 3rd, 2023, from https://pollen.tstebler.ch/MediaWiki/index.php?title=Datura_inoxia.

Waymire, J.C. (2023). Acetylcholine Neurotransmission. In: The Department of Neurobiology and Anatomy, McGovern Medical School at UTHealth: *Neuroscience online*. Section 1: cellular and Molecular Neurobiology. https://nba.uth.tmc.edu/neuroscience/m/s1/chapter11.html

Whittaker, V.P. (1990). The cell and molecular biology of the cholinergic synapse: twenty years of progress. In: Sten-Magnus Aquilonius, Per-Göran Gillberg (Editors), *Progress in Brain Research,* Volume 84. Elsevier, pp. 419-436. https://doi.org/10.1016/S0079-6123(08)60926-7.

Wink, M. (2010). Mode of action and toxicology of plant toxins and poisonous plants. Mitt. Julius Kühn-Inst. 421, pp. 93-112. https://core.ac.uk/download/pdf/235685921.pdf.

Wink, M. (2015). Modes of Action of Herbal Medicines and Plant Secondary Metabolites. *Medicines 2*, 251-286; doi:10.3390/medicines2030251.

Scientific Bulletin. Series F. Biotechnologies, Vol. XXVII, No. 2, 2023
ISSN 2285-1364, CD-ROM ISSN 2285-5521, ISSN Online 2285-1372, ISSN-L 2285-1364

BIODEGRADATION OF PLASTIC POLLUTANTS AND IDENTIFICATION OF MICROORGANISMS CAPABLE OF DEGRADING PLASTIC POLLUTANTS: REVIEW

Andreea TUDOR (DUMITRU)

University of Agronomic Sciences and Veterinary Medicine of Bucharest, 59 Marasti Blvd, District 1, Bucharest, Romania

Corresponding author email: eea.tudor@gmail.com

Abstract

Plastic can be a good solution for the industry and, even if plastic products might be designed and produced for circular usage, conventional methods production of it and waste management are not reliable solutions anymore.
Plastic pollution became a very serious problem for the environment around the world today. Several studies shown us that microplastic is a very dangerous form of pollution and increasing amounts of it can be found in animals and human's bodies. However, recent studies provide us important data about the involvement of microorganisms in biodegradation of polymer materials.
Biodegradation process is based on the ability of some microorganisms to degrade certain plastic pollutants through their metabolic activity. Many microorganisms have the ability to secrete specific degradation enzymes that participates in degrading processes of plastic.
In this review we discuss about various microorganisms and their role in plastic degradation. We review different types of approaches and applications of molecular biology used for identifying microorganisms capable of degrading polymers and key genes involved in polymers degradation.

Key words*: biodegradation, pollution, microorganism, polymers, molecular biology.*

INTRODUCTION

Although, in the last decades, new plastic products have been introduced to the market, claiming to be better for the environment, lately plastic waste poorly managed turned into a very serious threat: plastic pollution, which became a real problem for the environment and human health (Bahl et al., 2020). Numerous studies reveal that due to the massive pollution with plastic wastes, microplastic particles in increasingly large quantities are found in humans and animal's bodies (Zhang et al., 2020).
With the increasing accumulation of plastic pollutants, modern society is facing a serious environmental global problem (Ali et al., 2021). The effects of plastic pollution started to became important threats for the well-being of marine and earth environment, but also for human health too (Emmanuel-Akerele et al., 2022).
The reason for the massive accumulation of plastic on agricultural land and the ocean is the low rate of degradation of artificial polymer products (Emmanuel-Akerele et al., 2022).
Annually, over 380 million metric tons (MMT) of polymeric products are manufactured, with a significant portion of approximately 10 MMT contaminating water bodies and oceans (Edwards et al., 2022). It was estimated that by the year of 2010, 275 MMT of plastic wastes have been generated and between 4.8 and 12.7 MMT of it ended up in the ocean (Jambeck et al., 2015). Now is estimated that around 80% of the plastic ever created will be discarded in the nature and that's because half of the amount of produced plastic is designed for single use (Edwards et al., 2022).
Plastic products are mainly produced from polyethylene terephthalate (PET), polyvinylchloride (PVC), polyethylene (PE), polystyrene (PS), polypropylene (PP), polyurethane (PUR) and polyamide (PA) (Edwards et al., 2022). Recent data suggested that more than 500 billion tons of PE is produced every year to fulfil the global request (Mohanan et. al., 2020).
When plastic waste is discarded into rivers, it ultimately makes its way to the oceans, resulting

in the destruction of the ecosystem (Fleming et al., 2014; Rachmawati et al., 2021). Scientists worldwide have reported that 267 species from aquatic environments are impacted by plastic waste (Ru et al., 2020).

It is known that plastic waste is decomposed in nature by oxidation, abrasion, sunlight and/or sunlight, but these natural-occurring processes take around 100 up to 500 years to completely decompose the wastes and, during this process, microplastic is formed (Law and Thompson, 2014; Rachmawati et al., 2021). Hence, it can be concluded that the slow rate of plastic decomposition and the formation of microplastic may result in toxic particle ingestion by both land and aquatic animals, as well as humans thorough the food chain (Ru et al., 2020).

According to De Tender et al. (2017) study, because of the slow degrading rate of plastic, plastic wastes can serve as a perfect media for bacterial colonisation. Also, another study conducted by Harrison et al. (2014) shown that colonisation on plastic wastes with microorganisms is faster than on other surfaces and the colonizing communities are taxonomically distinct from those in the unpolluted ecosystems (Zettler et al., 2013).

In 2012 Yoon et al. isolated several species of microorganisms like *Moritella*, *Pseudomonas* sp., *Streptomyces* sp., *Bacillus* sp., *Staphylococcus* sp., *Shewanella*, *Psychrobacter* capable of degrading plastic. Furthermore, numerous studies isolated and shown that fungus like *Aurebasidium pullulans*, *Fusarium solani, Curvularia senegalensis* and *Aspergillus* sp. are capable of using plastic waste as a sole carbon source (Sivan, 2011; Pramila and Ramesh, 2011; Usha et al., 2011; Rachmawati et al., 2021).

Biodegradation of plastic wastes is very complicated because of the unpredictable behaviour of microorganisms and it depends on many factors like morphology or surface characteristics and also molecular weight of the polymers (Mohanan et al., 2020; Ru et al., 2020).

As mentioned earlier, the process of polymer biodegradation is complex, and the type of plastic pays a significant role in determining its biodegradability.

Several studies have indicated that *Phormidium lucidum* and *Oscillatoria subbrevis* cyanobacteria, as well as *Galleria mellonella* and *Achroia grisella* waxworms, and bacterial strains such as *Enterobacter asburiae* and *Bacillus* sp., are capable of breaking down polyethylene (PE) (Mohanan et al., 2020; Saeed et al., 2022). Metabolic activity of these microorganisms plays a key role in biodegradation by secreting polyester-degrading enzymes (Shinozaki et al., 2013; Sriyapai et al., 2018; Mohanan et al., 2020).

Due to the many variables involved in biodegradation, including changes in the chemical structure of polymers, carbon dioxide emissions and substrate weight loss, recent studies and experiments have been focused on examining microbial activity, as well as isolating and identifying microorganisms that are capable of degrading plastic (Mohanan et al., 2020).

POLYETHYLENE BIODEGRADATION FOR SUISTANABLE WASTE MANAGEMENT

Polyethylene (PE) is a polymer composed of long-chain ethylene monomers (Emmanuel-Akerele et al., 2022) and has an average molecular weight of 300,000 Daltons (Da). Due to this high molecular weight, the microbial degradation rate of PE has been found to be approximately 0.26% to 0.29% after a period of two years. However, pre-treating PE with chemical oxidizing agents, thermos-oxidation and UV light can enhance its biodegradation rate (Mohanan et al., 2020).

Efforts in research have been directed towards discovering microbial strains that have the ability to break down and degrade PE. These strains have been isolated from various environments, including soil, seawater, compost, active sludge and even the gut of insects such as the wax worm (Nowak et al., 2011; Kyaw et al., 2012; Gajendiran et al., 2016; Sen and Raut, 2016; Montazer et al., 2020; Mohanan et al., 2020). Some species of bacteria, such as *Bacillus spp*. (Mohanrasu et al., 2015; Abrusci et al., 2013), *Rhodococcus spp*. (Bonhomme et al., 2003; Gilan et al., 2004; Fontanella et al., 2020) and *Pseudomonas* spp. (Rajandas et al., 2012), as well as fungi like *Aspergillus* (Hasan et al., 2007; Sahebnazar et al., 2010) and *Fusarium*, have been demonstrated the ability to break down PE after

undergoing certain pre-treatments, such as UV and/or thermal treatments (Mohanan et al., 2020).

Several bacterial strains, such as Pseudomonas putida IRN22, *Acinetobacter pittii* IRN19 and *Micrococcus luteus* IRN20, have been found to degrade untreated PE. In addition, different species of *Pseudomonas*, *Comamonas*, *Delftia* and *Stenotrophomonas* have also demonstrated the ability to degrade PE (Kyaw et al., 2012; Yoon et al., 2022; Peixoto et al., 2017; Mohanan et al., 2020).

Emmanuel-Akerele et al., isolated several microorganisms from soil in a study conducted in 2022. Soil samples containing plastic and plastic materials were taken from a dumpsite and studied over six weeks in agar culture and broth under laboratory conditions and their capability of degrading PE was analysed by the weight loss determination method (Emmanuel-Akerele et al., 2022).

To identify microorganisms, present in the sample, Emmanuel-Akerele et al. (2022) used methods described in Bergey's Manual of Systemic Bacteriology, namely Gram staining reaction, biochemical tests like citrate, mannitol test, oxidase, glucose fermentation and coagulase test. A number of 38 microorganisms have been identified, but from all microorganisms identified from the samples, only *Staphylococcus aureus*, *Streptococcus* sp., *Bacillus* sp. and *Micrococcus* sp. shown significant results regarding plastic degradation with the following rates: 25%, 31.2%, 25% and 31.2% during the four to six weeks period of time (Emmanuel-Akerele et al., 2022). In order to determine the degradation rate weight loss method was used. Results shown that the most efficient microorganism with the ability of degrading plastic after 6 weeks was *Staphylococcus aureus* closely followed by *Proteus* sp., *Streptococcus* sp. and *Bacillus* sp. (Emmanuel-Akerele et al., 2022). *Staphylococcus aureus*, *Pseudomonas sp*., *Bacillus sp*. and *Micrococcus sp*. had the highest degradation rate in different types of plastic and plastic waste having significant weight loss percentages, as it follows: 27.7%, 25%, 14.2% and 25% (Emmanuel-Akerele et al., 2022).

In another study from 2022, Saeed et al. aimed to isolate and identify bacteria and fungi that can degrade PE from soil samples collected from dumping sites in Pakistan. After a preliminary screening of microbial isolates, they identified four strains (two bacterial and two fungal) that showed promising biodegradation properties (Saeed et al., 2022). Molecular methods, including 18s rRNA sequencing, were used to identify the strains mentioned. The bacterial strains were found to be *Bacillus licheniformis* and *Achromobacter xylosoxidans*, while the fungal strains were identified as *Aspergillus niger* and *Aspergillus glaucus* (Saeed et al., 2022). A phylogenetic analysis was conducted on MEGA7 using the Neighbour-joining method. Based on their biodegradation screening results, selected strain was chosen for further experimental studies (Saeed et al., 2022). Saeed et al. research demonstrates that these microorganisms can significantly degrade PE leading to a reduction in the polymer's mechanical strength and stability. The biodegradation was confirmed through various techniques, including scanning electron microscopy and Fourier-transform infrared spectroscopy (FTIR). The study highlights the potential of these microorganisms in the degradation of plastic both in vitro and in soil burial methods.

In 2021, Rachmawati et al. conducted a study that aimed to isolate bacteria capable of degrading PE waste from costal ecosystem of Marina Beach in Semarang. Their study aimed to contribute to the understanding of the microbial community involved in plastic waste degradation in the ecosystem and potentially provide new candidates for bioremediation strategies (Rachmawati et al., 2021).

It is interesting to note that each ecosystem has its own unique bacterial diversity, as evidenced by the variations in the numbers and appearances of the different colony morphologies (Rachmawati et al., 2021). The mangrove ecosystem had the highest number if isolates with a diverse range of colony forms and colours, while the costal sand and rock ecosystem had the fewer isolates with similar colour appearances (Rachmawati et al., 2021). This suggests that each ecosystem may harbour distinct bacterial communities with different abilities to degrade plastic waste.

It is promising that eight out of the twelve isolates were able to form bright zones around the colony in the presence of PE and Congo Red

(Rachmawati et al., 2021). The presence of a bright zone surrounding bacterial colonies suggests that the studies isolates have the ability to decompose PE (Rachmawati et al., 2021). The size of the bright zone varied among the isolates, with 3 (three) of them showing the largest brighter zone diameter (Rachmawati et al., 2021). This suggests that these three isolates have a higher ability to utilize PE as a carbon source compared with other isolates (Rachmawati et al., 2021).

Raziyafathima et al. (2016) reported that *Pseudomonas aeruginosa*, *Pseudomonas putida* and *Pseudomonas stutzeri* have been found to degrade PE. Furthermore, it has been reported that *Enterobacteriaceae*, including *Enterobacter cloacae* and *Escherichia coli*, are capable of breaking down PE (Urbanke et al., 2018). Borowski et al. (2019) also documented that *Moraxella* species can degrade PE. Based on the results of Rachmawati et al. (2021) study, as well as the findings of Urbanek et al. (2018) and Borowski et al. (2019), it could be inferred that the isolates obtained from the Marina Beach region possibly belong to *Pseudomonas*, *Enterobacteriaceae* and *Moraxella* species.

In a recent study from 2023, Nademo et al. isolated and screened bacteria for their ability to degrade low-density polyethylene (LDPE) bags using both clear zone and weight loss methods. The most efficient isolates, KS35, KS14 and KS19 were further analysed (Nademo et al., 2023). Molecular identification of the isolates was carried out by 16s rRNA sequencing and the results showed that KS35 had 99% similarity with *Methylobacterium radiotolerans* MN525302, KS119 had 100% similarity with *Methylobacterium fujisawaense* KT720189 and KS14 had 99% similarity with species of *Lysinibacillus fusiformis* (Nademo et al., 2023). Their study suggested that these isolates have a high potential for degrading LDPE bags, which is significant given the global plastic problem.

BIODEGRADING POLYESTER FOR SUSTAINABILITY

In additional to microbial biodegradation, other methods have been explored for the disposal of polystyrene (PS) waste. These include chemical recycling and physical recycling. The process of chemical recycling entails the decomposition of PS into its constituent monomer, styrene, which can be then utilized to create fresh plastic goods. In contrast, physical recycling involves the melting and reprocessing of PS waste to manufacture new products. Both methods have their limitations and challenges, including high energy requirements and the generation of hazardous by-products. Therefore, microbial biodegradation remains a promising approach for the sustainable disposal of PS waste.

Studies have demonstrated that various microbes and microbial enzymes are capable of biodegrading PS, whether is pure PS, modified PS or PS blended with other polymers such as PLA or starch (Mohana et al., 2020). It has also been observed that the biodegradation rate of PS can be improved by blending it with other polymers. Specifically, *Pseudomonas aeruginosa*, *Curvularia species* and *Rhodococcus ruber* have shown the ability to degrade PS in various forms (Shimpi et al., 2012; Motta et al., 2009; Mohanan et al., 2020). Moreover, *Pseudomonas putida* CA-3 has been found to utilize styrene oil as its sole source of carbon and energy to produce PHAs (Ward et al., 2006; Mohanan et al., 2020).

In the same study from 2022, Emmanuel-Akerele et al. used the identified microorganisms to observe their degrading capacity on PS samples. After four, respectively six weeks it was observed that *Staphylococcus* sp. had the highest degrading ability after six weeks, while *Pseudomonas* sp. and *Lactobacillus* sp. did not degrade PS (Emmanuel-Akerele, 2022).

Jiang et al. (2021) reported the isolation of a PS-degrading bacterium identified as *Massilia sp*. FS1903 from the gut of *Galleria mellonella* (Lepidoptera: Pyralidae) larvae that were fed with PS foam. *Galleria mellonella*, a common agricultural pest, has been found to have the ability to biodegrade PE and PS and this phenomenon is likely related to the gut microorganisms of these insects (Yang et al., 2015; Peng et al., 2019; Yang et al., 2020; Jiang et al., 2021). Prior research has established the existence of microbes in the gut of G. *mellonella* larvae that are involved in the degradation of PE, with the identification of the PE-degrading *Enterobacter* sp. D1 strain (Bombelli et al., 2017; Ren et al., 2019; Cassone et al., 2020; Jiang et al., 2021). However, studies focusing on PS-degrading bacteria from the gut of G.

mellonella larvae are relatively scarce. In Jiang et al. (2021) investigation, a PS-degrading bacterium was successfully isolated from the larval gut and identified through a combination of phylogenetic analysis and physiological and biochemical indicators. The study involved feeding *G. mellonella* larvae with PS foam for 21 days, after which their intestinal tissue was employed as a bacterial inoculum to enrich PS-degrading bacteria.

The intestinal cell suspension was placed into a flask with MSM and PS film and shaken for 60 days (Jiang et al., 2021). The remaining PS film was removed and dispersed the resulting enrichment culture onto LB agar plates, which led to isolation of pure bacterial strains (Jiang et al., 2021). During the logarithmic growth stage, genomic DNA was extracted from the bacterial cells using a commercially available kit (Jiang et al., 2021). Universal primers targeting the 16s rRNA gene were used for PCR amplification, and the resulting amplicons were visualized using agarose gel electrophoresis (Jiang et al., 2021). Finally, a phylogenetic tree was constructed using MEGA 5.0 software and Neighbour-joining method, with bootstrap values calculated from 1,000 replications (Jiang et al., 2021).

Based on its physiological and biochemical characteristics, the strains were identified as similar to microorganisms in the genus *Massilia*, which are commonly found in the rumen or large intestine of both humans and animals (Jiang et al., 2021). Phylogenetic analysis of the 16s rRNA gene showed that the tested strains FS1903 and *Massilia suwonensis* 5414s-25 had a high sequence similarity of 79% (Jiang et al., 2021). Considering the results of the physiological and biochemical tests, the strain has been deposited in the GenBank database under the accession number MW138062 (Jiang et al., 2021).

Jiang et al. (2021) study was the first study reporting the identification of a PS-degrading bacterial strain, FS1903, isolated from the gut of *G. mellonella* larvae. Further investigations are also necessary to assess the ability of the larvae to degrade other common plastic types, including PE, PP, PVS and PET, and to identify the underlying mechanisms and pathways responsible for this biodegradation.

EXPLORING THE BIODEGRADATION OF PVC

While there have been some reports of microbial consortia being able to biodegrade PVC materials, the research progress on PVC biodegradation is not as advanced as that for PE (Yang et al., 2014; Restrepo-Florez et al., 2014; Kumar Sen et al., 2016; Montazer et al., 2020; Ru et al., 2020), PET (Yoshida et al., 2016; Tournier et al., 2020; Ma et al., 2018; Edwards et al., 2022) and PS (Peng et al., 2019; Brandon et al., 2018). The degradation of PVC is more difficult compared to other plastics because PVC lacks a hydrolysable ester bond (Zhang et al., 2022). This suggests that there is a significant knowledge gap in understanding the microorganisms and mechanisms involved in PVC biodegradation (Peng et al., 2020; Giacomucci et al., 2019; Zhang et al., 2022).

Zhang et al. (2022) conducted an experiment to investigate whether *Spodoptera frugiperda* larvae could survive solely on PVC film and whether the larval intestinal microbiota could aid in PVC film digestion. Their study led to the isolation of *Klebsiella* strain EMBL-1, which was found to be capable of using PVC films as a sole source of energy and carbon. This finding is significant as it represents the first report of a bacterium capable of degrading PVC, and provides a potential avenue for developing bioremediation strategies to address PVC pollution (Zhang et al., 2022).

Zhang et al., in 2022, conducted an experiment, in triplicate, to verify the discovery that larvae of *S. frugiperda* can consume PVC film for survival. They compared the key physiological indexes (survival rate and body weight) and intestinal microbiota among larvae under three different conditions: starvation, feeding solely on PVC film and feeding normally on corn leaves (Zhang et al., 2022). After 5 days of cultivation, the survival rate of the larvae in the PVC group was found to be significantly higher compared to the starvation group, but still lower than the corn group (Zhang et al., 2022). The body weight of larvae groups followed the order: starvation < PVC < corn, indicating that PVC film can provide energy and sustain the survival of the larvae, although the growth efficiency on this specialized feeding on PVC films was lower than that with normal feeding on corn leaves (Zhang et al., 2022). To further

test their hypothesis that the intestinal microbiota is essential for PVC film degradation by *S. frugiperda* larva, Zhang et al. set up an antibiotic group in which gentamicin was used to inactivate most intestinal microbes of the larvae. Based on scanning electron microscopy (SEM) analysis, they found that PVC fragments recovered from excretion products in the PVC group showed a strong surface damage in contrast with antibiotic group, revealing the importance of intestinal microbiota for PVC degradation. These results shown the importance of the intestinal microbiota in the PVC biodegradation process by *S. frugiperda* larvae.

PVC film degradation by intestinal microbiota of *S. frugiperda* laervae is likely to create new ecological niches for microbiome selection through cross-feeding and this is because the degradation process leads to the release of transformation products (Zhang et al., 2022). (Zhang et al., 2022). The study by Zhang et al. (2022) showed that the degradation of PVC film by *Spodoptera frugiperda* larvae was accompanied by a significant shift in the composition of the larvae's intestine microbiota. Specially, the researchers found that the dominant phylum *Proteobacteria* was replaced bt a co-dominance of *Firmicutes* and that PVC feeding increased the diversity of bacterial amplicon sequence variants (ASVs) in the gut microbiota (Zhang et al., 2022). Certain bacteria such as *Enterococcus*, *Ochrobactrum*, *Falsochrobactrum*, *Microbacterium*, *Sphingobacterium* and *Klebsiella* were selectively enriched by the gut of microbiota upon feeding on PVC (Zhang et al., 2022). The study suggests that the intestinal microbiota of the larvae may pay a role in the degradation of PVC film (Zhang et al., 2022).

In the same study from 2022, Zhang et al. found that the larvae intestinal microbiota of *S. frugiperda* serves a significant source of PVC-degrading strains. Through laboratory screening, they identified a gram-negative strain called *Klebsiella* sp. EMBL-a, which formed a biofilm on the surface of the PVC film after 10 days of incubation (Zhang et al., 2022). Based on the given information, it can be inferred that the researchers observed visible cracks on the surface of the PVC film and an increase in biomass concentration, indicated by an OD600 rise from 0.20 to 0.60 (Zhang et al., 2022). This strain was identified thorough PCR cloning, sequencing and phylogenetic analysis of the 16s rRNA gene, and was found to be closely related to *Klebsiella variicola* and *Klebsiella pneumoniae* (Zhang et al., 2022). The researchers also discovered that the strain was able to alter the surface hydrophobicity and tensile strength of the PVC film, resulting in a weight loss of 19.57% after 90 days of incubation (Zhang et al., 2022).

According to the provided information, the use of advanced polymer chromatography (APC) indicated that the strain EMBL-1 was able to depolymerize the long-chain structure of PVC and generate fragments with lower molecular weight (Zhang et al., 2022). Furthermore, thermogravimetric analysis (TGA/DSC) demonstrated that the strain could attack the polymer chain of PVC and reduced its chemical stability (Zhang et al., 2022). These findings suggest that strain EMBL-a possesses the capacity to degrade PVC and modify its physical and chemical characteristics (Zhang et al., 2022).

The analysis of the strain EMBL-1's genome revealed that it belongs to the *Klebsiella* genus and has a closer relationship with *Kebsiella variicola* based on average nucleotide identity (ANI), which supports the findings from the phylogenetic analysis of the 16s rRNA gene (Zhang et al., 2022). The genomic analysis of strain EMBL-a revealed the presence of various genes that may be involved in the degradation of PVC (Zhang et al., 2022). With 5,646 predicted protein-coding genes, the strain's genome could provide valuable information about the biodegradation mechanism of PVC films (Zhang et al., 2022). These genes include those responsible for the degradation of aromatic compounds, as well as those involved in the metabolism of fatty acids and other organic compounds, indicating the strain's potential for PVC degradation and bioremediation purpose (Zhang et al., 2022).

The presence of these genes suggests that strain EMBL-1 is well adapted to degrading PVC, but further research is necessary to understand the underlying molecular mechanism responsible for PVC degradation by this strain.

RECENT ADVANCES IN PET BIODEGRADATION

Polyethylene terephthalate (PET) is an aliphatic polyester synthetized by the polycondensation reaction of monomers derived from the esterification of terephthalic acid and ethylene glycol, or the trans-esterification of ethylene glycol and dimethyl terephthalate. Due to its durability against solvents, impact, alcohols and moisture, PET is widely utilized in the packaging industry. Additionally, PET can be easily recycled, making it a popular choice for sustainability efforts.

In 2016, Yoshida et al. discovered *Ideonella sakaiensis*, a bacterium isolated from a plastic recycling plant in Japan, as part of ongoing efforts to develop more effective solutions for plastic pollution (Edwards et al., 2022). During their study, Yoshida et al. determined that *Ideonella sakaiensis* was able to use PET as a sole source of carbon along with other microorganisms. After their study it was concluded that using a bacterial consortium for biodegradation of polymers shows numerous benefits because of their mixed metabolism which can create a synergic effect in the process of degradation (Edwards et al., 2022).

In another study in 2020, Roberts et al. have been able to isolate, from soils contaminated with petroleum, a microbial consortium of *Psedomonas* and *Bacillus* sp. that was able to degrade PET in a synergic activity (Edwards et al., 2022). Previously, in a study from 2019, Leon-Zayas et al. revealed that microbial strains have "unique and diverse genome" (Edwards et al., 2022). Based on the study of Leon-Zayas et al. from 2019, a new study based on the synergy of microbial consortia, was conducted in 2022 by Edwards et al. trying to decipher the pangenome of microbial consortia and how, genetically and metabolically, PET can be degraded. Using the same type of microbial consortia isolated in 2019 by Robert et al. (*Pseudomonas* and *Bacillus* sp.), Edwards et al. analysed the pangenome gene cluster using MicroScope gene families (MICFAM) computed with the SiLiX software. During the analysis 232 core genomes from "all Pseudomonas and Bacillus were excluded and 259 different gene groups were found to be shared in the core genome with the pangenome of these five strains" (Edwards et al., 2022).

The study's findings revealed that *Bacillus albus* PFN01 strain 13.1 and Pseudomonas sp. B10 strain 9.2 had the most diverse collection of accessory genes compared to the other strain examined (Edwards et al., 2022). In particular, *Bacillus* strain 9.1 and 13.1 shared more than 3305 genes, while *Pseudomonas* strains shared over 22192 genes (Edwards et al., 2022). It was assumed that aldehyde dehydrogenases, esterase and alcohol dehydrogenases gene groups were involved in degradation of PET as they proved to be involved in PET monomer and oligomer's degradation (Edwards et al., 2022). Additionally, the study by Edwards et al. (2022) utilized RNA sequencing (RNAseq) to investigate the potential synergistic effect of microbial consortia. By comparing a consortium grown on L-asparagine as a control to one grown on PET, researchers observed an upregulation of genes associated with the initial degradation of PET, providing a possible explanation for the enhanced PET degradation observed in microbial consortia. Kumari et al. in 2021, after performing a transcriptomic analysis, concluded that aldehyde dehydrogenases found in Bacillus can be effective in PET degradation, "generating 4-[(2-hydroxyethoxy)carbonyl]benzoate from the deprotonation of free carboxy group of MHET".

During RNA extraction, an analysis comparing RNAseq transcripts from strain 10 and 13.2 revealed that TPA was being degraded (Edwards et al., 2022). The analysis also showed the presence of dioxygenases and decarboxylases that share similarity with phthalate oxireductases, such as 1.2-dioxygenase (Figure 10) (Edwards et al., 2022).

Both *Pseudomonas* strains 10 and 13.2 had upregulated genes related to PHA biosynthesis and carboxylesterase NlhH, which previously was identified in the pangenome as a potential PHA/PHB depolymerase (Jendrossek et al., 2002; Edwards et al., 2022). However, the deletion of NlhH in strain 9.2 did not show any reduction in np-butyrate hydrolysis, suggesting that there may be differences in esterase activity compared to EstB and NlhH may or may not be directly involved in PET polymer depolymerization (Edwards et al., 2022). Only strain 10 exhibited increased transcriptional levels of surfactin, and it had 113 upregulated

"hypothetical proteins", whereas only 77 were upregulated in strain 13.2 (Edwards et al., 2022). With the aid of pangenome of the complete consortium comprising three *Pseudomonas* and two *Bacillus* strains, Edwards et al. could anticipate which gene has the potential to degrade PET and identify many hydrolases, dehydrogenases and oxidoreductases.

After identifying EstB within pangenome, Edwards et al. proposed that this enzyme from *Pseudomonas* strain may be capable of hydrolysing PET/BHET. Edwards et al. compared the structure of IsPETase with the predicted structure of EstB and found significant similarities, including a binding cleft, catalytic triad and the absence of a lid structure, which suggests that EstB could be a PETase. Their observation of similar active sites and enzymatic activities, combined with a relatively low primary amino acid sequence identity between IsPETase and EstB, support the concept of convergent evolution in bacteria from different locations, allowing them to degrade PET and other plastics (Edwards et al., 2022). Additionally, the authors predicted that EstB could also have feruloyl esterase activity based on its alignment with the PMBD and other potential PET-degrading enzymes identified in the study (Edwards et al., 2022).

In 2021, Qi et al. tried an innovative approach for using artificial microbiota consortia to biodegrade PET or other types of polymers. In this study they created three artificial consortia to break down PET (Qi et al., 2021). Qi et al. (2021) genetically modified two strains of Bacillus subtilis to secrete PETase and MHTase, respectively. In addition, the included wild strain of Rhodococcus jostii and Pseudomonas putida to consume PET monomers TPA and EG (Qi et al., 2021). The researchers formed a four-species microbial consortium comprising Bs-PETase, Bs-MHETase, *R. jostii* and *P. putida*, which directly broke down PET into monomers and converted them into carbon dioxide and water through that tricarboxylic acid cycle (Qi et al., 2021). By doing so, they effectively improve the degradation rate by alleviating the metabolic inhibition of TPA and EG (Qi et al., 2021). This study presents a novel approach for using artificial microbial consortia to biodegrade PET and potentially other types of polymers in the future.

PU BIODEGRADATION

PU are commonly used plastic polymers that can be difficult to degrade. They are synthesized from polyols and polysiocyanates and can be classified into two types, polyester PUs and polyether PUs (Howard et al., 2000). Polyester PUs are more susceptible to microbial degradation (Pathirana and Seal, 1985; Howard et al., 2000).

Biodegradation of PU primarily occurs through hydrolytic cleavage of urethane bonds, but only a few microbial strains have been reported to efficiently degrade it (Nakajima-Kambe et al., 1999; Mohanan et al., 2020). While some fungal and bacterial species can degrade PU through enzymatic hydrolysis of ester linkages (Nakajima-Kambe et al., 1999; Howard, 2002; Mohanan et al., 2020), the efficiency of this process varies among different strains. Examples of fungal species that can degrade PU include *Aureobasidium pullulans*, *Cladosporium* sp., *Curvularia senegalensis* and *Fusarium solani*, while bacterial strains such as *Pseudomonas aeruginosa*, *Corynebacterium* sp., *Comamonas acidovorans*, *Pseudomonas fluorescens*, *Acinetobacter calcoaceticus* and *Bacillus subtilis* have been shown to use PU as a carbon, nitrogen and energy source for growth (Mohanan et al., 2020).

Cardenas Espinosa et al. conducted a study in 2020 to investigate the biodegradability of PU by a soil bacterium isolated from an area with brittle plastic waste. Through 16s rRNA gene sequencing and membrane fatty acid profile analysis, the strain was identified as *Pseudomonas* sp. (Cardenas Espinosa et al., 2020). It was found that the strain could use a PU-diol solution and a PU oligomer as the sole source of carbon and energy, and 2,4-diaminotiluene as the sole source of energy, carbon and nitrogen (Cardenas Espinosa et al., 2020). The researchers identified selected bacterial strains using the membrane fatty acid profile, while PLFA extraction was performed using the Blight and Dyer method, and identification and quantification of the fatty acid methyl esterase (FAME) were done using gas chromatography with flame ionization detector (GC-FID) (Cardenas Espinosa et al., 2020). Whole-genome sequencing revealed the presence of various catabolic genes for aromatic

compounds in the strain (Cardenas Espinosa et al., 2020).

For the genomic DNA extraction, the DNeasy R Blood & Tissue Kit from QIAGEN was used and the quantity of extracted DNA was checked by nanodrop (Cardenas Espinosa at el., 2020). The library preparation was performed with the NExtera XT DNA library kit, and the paired-end libraries were sequenced using Illumina v3 chemistry on Illumina MiSeq sequencer with a 250-bp paired-end protocol (Cardenas Espinosa et al., 2020).

To reveal similarities to known enzymes mono and dioxygenases, enzymes involved in aromatic degradation, amino acid sequences of genes present in the genome of TDA1 were compared to UniprotKB database or by using the basic local alignment search tool (BLAST) database in NCBI. The AROMADEG database was also used for the annotation of dioxygenases (Cardenas Espinosa et al., 2020). The proposed genes potentially involved in the degradation were identified through significant similarities in amino acid sequences, with high coverage and similarity and a low E value (Cardenas Espinosa et al., 2020).

In their study, Cardenas Espinosa et al. (2020) isolated two bacterial strains based on their growth rate on agar plates and in liquid media containing 2,4-TDA as the only carbon and energy source and was identified as *Pseudomonas sp*. TDA1. The isolated strain demonstrated the ability to utilize an oligomeric PU substrate and 2,4-TDA as a sole source of carbon, nitrogen and energy, along with other aromatic compounds, indicating its strong metabolic potential (Cardenas Espinosa et al., 2020). The identification of the strain was performed through analysis of its complete 16s rRNA gene sequences and phospholipid fatty acid profile, which resembled that of *Pseudomonas putida* KT2440, a *Pseudomonas* marker gene (Cardenas Espinosa et al., 2020). The findings support previous reports that *Pseudomonas* sp. is capable of degrading PU (Howard and Blake, 1998; Howard, 2002; Gautam et al., 2007; Hung et al., 2019; Cardenas Espinosa et al., 2020). Based on the genomic potential and substrate spectrum of the strain, the study proposes a degradation pathway for 2,4-TFA and identifies candidates genes encoding the enzymes involved (Cardenas Espinosa et al., 2020).

CONCLUSIONS

In recent years, the issue of plastic pollution has become a major concern, and finding effective and sustainable methods for plastic waste management has become urgent. Biodegradation of plastic waste is a complicated process that is affected by various factors, including morphology, surface characteristics and molecular weight of polymers (Mohanan et al., 2020). The microbial degradation of PE has been demonstrated to be feasible, with several bacterial strains such as *Bacillus spp*., *Rhodococcus* spp. and *Pseudomonal* spp., as well as fungi like *Aspergillus* and *Fusarium* showing potential for breaking down PE following specific pre-treatment methods (Mohanan et al., 2020). Microbial biodegradation, chemical recycling and physical recycling are some methods for the disposal of PS waste. However, microbial degradation of PS waste is a promising approach that can utilize PS waste as a carbon source and produce less harmful by-products compared to traditional recycling methods (Shimpi et al., 2012; Motta et al., 2009; Mohanan et al., 2020).

PVC is more challenging to biodegrade due its lack of hydrolysable ester bonds. Recent research has identified the gut microbiota of *Spodoptera frugiperda* larvae as playing a role in the digestion of PVC film (Zhang et al., 2022). Moreover, certain bacterial strains, including *Enterococcus*, *Ochrobactrum*, *Falsochrobactrum*, *Microbacterium*, *Sphingobacterium* and *Klebsiella* have been found to have the ability to degrade PVC (Zhang et al., 2022).

PP is highly resistant to biodegradation and while pre-treatment and blending with other materials can improve its biodegradability, recycling and reuse are more effective and sustainable solutions for PP disposal (Iwamoto and Tokiwa, 1994; Huang et al., 2005; Sameh et al., 2006; Mohanan et al., 2020).

Studies have used various techniques such as scanning electron microscopy and Fourier-transform infrared spectroscopy to confirm the biodegradation of plastic by microorganisms. The potential use of bacterial and fungal strains

in managing plastic waste highlights the potential of these microorganisms in the degradation process of plastic both in vitro and in soil burial methods and in the future could provide a framework for developing effective bioremediation strategies for plastic waste.

REFERENCES

Abrusci C., Pablos J. L., Marin I., Espi E., Corrales F. & Catalina F. (2013). Comparative effect of metal stearates of pro-oxidant additives on bacterial biodegradation of thermal- and photo-degraded low density polyethylene mulching films. *International Biodeterioration & Biodegradation*, 83, 25-32.

Ali S. S., Elsamahy T., Al-Tohamy R., Zhu D., Mahmoud Y. A.-G., Koutra E., Metwally M. A., Kornaros M. & Sun J. (2021). Plastic wastes biodegradation: Mechanisms, challenges and future prospects. *Science of the Total Environment*, 780, 146590.

Bahl S., Dolma J., Singh J. J. & Sehgal S. (2020). Biodegradation of plastics: A state of the art review. *Materials Today: Proceedings.*

Bombelli P., Howe C. J. & Bertocchini F. (2017). Polyethylene bio-degradation by caterpillar of wax moth *Galleria mellonella*. *Current biology*, 27(8), R292-293.

Bonhomme S., Cuer A., Delort A-M., Lemair J., Sancelme M. & Scott G. (2003). Environmental biodegradation of polyethylene. *Polymer Degradation and Stability*, 81(3), 441-452.

Borowski G., Klepka T., Pawlowska M., Lavagnolo M. C., Oniszczuk T., Wojtowicz A. & Combrzynski M. (2020). Effect of flax addition on the mechanical properties and biodegradability of biocomposites based on thermoplastic starch. *Archives of Environmental Protection*, 46(2), 74-82.

Brandon A. M., Gao S. H., Tian R., Ning D., Yang S. S. & Zhou J. (2018). Biodegradation of polyethylene and plastic mixtures in mealworms (Larvae of *Tenebrio molitor*) and effects in the gut microbiome. *Environmental Science & Technology*, 52(11), 6526-6533.

Cardenas Espinosa M. J., Colina Blanco A., Schmidgall T., Atanasoff-Kardjalieff A. K., Kappelmeyer U., Tischler D., Pieper D. H., Heipieper H. J. & Eberlein C. (2020). Toward Biorecycling: Isolation of a Soil Bacterium That Grows on a Polyurethane Oligomer and Monomer. *Frontiers in Microbiology*, 11:404.

Cassone B. J., Grove H. C., Elebute O., Villanueva S. M. P. & LeMoine C. M. R. (2020). Role of the intestinal microbiome in low-density polyethylene degradation by caterpillar larvae of the greater wax moth *Galleria mellona*. *Proceedings of the Royal Society B: Biological Sciences*, 287(1922).

Edwards S., Leon-Zayas R., Ditter R., Laster H., Sheehan G., Anderson O., Beattie T. & Mellies J. L. (2022). Microbial Consortia and Mixed Plastic Waste: Pangenomic Analysis Reveals Potential for Degradation of Multiple Plastic Types via Previously Identified PET Degrading Bacteria. *International Journal of Molecular Sciences*, 23, 5612.

Emmanuel-Akerele H. A., Akintemi P. O. & Igbogbo-Ekpunobi O. E. (2022). Isolation and identification of plastic degrading bacteria from dumpsites Lagos. *Advances in Environmental Technology* 1, 59-71.

Fontanella S., Bonhomme S., Koutny M., Husarov L., Brusso J. M., Courdavault J. P., Pitteri S., Samujel G., Pichon G., Lemaire J. & Delort A. M. (2010). Comparison of the biodegradability of various polyethylene films containing pro-oxidants additive. *Polymer Degradation and Stability*, 95(6), 1011-1021.

Gajendiran A., Krishamoorthy S. & Abraham J. (2016). Microbial degradation of low-density polyethylene (LDPE) by *Aspergillus clavatus* strain JASK1 isolated from landfill soil. *3 Biotech Journal*, 6(1).

Gautam R., Bassi A. S. & Yanful E. K. (2007). A review of biodegradation of synthetic plastic and foams. *Applied Biochemistry and Biotechnology*, 141(1), 85-108.

Giacomucci L., Raddadin N., Soccio M., Lotti N. & Fava F. (2019). Polyvinyl chloride biodegradation by *Pseudomonas citronellolis* and *Bacillus flexus*. New Biotechnology, 25, 52:35-41.

Gilan (Orr) I., Hadar Y. & Sivan A. (2004). Colonization, biofilm formation and biodegradation of polyethylene by a strain of *Rhodococcus ruber*. *Applied Microbiology and Biotechnology*, 65(1).

Hasan F., Shah A. A., Hameed A. & Ahmed S. (2007). Synergistic effect of photo and chemical treatment on the rate of biodegradation of low-density polyurethane by *Fusarium sp*. AF4. *Journal of Applied Polymer Science*, 105(3), 1466-1470.

Howard G. T., Vicknair J. & Mackie R. I. (2001). Sensitive plate assay for screening and detection of bacterial polyurethanase activity. *Letters in Applied Microbiology*, 32, 211-214.

Hung C.-S., Barlow D. E., Varaljay V. A., Drake C. A., Crouch A. L., Russell J. N., Nadeau L. J., Crookes-Goodson W. J. & Biffinger J. C. (2019). The Biodegradation of Polyester and Polyester Polyurethane coating using *Papiliotrema laurentii*. *International Biodeterioration & Biodegradation*, 139, 34-43

Jendrossek D. & Handrick R. (2019). Microbial degradation of polyhydroxyalanoates. *Annual Review of Microbiology*, 56(1), 403-432.

Jiang S., Su T., Zhao J. & Wang Z. (2021). Isolation, Identification, and Characterization of Plystyrene-Degrading Bacteria from the Gut of *Galleria Mellonella (Lepidoptera: Pyralidae)* Larvae. *Frontiers in Bioengineering and Biotechnology*, 9:736062.

Kumar Sen S. & Raut S. (2015). Microbial degradation of low-density polyethylene (LDPE): A review. *Journal of Environmental Chemical Engineering*, 3(1), 462-473.

Kyaw B. M., Champakalakshmi R., Sakharka M. N., Lim C. S. & Sakharkar K R. (2012). Biodegradation of Low-Density Polythene (LDPE) by *Pseudomonas Species*. *Indian Journal of Microbiology*, 52(3): 411-419.

Leon-Zayas R., Roberts C., Vague M. & Mellies J. L. (2019). Draft Genome Sequences of Five Environmental Bacteria Isolates That Degrade Polyethylene Terephthalate Plastic. *Microbiology Resource Announcements*, 8(25).

Ma Q., Zhang X. & Qu Y. (2018). Biodegradation and Biotransformation of Indole: Advances and Perspectives. *Frontiers in Microbiology*, 9.

Mohanan N., Montazer Z. & Levin D. B. (2020). Microbial and Enzymatic Degradation of Synthetic Plastics. *Frontiers in Microbiology*, 11:2837.

Mohanrasu K., Premnath N., Peakash G. S., Sudhakar M., Boobalan T. & Arun A. (2018). Exploring multi potential use of marine bacteria; an integrated approach for PHB production, PAHs and polyethylene biodegradation. *Journal of Photochemistry and Photobiology B: Biology*, 185, 55-65.

Montazer Z., Habibi-Najafi M. B. & Mohebbi M., Oromiehei A. (2018). Microbial Degradation of UV-Pretreated Low-Density Polyethylene Films by Novel Polyethylene-Degrading Bacteria Isolated from Plastic-Dump Soil. *Journal of Polymers and the Environment*, 26:3613-3625.

Motta O., Proto A., De Carlo F., De Caro F., Santoro E., Brunetti L. & Capunzo M. (2009). Utilization of chemically oxidized polystyrene as co-substrate by filamentous fungi, *International Journal of Hygiene and Environmental Health*, 212(1), 61-66.

Nademo Z. M., Shibeshi N. T. & Gemeda M. T. (2023). Isolation and screening of low-density polyethylene (LDPE) bags degrading bacteria from Addis Ababa municipal solid waste disposal site "Koshe". *Annals of Microbiology*, 73:6.

Nakajima-Kambe T., Shigeno-Akutsu Y., Nomura N., Onuma F. & Nakahara R. (1999). Microbial degradation of polyurethane, polyester, polyurethanes and polyether polyurethanes. *Applied Microbiology and Biotechnology*, 51(2), 134-140.

Nowak B., Pajak J., Drozda -Bratkowicz M. & Rymarz G. (2011). Microorganisms participating in the biodegradation of modified polyethylene films in different soils, under laboratory conditions. *International Biodegradation & Biodeterioration*, 65(6), 757-767.

Pathirana R. A. & Seal K. J. (1985). Studies on polyurethane deterioration fungi. *International Biodeterioration and Biodegradation*, 21:41-19.

Peixoto J., Silva L. P. & Kruger R. H. (2017). Brazilian Cerrato soil reveals an untapped microbial potential for unpretreated polyethylene biodegradation. *Journal of Hazardous Materials*, 324, 634-644.

Peng B. Y., Su Y., Chen J., Zhou X. & Benbow M. E. (2019). Biodegradation of polystyrene by dark (*Tenebrio obscurus*) and yellow (*Tenebrio molitor*) mealworms) and effects on the gut microbiome. *Environmental Science & Technology*, 52(11), 6526-6533.

Peng B-Y., Chen Z., Chen J., Yu H., Zhou X., Criddle C. S., Wu W-M. & Zhang Y. (2020). Biodegradation of Polyvinyl Chloride (PVC) in *Tenebrio molitor (Coleoptera: Tenebrionidae)* larvae. *Environmental International*, 145, 106106.

Qi X., Ma Y., Chang H., Li B., Ding M. & Yuan Y. (2021). Evaluation of PET Degradation Using Artificial Microbial Consortia. *Frontiers in Microbiology*, 12:778828.

Qi Z., Ma Y., Chang H., Li B., Ding M. & Y Yingjin (2021). Evaluation of PET Degradation Using Artificial Microbial Consortia. *Frontiers in Microbiology*, 12:778828.

Rachmawati A. C., Mahardika A., Djohan, Susanto A. B. & Andriana B. B (2021). Exploration of Plastic-Degrading Bacteria from Marina Beach, Semarang, Central Java. *Indonesian Journal of Marine Sciences*, Vol. 26(4): 247-253.

Rajandas H., Parimannan S., Sathsivam K., Ravichandram M. & Su Yin L. (2012). A novel FTIR-ATR spectroscopy-based techniques for the estimation of low-density polyethylene biodegradation. *Polymer Testing*, 31(8), 1094-1099.

Raziyafathima M., Praseetha P. K. & Rimal Isaac R. S. (2016). Microbial Degradation of Plastic Waste: A review. *Journal of Pharmaceutical, Chemical and Biological Sciences*, 4(2): 231-242.

Ren L., Men L., Zhang Z., Guan F., Tian J., Wang B., Zhang Y. & Zhang W. (2019). Biodegradation of Polyethylene by *Enterobacter sp*. D1 from the Guts of Wax Moth *Galleria mellonella*. *International Journal of Environmental Research and Public Health*, 16(11): 1941.

Restrepo-Florez J-M., Bassi A. & Thompson M. R. (2014). Microbial degradation and deterioration of polyethylene. A review. *International Biodeterioration & Biodegradation*, 88, 83-90.

Ru J., Huo Y. & Yang Y. (2020). Microbial Degradation and Valorisation of Plastic Wastes. *Frontiers in Microbiology*, 11.

Saeed S., Iqbal A. & Deeba F. (2022). Biodegradation study of Polyethylene and PVC using naturally occurring plastic degrading microbes. *Archives of Microbiology*, 204:497.

Sahebnazar Z., Abbas S. S., Mahsa M-T. & Mohsen N. (2010). Biodegradation of low-density polyethylene (LDPE) by isolated fungi in solid waste medium. *Waste Management*, 30(3), 396-401.

Sameh A. S., Alariqi, Pradeep Kumar A., Rao B. S. M. & Singh R. P. (2006). Biodegradation of γ-sterilized biomedical polyolefins under composting and fungal culture environments. *Polymer Degradation and Stability*, 91(5), 1105-1116.

Sen S. K., Raut S. & Bandyopadhyay (2016). Fungal decolouration and degradation of azo dyes: A review. *Fungal Biology Reviews*, 30(3), 112-133.

Shimpi N., Borane M., Mishra S. & Kadam M. (2012). Biodegradation of polystyrene (PS)-poly(lactic acid) (PLA) nanocomposites using *Pseudomonas aeruginosa*. *Macromolecular Research*, 20(2), 181-187.

Sriyapai P., Chansiri K. & Sriypai T. (2018). Isolation and Characterization of Polyester-Based Plastic-Degrading Bacteria from Compost Soils. *Microbiology*, Vol. 87, No. 2, pp. 290-300.

Tournier V., Topham C. M., Gilles A., David B., Folgoas C., Moya-Leclair E., Kamionka E., Desrousseaux M-L., Texier H., Gavalda S., Cot M., Guemard E.,

Dalibely M., Nomme J., Cioci G., Barbe S., Chateau M., Andre I., Suquesne S. & Marty A. (2020). An engineered PET depolymerase to break down and recycle plastic bottles. *Nature*, 580(7802), 216-219.

Urbanek A. K., Rymoewicz W. & Mironczuk A. M. (2018). Degradation of plastic and plastic degrading bacteria in cold marine habitats. *Applied Microbiology and Biotechnology*, 102(18), 7669-7678.

Ward P. G., Goff M., Donner M., Kaminsky W. & O'Conner K. E. (2006). A two-step chemo-biotechnological conversion of polystyrene to a biodegradable thermoplastic. *Environmental Sciences & Technology*, 40(7), 2433-2437.

Yang J., Yang Y., Wu W. M., Zhao J. & Jiang L. (2014). Evidence of polyethylene biodegradation by bacterial strains from the guts of plastic-eating worms. *Environmental Science & Technology*, 48(23), 13776-13784.

Yang Y., Ghatge S., Ahn J-H. & Hur H-G. (2020). Biodegradation of polyethylene: a brief review. *Applied Biological Chemistry*, 63(1).

Yang Y., Yang J., Wu W-M., Zhao K., Song Y., Gao L., Yang R. & Jiang L. (2015). Biodegradation and Mineralization of Polystyrene by Plastic-Eating Mealworms: Part 2. Role of Gut Microorganisms. *Environmental Science & Technology*, 49(20), 12087-12093.

Yoon G. M., Jeong Jeon H. & Nam Kim M. (2012). Biodegradation of Polyethylene by a Soil Bacterium and AlkB Cloned Recombinant Cell. *Journal of Bioremediation & Biodegradation*, 03(04).

Yoshida S., Hiraga L., Takehara T. & Oda K. (2016). A bacterium that degrades and assimilates poly(ethylene terephthalate). *Science*, 351(6278), 1196-1199.

Zhang N., Ding M. & Yuan Y. (2022). Current Advances in Biodegradation of Polyolefins. *Microorganisms*, 10(8):1537.

Zhang Z., Peng H., Yang D., Zhang G., Zhang J. & Ju F. (2022). Polyvinyl chloride degradation by a bacterium isolated from the gut of insect larvae. *Nature Communications*, 13:5360.

Scientific Bulletin. Series F. Biotechnologies, Vol. XXVII, No. 2, 2023
ISSN 2285-1364, CD-ROM ISSN 2285-5521, ISSN Online 2285-1372, ISSN-L 2285-1364

MULTIDRUG-RESISTANT BACTERIA ASSOCIATED TO STRAWBERRIES AND GOOSEBERRIES AT VARIOUS STAGES OF RIPENESS

Pamela REYES, Diana MOLINA, Gabriela N. TENEA

Biofood and Nutraceutics Research and Development Group,
Faculty of Engineering in Agricultural and Environmental Sciences, Universidad Tecnica del Norte,
Av. 17 de Julio s-21. Barrio El Olivo, Postcode: 100150, Ibarra, Ecuador

Corresponding autor email: gntenea@utn.edu.ec

Abstract

The lack of understanding about proper food manufacturing, transport, storage, and marketing methods has resulted in a high level of contamination with pathogenic bacteria that cause gastrointestinal disorders and induce drug resistance, which is a serious issue for the food sector. Due to their popularity as a fruit in the province of Imbabura, Ecuador, this issue has sparked a lot of interest in evaluating the quality and microbiological safety of strawberries and cape gooseberries at different ripening stages. According to bacteriological analyses, the microbiota of strawberries was dominated by Escherichia coli (1.61 x 10^3 CFU/g) at ripeness stages 4 (three-quarters of the fruit has turned red) and 6 (red colour, they feel slightly soft when squeezed, ready to eat), while the microbiota of gooseberries was predominated by Enterobacter spp. (1.11 x 10^2 CFU/g) at ripe stage 6 (intense yellow, ready to eat). The differences in the physiochemical characteristics (pH, total solids, acidity) at the time of harvest results in a difference in some pathogenic bacteria's adaptation. The selected clones per each ripe stage showed multi-antibiotic resistance (MAR). The calculated MAR index varies from 0.17 to 1.00 and was ripe stage-, and fruit dependent. The most resistant clones were E. coli for strawberries and Enterobacter spp. for gooseberries. Thus, it is crucial to set up efficient control measures and develop coordinated strategies to guarantee the microbiological quality of these foods.

Key words*: Strawberry, gooseberry, food safety, pathogens, antibiotic resistance.*

INTRODUCTION

The promotion of healthier lifestyles increases the consumption of fresh products such as tropical fruits. They offer bioactive phytochemicals such as flavonoids and phenolic compounds, which have been linked to several health advantages (Septembre-Malaterre et al., 2018). Worldwide, health organizations launched campaigns encouraging people to eat at least "5 a day" portions of fruit and vegetables every day (Lafarga et al., 2019). In fact, consuming fruit on a regular basis appears to help avoid a wide range of chronic disorders (Samtiya et al., 2021). Strawberry (*Fragaria x ananassa*) and cape gooseberry or uvilla (*Physalis peruviana*) are the most consumed fruits valued for their flavour, aroma, and nutritional quality. Eating fresh fruits is a healthy habit that should be adopted by everyone. Contrarily, fruits do not go through any stage of processing to guarantee the effective eradication or inactivation of pathogenic bacteria, so the customer is exposed to a higher risk of illness (Drobek et al., 2021). Fruit-associated pathogens pose safety concerns as they may carry antibiotic-resistance genes (ARG). Recent studies indicated that strawberries' ARG diversity and abundance decreased when they were fertilized with aerobic and anaerobic composting manure (Zhang et al., 2022). In addition, despite growing interest in plant microbiomes, perennial fruit trees' associated microbial communities continue to be underappreciated (Sangiorgio et al., 2022). Compared to fruits off the ground, strawberry plants crawl on the ground, making their fruits more susceptible to soil ARG contamination (Zhang et al., 2022). Thus, due to their production in the external environment, there is an increased risk of contamination with various pathogenic microorganisms, some of which cause foodborne illnesses (Pangahl et al., 2022). Postharvest losses of between 20 and 45% have been recorded for gooseberries. Up to 50% of strawberries can be lost due to the

Botrytis cinerea fungus's influence (Azam et al., 2019). The fruits start to degrade after harvest because of mechanical damage, increased rates of respiration and transpiration, and mold infection. Early, we prospect the quality of strawberries and gooseberries purchased from a local retail market and a local farm in Ecuador (Tenea et al., 2023). The results indicated the presence of a high number of microorganisms in fruits independently of their origin. However, the composition of the raw materials and their physicochemical characteristics determine the degree of contamination and the existence of a specific indicator. We also assessed the selected clones' antibiotic resistance profiles, which showed strong resistance to several drugs. This could be explained by the fact that the fruits came from a field where the farmers used animal waste as fertilizer. In this context, to understand how the microorganisms might spread from farm to table, the present study presents a comparative bacteriological analysis and antibiotic resistance patterns of microorganisms isolated from both gooseberries and strawberries collected from fields at the early ripeness stage of 2, 4, and 6 (ready-to-eat) fruits.

MATERIALS AND METHODS

Field fruit collection

Gooseberries (*Physalis peruviana,* variety Colombiana) at ripe stage 2 (green) and 4 (light yellow), and 6 (intense yellow, ready to eat) samples (20 fruits with calix/ field raw/ stage), and strawberries (*Fragaria* x *ananassa* variety Monterey) at the ripe stage 2 (3/4 white and 1/4 red color), 4 (three-quarters of the fruit has turned red) and 6 (red color, they feel slightly soft when squeezed, ready to eat) with no visible damages were used. This study was realized between October 2021-May 2023, regardless of the season, and the fruits were collected from the open fields of farmers from Imbabura Provence, Northern Ecuador. A total of 20 x 6 fields =120 fruits/ stage/ variety). The samples were translated to the laboratory and immediately proceeded to the bacteriological analysis. Three independent fruit harvests have been considered.

Bacteriological analysis

The microbiological quality was performed as previously described (Tenea et al., 2023). Briefly, the fruits (20 fruits/field raw/stage) were placed in a sterile zip bag containing buffered peptone water (0.1 %) and mixed vigorously by hand for 30s. The bags were placed at 37°C for 1.30 h and mixed gently at a 15 min interval, followed by centrifugation and recollection of the cells in 1 X PBS buffer. The presence of total coliforms, total aerobes, *E. coli*, *Salmonella* spp., *Shigella* spp., *Enterobacter* spp., yeasts, and molds was assessed in both Petrifilm (3M Science Applied to Life, MI, USA) and chromogenic media as described early (Tenea et al., 2023). CFU/(g) was used to express microbial counts. To test for antibiotic susceptibility, at least 10 colonies, stages, fruits, or biological markers (grouped by genus-level classification) were randomly selected, extracted, and purified.

Physicochemical analysis

Using a pH meter (Seven Compact S210, Mettler Toledo LCC, Columbus, OH, USA), 50 g of strawberry/gooseberry fruits were blended for 1 minute with 150 mL of distilled water to determine the pH. By titrating 10 mL of strawberry/gooseberry juice with 0.1 mol/L NaOH and using phenolphthalein as an indicator, titratable acidity (TA) was determined (AOAC, 1990). The tests were carried out three times. The data are given as a percentage of citric acid determined as previously described (Alharaty et al., 2020). A digital refractometer was used to calculate the fruit's total soluble solids (°Brix) and total sugar content (TSS).

Antibiotic susceptibility testing

A total of 145 and 142 random colonies from strawberries and gooseberries were selected and used for antibiotic susceptibility tests using commercial discs: Amoxicillin (AMX25: 25µg), Ampicillin (AN10: 10 µg), Gentamicin (CN10: 10 µg), Kanamycin (K30: 30 µg), Tetracycline (TE30: 30 µg), and Cefuroxime (CXM30: 30 µg). We employed the concentrations advised by the Scientific Committee on Animal Nutrition for the disk diffusion experiment (discs supplied by Merck, USA). According to the microbiological breakpoints specified by the FEEDAP guidelines, the Scan500 (Interscience, Fr) was used to automatically identify the inhibitory halos and grade them as susceptible, medium, or resistant (EFSA, 2012). As a reference, *E. coli* ATCC25922, *S. enterica* subsp. *enterica* ATCC51741, and *Enterobacter* spp. (lab strain) were used. The % of antibiotic resistance was determined as the number of total

bacteria resistant / the number of total isolates tested. MAR index was calculated as the ratio between the number of antibiotics that an isolate is resistant to and the total number of antibiotics the organism is exposed to (Davis and Brown, 2016).

Hemolysis and catalase assay

The clones showing resistance to more than 3 antibiotics were evaluated for their hemolytic activity on Columbia agar containing 5% (w/v) human blood as described (Tenea et al., 2023). The isolates were divided into three groups based on the number of red blood cells that were lysed in the medium next to the colonies: alfa-hemolytic (green region near colony), beta-hemolytic (clear region near colony), and gamma-hemolytic (no region). The catalase assay was performed using hydrogen peroxide (H_2O_2) as substrate. An overnight culture of the selected colonies was obtained, and one drop was applied on a glass slide containing three drops of H_2O_2. The presence of bubbles indicates that the test is positive.

Statistical analysis

All experiments were run in triplicate and the results were reported as mean ± standard deviation. To identify significant differences between the means, the Fisher Least Significant Difference test was used (SPSS 13.0, Inc., Chicago, IL).

RESULTS AND DISCUSSIONS

A new trend in the food industry, rising consumer demand for natural products, contributed to an increase in the consumption of fresh foods over the past ten years (Melo and Quintas, 2023). Microbiological safety has raised significant concerns for the food industry and public health agencies despite being one of the most rapidly expanding sectors in recent years. Due to the lack of preceding microbiological lethal techniques, such food products may expose consumers to a risk of contracting a foodborne infection (Drobek et al., 2021). For example, enterococci can be found in the gastrointestinal tracts of both humans and animals as well as in foods that have either animal or plant origins. They have contributed to nosocomial infections, food poisoning, and the escalation of antibiotic resistance (Al-Kharousi et al., 2022). In this study, the bacteriological analysis indicated a comparable number of total aerobes in both fruits, which varies with the ripening stage (Figure 1).

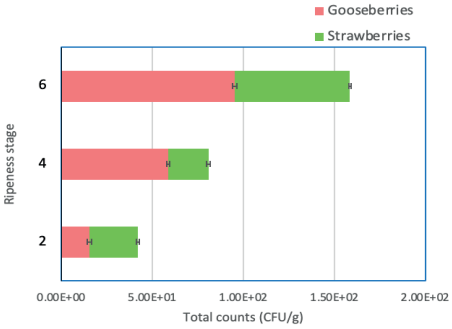
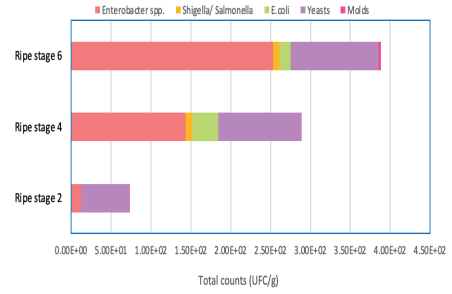
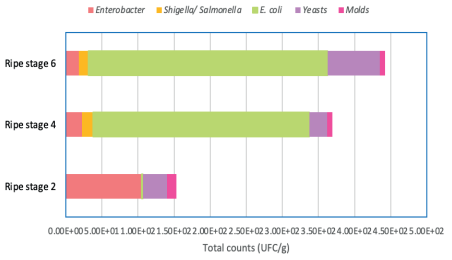


Figure 1. Total counts (UFC/g) enumerated in gooseberries and strawberries

Although gooseberries were bought with calix in this study, a greater number of indicators, *Enterobacter* spp., and *Shigella* spp., were detected at ripe stages 4 and 6 (Figure 2A). There were no *Salmonella* colonies detected, while a significant increase ($p < 0.05$) of *E. coli* and yeasts were detected at stage 4. The strong ability of some pathogenic bacteria to adapt and colonize the raw material to assure their metabolic activities may be related to the physiological characteristics at the maturity stage.



A).



B)

Figure 2. Total counts (UFC/g) of the indicator microorganisms detected in: A) - gooseberries and B) -strawberries at different ripe stages

Similarly, in strawberries, a high amount of indicator *E. coli* was detected at ripe 4 and 6, while *Enterobacter* was at stage 2 (Figure 2B). An increase in yeasts was observed at stage 6, which might have something to do with the fruit fermentation. According to Jensen et al. (2013), the microbiota found in healthy strawberries is diverse and contains potential plant pathogens, human pathogens, molds that produce mycotoxins, and biocontrol agents for plant diseases. The number of molds was higher in strawberries than in gooseberries at the sixth stage of ripeness. Although the fungus is usually present during the strawberry growing season and cannot be avoided, the amount of inoculum in each field can be reduced by removing dead leaves and ill fruit. After harvest, the fungus lives on tilled-in leaves and fruit as tiny, black, dormant sclerotia (Sengupta et al., 2020). The differences in the physiochemical characteristics (Table 1) at the time of harvest results in a difference in some pathogenic bacteria's adaptation. The proportion of Brix to titratable acidity (TSS/TA), which measures the amount of soluble solids and acidity in a substance, appears to be a good indicator of how sweet a fruit is and its overall acceptance (Martínez-Bolaños et al., 2008). However, in this study, the values were higher in strawberries than gooseberries indicating that the fruits were sweet. According to other studies, these values are cultivar-dependent and are impacted by the growing environment (Ikegaya et al., 2019).

Table 1. Physicochemical characteristics of fruits

Sample	Ripe stage	TSS (°Brix)	pH	TA (citric acid %)	TSS/TA*
Gooseberries	2	8.41 ± 0.04	3.62 ± 0.04	1.89 ± 0.15	4.44 ± 0.05
	4	12.84 ± 0.02	3.46 ± 0.03	2.36 ± 0.12	5.50 ± 0.12
	6	13.65 ± 0.06	3.51 ± 0.07	2.06 ± 0.03	6.79 ± 0.03
Strawberries	2	6.87 ± 0.02	3.32 ± 0.02	0.50 ± 0.06	13.74 ± 0.06
	4	7.43 ± 0.08	3.36 ± 0.02	0.46 ± 0.21	16.15 ± 0.21
	6	7.36 ± 0.05	3.53 ± 0.02	0.25 ± 0.03	29.93 ± 0.03

*was calculated as the ratio between Brix and titratable acidity.

Raw fresh vegetables may include antimicrobial residues, antibiotic-resistant bacteria, and clinically significant antimicrobial resistance genes (Rahman et al., 2021). The development of antibiotic resistance associated with food-borne microbes has a harmful effect on human health (Krahulcová et al., 2021). According to Davis and Brown (2016), MAR index provides a reliable, efficient, and effective way to identify the origins of bacteria that are resistant to antibiotics. A MAR greater than 0.2 indicates a source of contamination with a significant risk (Davis and Brown, 2016). However, this study found that for gooseberries the MAR index was greater than 0.25 for more than 50% of the selected clones independently of their ripe stage (Figure 3). Instead, for strawberries at stage 2, more than 79% of clones showed a MAR index above 0.25. Interesting, 70% and 90% of the *E. coli* selected clones were resistant to tetracycline and kanamycin, respectively. The antibiotics from the tetracycline family are most frequently found in various types of manure.

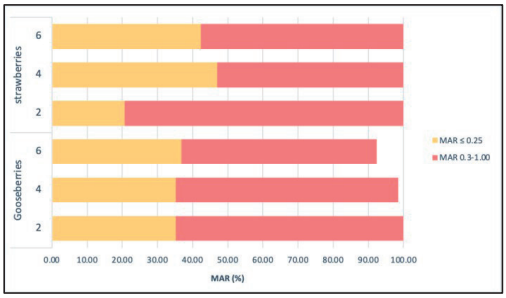


Figure 3. MAR index (%) calculated for a pool of indicator clones selected for gooseberries and strawberries.

According to previous research, the size of the farm and the concentrations of tetracycline found in the fertilized soils were directly connected with the use of tetracycline (Carballo et al., 2016). As a result, worries concerning the contribution of antibiotic residues to the antibiotic resistance reservoir are growing as they are released into the environment through the usage of animal wastes (Amador et al.,

2019). Although in lower amounts, 8.33% *E. coli* and 11.11% of *Enterobacter* spp. clones at ripe stages 2 and 4, as well as 5.5% *E. coli* at ripe stage 6, were resistant to all antibiotics tested. Early studies indicate that despite being regarded as opportunistic pathogens, enterococci can act as a source of antibiotic resistance (McGowan et al., 2006). Additionally, strawberry fruits obtained from soil enriched with livestock manure, which is often used by farmers to increase productivity and fruit quality, could easily acquire antibiotic-resistance genes (Zhang et al., 2022). In addition, 84% of the selected *E. coli* and *Enterobacter* spp. clones from strawberries at stage 6 were beta-hemolytic, while *Enterobacter* spp, but not *E. coli* from gooseberries were catalase positive and beta-hemolytic regardless of their level of ripeness. Early research indicated that beta-hemolytic phenotypes of isolates from environmental conditions are considered as virulence-associated determinants and of clinical relevance (Mogrovejo-Arias et al., 2020).

CONCLUSIONS

To date, there are no studies evaluating the microbiota associated with gooseberries and strawberries at different ripeness stages and their antibiotic resistance patterns. The prevalence of microorganisms was higher at the maturity stage (ready-to-eat) in both fruits. Nonetheless, several AR microorganisms were detected in all clones independently of the ripe stage, suggesting that adequate handling and high cleanliness of fruits are required to reduce the possibility of AR bacterial propagation. These data may also have potential applications in the treatment of strawberry and gooseberry diseases, particularly those caused by economically significant infections which can affect fruit yield and quality postharvest.

ACKNOWLEDGEMENTS

This research work was carried out with the support of Universidad Tecnica del Norte and was financed by Project Investiga UTN 2022 No. 1034/2022.

REFERENCES

Alharaty, G., and Ramaswamy, H.S. (2020). The Effect of Sodium Alginate-Calcium Chloride Coating on the Quality Parameters and Shelf Life of Strawberry Cut Fruits. *J. Compos. Sci*. 4(3):123. https://doi.org/10.3390/jcs4030123AOAC.

Al-Kharousi, Z. S., Guizani, N., Al-Sadi, A. M., and Al-Bulushi, I. M. (2022). Antimicrobial Susceptibility of Fresh Produce-Associated Enterobacteriaceae and Enterococci in Oman. *Foods (Basel, Switzerland)*, *11*(19), 3085. https://doi.org/10.3390/foods11193085.

Amador, P., Fernandes, R., Prudêncio, C., and Duarte, I. (2019). Prevalence of antibiotic resistance genes in multidrug-resistant *Enterobacteriaceae* on Portuguese livestock manure. Antibiotics 8:23. doi: 10.3390/antibiotics8010023

Association of Official Analytical Chemists. (1990). Official Method of Analysis, Titrimetric Method for Fruits; AOAC: Rockville, MD, USA.

Azam, M., Ejaz, S., Naveed, R., Rehman, U., Khan,M., and Qadri, R. (2019). Postharvest quality management of strawberries. Strawberry-Pre-and Post-Harvest Management Techniques for Higher Fruit Quality. https://www.intechopen.com/chapters/6668.

Carballo, M., Aguayo, S., González, M., Esperon, F., and Torre, A. (2016). Environmental assessment of Tetracycline's residues detected in pig slurry and poultry manure. *J. Environ. Prot*. 7, 82–92. doi: 10.4236/jep.2016.71008.

Davis, R., and Brown, P.D. (2016). Multiple antibiotic resistance index, fitness and virulence potential in respiratory Pseudomonas aeruginosa from Jamaica. *J. Med. Microbiol.* 65(4): 261-271. doi: 10.1099/jmm.0.000229.

Drobek, M., Cybulska, J., Gałązka, A., Feledyn-Szewczyk, B., Marzec-Grządziel, A., Sas-Paszt, L., Gryta, A., Trzciński, P., Zdunek, A., and Frąc, M. (2021). The Use of Interactions Between Microorganisms in Strawberry Cultivation (*Fragaria* x *ananassa* Duch.). *Front. Plant Sci*. 12, 780099. https://doi.org/10.3389/fpls.2021.780099.

European Food Safety Authority (2012). Guidance on the assessment of bacterial susceptibility to antimicrobials of human and veterinary importance. *EFSA J.* 10, 2740.

Ikegaya, A., Toyoizumi, T., Ohba, S., Nakajima, T., Kawata, T., Ito, S., and Arai, E. (2019). Effects of distribution of sugars and organic acids on the taste of strawberries. *Food Sci Nutr.* 7:2419–2426. https://doi.org/10.1002/fsn3.1109.

Jensen B., Knudsen I. M. B., Andersen B., Nielsen K. F., Thrane U., Jensen D. F., and Larsen, J. (2013). Characterization of microbial communities and fungal metabolites on field grown strawberries from organic and conventional production. *Int. J. Food Microbiol*. 160, 313–322. 10.1016/j.ijfoodmicro.2012.11.005.

Krahulcová, M., Micajová, B., Olejníková, P., Cverenkárová, K., and Bírošová, L. (2021). Microbial Safety of Smoothie Drinks from Fresh Bars Collected

in Slovakia. *Foods,* 10, 551. https://doi.org/10.3390/foods10030551.

Lafarga, T., Colás-Medá, P., Abadias, M., Aguiló-Aguayo,I., Bobo, G., and Viñas, I. (2019) Strategies to reduce microbial risk and improve quality of fresh and processed strawberries. *Inn. Food Sci. Emer. Tech.* 52: 197–212. https://doi.org/10.1016/j.ifset.2018.12.012.

Martínez-Bolaños, M., Nieto-Angel, D., Téliz-Ortiz, D., Rodríguez-Alcazar, J., Martínez-Damian, Ma. T., Vaquera-Huerta, H., and Carrillo Mendoza, O. (2008). Comparación cualitativa de fresas (Fragaria x ananassa Duch.) de cultivares mexicanos y estadounidenses. *Revista Chapingo. Serie horticultura*, *14*(2), 113-119. Available from http://www.scielo.org.mx/scielo.php?script=sci_arttext&pid=S1027-152X2008000200003&lng=es&tlng=es.

McGowan, L. L., Jackson, C. R., Barrett, J. B., Hiott, L. M., and Fedorka-Cray, P. J. (2006). Prevalence and antimicrobial resistance of enterococci isolated from retail fruits, vegetables, and meats. *J. Food Protect*. *69*(12), 2976–2982. https://doi.org/10.4315/0362-028x-69.12.2976.

Melo, J., and Quintas, C. (2023). Minimally processed fruits as vehicles for foodborne pathogens. *AIMS microbiology*, 9(1), 1–19. https://doi.org/10.3934/microbiol.2023001.

Mogrovejo-Arias, D. C., Brill, F. H. H., and Wagner, D. (2020). Potentially pathogenic bacteria isolated from diverse habitats in Spitsbergen, Svalbard. *Environ. Earth Sci.* 79:109. doi: 10.1007/s12665-020-8853-4.

Panghal, A., Mor, R. S., Kamble, S. S., Khan, S. A. R., Kumar, D., and Soni, G. (2022). Global food security post COVID-19: Dearth or dwell in the developing world?. *Agron J*. 114(1), 878–884.

Rahman, M., Alam, M. U., Luies, S. K., Kamal, A., Ferdous, S., Lin, A., Sharior, F., Khan, R., Rahman, Z., Parvez, S. M., Amin, N., Hasan, R., Tadesse, B. T., Taneja, N., Islam, M. A., and Ercumen, A. (2021). Contamination of Fresh Produce with Antibiotic-Resistant Bacteria and Associated Risks to Human Health: A Scoping Review. *Int. J. Environ. Res. Public Health*.19, 360. https://doi.org/10.3390/ijerph19010360.

Samtiya, M., Aluko, R.E., Dhewa, T., and Moreno-Rojas, G.M. (2021). Potential health benefits of food-plant derived bioactive components: An Overview. *Foods* 10: 839–864. https://doi.org/10.3390/foods10040839.

Sangiorgio, D., Cellini, A., Donati, I., Ferrari, E., Tanunchai, B., Fareed Mohamed Wahdan, S., Sadubsarn, D., Farneti, B., Checcucci, A., Buscot, F., Spinelli, F., and Purahong, W. (2022). Taxonomical and functional composition of strawberry microbiome is genotype-dependent. *J. Adv. Res.* 42, 189–204. https://doi.org/10.1016/j.jare.2022.02.009.

Sengupta, P., Sen, S., Mukherjee, K., and Acharya, K. (2020) Postharvest Diseases of Indian Gooseberry and Their Management: A Review. *Int. J. Fruit Sci.* 20:2, 178-190, doi: 10.1080/15538362.2019.1608889.

Septembre-Malaterre A, Remize F, and Poucheret P (2018) Fruits and vegetables, as a source of nutritional compounds and phytochemicals: Changes in bioactive compounds during lactic fermentation. *Food Res. Int.* 104: 86–99. https://doi.org/10.1016/j.foodres.2017.09.031.

Tenea, G. N., Reyes, P., Molina, D., and Ortega, C. (2023). Pathogenic Microorganisms Linked to Fresh Fruits and Juices Purchased at Low-Cost Markets in Ecuador, Potential Carriers of Antibiotic Resistance. *Antibiotics*, 12(2), 236. https://doi.org/10.3390/antibiotics12020236.

Wei, X., Hou, S., Pan, X., Xu, C., Li, J., Yu, H., Chase, J., Atwill, E. R., Li, X., Chen, K., and Chen, S. (2019) Microbiological Contamination of Strawberries from U-Pick Farms in Guangzhou, China. *Int. J. Environ. Res. Pub. Health.* 16(24), 4910. https://doi.org/10.3390/ijerph16244910.

Zhang, W-G., Zhang, M-S., and Li, W. (2022). Antibiotic Resistance Genes Contamination of Strawberries With the Long-Term Use of Raw, Aerobic Composting, and Anaerobic Composting Livestock Manure: A Comparative Study. *Front. Environ. Sci.* 10-2022, https://doi.org/10.3389/fenvs.2022.902321.

Scientific Bulletin. Series F. Biotechnologies, Vol. XXVII, No. 2, 2023
ISSN 2285-1364, CD-ROM ISSN 2285-5521, ISSN Online 2285-1372, ISSN-L 2285-1364

EXTRACTION AND EVALUATION OF TOTAL PHENOLICS CONTENT FROM RED CORN BRAN

Andrei Constantin Ioan ARLET[1, 2], Mihaela TOCIU[3], Brînduşa BALANUCA[3], Florentina ISRAEL-ROMING[2]

[1]Ministry of Education, 28-30 Gen. Berthelot Street, Bucharest, Romania
[2]University of Agronomic Sciences and Veterinary Medicine from Bucharest, 59 Marasti Blvd, District 1, Bucharest, Romania
[3]Department of Organic Chemistry "C. Nenițescu", National University of Science and Technology Politehnica Bucharest, 1-7 Polizu Street, Bucharest, Romania

Corresponding author email: mihaela.tociu@upb.ro

Abstract

Red and purple corn varieties were well studied in terms of their profile of biological active compounds, trying to explore the great potential of agricultural resources in different advanced applications, in food, pharmaceutic or medical fields. In this research paper, Bloody Butcher red corn variety was studied related to its total phenolics content and antioxidant activity. The research was conducted with milled corn and sifted milled corn, analyzing the use of several solvents for the extraction experiments and different methods (magnetic stirring, ultrasound, microwaves). Water-ethanol (1/1) was found to be the optimal extraction solvent mixture and ultrasound treatment was chosen as the best method to obtain extracts with high polyphenolic content (290 mg GAE/100 g DM). Microwave assisted extraction lead to similar results at 700W power level and 20 seconds exposure. Also, the highest antioxidant activity resulted for sifted milled corn after 60 minutes of magnetic stirring extraction with aqueous-ethanol solvent (451.71 mM TE/100 g DM).

Key words*: antioxidant, magnetic stirring, phenolics, purple corn, ultrasound*

INTRODUCTION

Important efforts are made all over the world to harness agricultural biomass (cereals and waste) in a progressive manner, by obtaining extracts of biological interest (Ursu et al., 2023; Romano et al., 2023; Francavilla and Joye, 2020). In this context, pigmented grains (corn, rice, barley, wheat, millet, in different colours as black, purple, blue, red, etc.) are well known for their great content of phytochemicals as phenolics, being explored in different ways to obtain active molecules for advanced application, in food, nutraceutical or biomedical fields (Francavilla and Joye, 2020; Bangar et al., 2023; Ed Nignpense et al., 2022).

Due to its worldwide availability and its easy cultivation, from an economic point of view and the necessary climatic conditions, the use of corn and its numerous by-products were considered in different research studies (Francavilla and Joye, 2020; Kim et al., 2023).

Natural dyes attract great interest among different industries and the recovery of pigments from cereals and agricultural waste is one of the best solutions for a sustainable economy. Recently, biorefining methods of dry purple corn cobs have been proposed, in order to recover anthocyanins and obtain pigments used in dyeing natural fibers (De Nisi et al., 2021). Lau et al. (2019) investigated the composition of sweet corn cobs, identifying high contents of minerals, proteins, and phenolic compounds in their waste. Phenolics play different roles in promoting human health, such as detoxification, inhibition of enzymes involved in inflammatory processes, antibiotic action against pathogen (fungi or bacteria) (Drăgan et al., 2022). In addition, natural compounds that possess antioxidant and anti-inflammatory (through phenolic compounds) properties are deeply involved in promoting healthy aging and combating senescence (Miu et al., 2023). Of all the coloured varieties of corn, purple corn attracts attention, and several studies were focused on the extraction of anthocyanin pigments (Kim et al., 2023). However, because there are different varieties of purple corn, adapted to cultivation in different climatic

zones, this natural resource is still not fully exploited.

Conventional and non-conventional methods were studied for the extraction of phenolics from different materials, including purple corn. The energy consumption, considerable volumes of solvents and the risk of degrading those compounds of interest (some of them sensitive under certain conditions) are the main drawbacks related to the conventional extractive methods, such as Soxhlet / solvent extraction, maceration and other methods currently used (Ivanovic et al., 2014; Cristianini and Guillén Sánchez, 2020). In the context of the global concern regarding energy consumption and a clear requirement for sustainable processes, non-conventional extraction technologies were considered. In this context microwave assisted extraction is one of the simplest and most efficient method for obtaining extracts rich in bioactive molecules; in addition, this process does not affect the integrity and quality of the targeted compounds (Cristianini and Guillén Sánchez, 2020; Piyapanrungrueang et al., 2016). This paper focuses on the extraction of phenolic compounds from Bloody Butcher red corn variety cultivated in Romania. The grinded purple corn was subjected to extraction, either in the form of bran or sifted bran. Different conventional and non-conventional methods were used: extraction under magnetic stirring, ultrasonic-assisted extraction, or microwave-assisted extraction. In order to select the best method for extracting phenolics, several extraction parameters were tested such as: types of solvent or extraction time period. The total polyphenolic content (TPC) and total antioxidant activity (AA) were evaluated for the studied corn extracts.

MATERIALS AND METHODS

Samples

The raw material, Bloody Butcher red corn variety, was cultivated and harvested in 2022 from a region in Brăila county, Romania. The dried corn kernels were processed by grinding, the obtained product (milled corn, MC) being further subjected to different extraction methods tested in the study. Also, the MC product was sieved (1 mm sieve mesh size.), and the resulting sieved material (sifted milled corn, SMC) was subjected to the same extraction methods.

Materials

Several analytical grade solvents were used either for extraction or for different analysis: ethanol (96% purity, Sigma Aldrich), hydrochloric acid (36.5-38.0%, Sigma Aldrich), distilled water (Milli-Q), 2,2´-Azino-bis (3-ethylbenzo-thiazoline-6-sulphonic acid) (ABTS), 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox), gallic acid (GA), sodium carbonate, sodium acetate, potassium persulfate (Sigma-Aldrich), Folin-Ciocâlteu reagent (Merck).

Phenolics extraction

Purple corn extracts were obtained either starting from the milled corn (MC) or from the sifted milled corn (SMC). Based on literature review (Cristianini and Guillén Sánchez, 2020; Lao and Giusti, 2018; Muangrat et al., 2018; Magni et al., 2018), different solvents were selected for this research, as shown in Table 1. Aqueous and hydro-alcoholic extracts were obtained, using distilled water (DW) and distilled water-ethanol mixtures (DW-EtOH), with or without acid addition (1% (v/v) addition of 1N HCl solution,), in a ratio of MC or SMC vegetal matter/solvent of 1/10.

The samples were subjected to two different types of treatment such as: magnetic stirring (1000 rpm) or ultrasound (30W power) for 60 minutes (Table 1).

In the second phase of the experiment, the different parameters were tested such as extraction time (1-45 minutes) and treatments (magnetic stirring or ultrasound), in order to select the optimal extraction method based on the total amount of phenolics obtained related to minimal energy consumption (economic aspects). In this stage of the research 28 extraction experiments were performed, 14 for the MC material and 14 for SMC material (stirring/ultrasounds), all of them with the optimal solvent.

The third experimental setup involved microwaves as the extraction technique for the phenolic compounds from the vegetable raw material. The microwave oven had 6 power levels and a maximum of 700 W, mechanical control (Hansa, Germany). Thus, 4 extraction times were tested (5, 10, 15 and 20 seconds), on 2 power levels (350 and 700 W) for the optimal solvent (based on E1-E8 experiment according to Table 1), for both MC and SMC products, resulting in a total number of 16 extraction experiments.

Table 1. Phenolics extraction experiments from purple corn

Experiment	Solvent	Solvent composition %	Extraction parameters
E1	DW	100	MC and SMC materials ***magnetic stirring*** 60 minutes | RT, 1000 RPM
E2	DW/ HCl	99/1 (v/v)	
E3	DW-EtOH	50/50 (v/v)	
E4	DW-EtOH/HCl	99/1 (v/v)	
E5	DW	100	MC and SMC materials ***ultrasounds treatment*** 60 minutes | RT, 30W power
E6	DW/HCl	99/1 (v/v)	
E7	DW-EtOH	50/50 (v/v)	
E8	DW-EtOH/HCl	99/1 (v/v)	

All the obtained extracts were filtered (Whatman filter paper, Grade 1) and the recovered filtrates were further subjected to different analysis. Each experimental phase was performed in triplicate and the reported results represent the average value of the three determinations.

The dry matter was calculated using freeze drying method. The samples were frozen and ground with dry ice and kept in a Freeze Dryers, D-37520 (Osterode am Harz, Germany) for 24 h. The samples were weighed in duplicate.

Quantification of the total polyphenolic content

The obtained purple corn extracts were evaluated in terms of the total phenolic content (TPC) using the well-known Folin-Ciocâlteu method (Munteanu and Apetrei, 2021; Ramos-Escudero et al., 2012). Briefly, 0.1 mL extract, 0.5 mL Folin-Ciocâlteu reagent and 1.5 mL of distilled water were mixed and maintained for 10 minutes at RT in a dark place. Then, 1.5 mL Na_2CO_3 solution (20%) was added, and the resulting samples are kept under the same conditions to reach a total incubation time of 2 hours, to complete the reaction and to generate the blue complex. The absorbance was measured at 750 nm for each studied extract using a Helios Beta UV-Vis spectrophotometer with Vision software (Thermo Electron Corporation, Waltham, MA, United States). Aqueous solutions of gallic acid (GA) with known concentration were employed for the calibration curve further used to assess the phenol content of the purple corn studied extracts (Ballus et al., 2015; Tociu et al., 2019; Odumose et al., 2015). TPC was expressed as mg of gallic acid equivalent (GAE) per 100g dry matter (DM).

Assessment of antioxidant capacity

To evaluate the total antioxidant capacity of the studied extracts from purple corn, TEAC method (Trolox equivalent antioxidant capacity) was considered (Lungu et al., 2016; Kim et al., 2022). Briefly, the ABTS•+ radical solution was obtained by mixing appropriate volumes of ABTS and $K_2S_2O_8$ in order to achieve final concentrations of 7 mM and 2.45 mM respectively, due to the rapid reaction of the antioxidant compounds within the tested samples and the blue-green ABTS•+ radical cation. The mixture was kept at room temperature, protected from the light, for 16 h. Before use, the ABTS•+ stock solution was diluted with ethanol to a final absorbance of 0.70 ± 0.02 at 734 nm. To a volume of 990 µL of this diluted solution were added 10 µL of sample or Trolox solution prepared in ethanol. The absorbance was recorded exactly after 1 minute. An appropriate solvent blank was run for each assay.

The results were calculated using a calibration curve made with Trolox solutions with concentrations ranging from 0.5 mM to 2.5 mM. The percentage of inhibition of absorbance at 734 nm was calculated for each Trolox solution and plotted as a function of concentration. Also, the percentage of inhibition of absorbance was calculated for each sample ($y = 34.8x + 12.944$, where y = percentage of inhibition, x = concentration of standard) and $R2 = 0.9946$). Antioxidant activity values of extracts were expressed as mmol/L Trolox equivalents, and the final results were expressed as Trolox equivalents/ 100g dry matter (mM TE/ 100g DM). All determinations were performed in triplicate.

RESULTS AND DISCUSSION

Evaluation of the extraction solvents based on TPC assays

Each selected solvent was involved in the extraction experiments for MC and SMC raw materials, by employing magnetic stirring and

ultrasound treatment (Table 1). The freshly obtained extracts were evaluated in terms of the TPC through Folin-Ciocâlteu spectrophotometric method, using a gallic acid (GA) calibration curve. The absorption of the samples prepared as described in the Methods section were measured at 750 nm against a blank sample, the obtained results being - presented in Table 2.

Table 2. TPC of the purple corn extracts in different solvents (extraction time, 60 minutes)

Extraction solvent	Magnetic stirring		Ultrasound treatment	
	TPC (mg GAE/100 g DM)		TPC (mg GAE/100 g DM)	
	MC	SMC	MC	SMC
DW	194.98 ± 19.00	153.53 ± 30.46	152.80 ± 28.84	171.71 ± 26.25
DW/HCl	143.35 ± 20.16	146.98 ± 33.18	98.98 ± 22.05	202.25 ± 41.36
DW-EtOH	202.98 ± 27.08	216.07 ± 38.85	290.25 ± 25.74	275.71 ± 49.01
DW-EtOH/HCl	115.71 ± 22.75	96.80 ± 29.41	291.71 ± 32.72	237.16 ± 33.86

According to the data presented in Table 2, TPC of the studied extracts ranges in a large interval for the different extraction solvents. The lowest values related to the phenolic content (TPC, as mg GAE/100 g dry matter) was registered for those extracts in acidified hydro-alcoholic medium, for both MC and SMC vegetal products, when magnetic stirring was employed, since the greater content of phenolics were obtained for the same solvent mixture, free of acid (DW-EtOH). The high extraction capacity of the phenolic compounds, associated with the DW-EtOH mixture is also validated when using ultrasounds (for MC and SMC also), while the HCl-aqueous media seems to have the lowest potential in this case, slightly higher results were observed for SMC under ultrasound treatment. From these experimental results the first conclusion that may be drawn is that the mixture of DW-EtOH (1:1 v/v) is the optimal extraction medium for the purple corn variety explored in this study.

The extraction method influences the solvent extraction capacity, ultrasound treatment being more efficient, conducing to the higher values of the calculated TPC. Also, ultrasound treatment seems to influence positively the phenolics extractive processes for MC vegetal material. Probably, in this case a much better contact of the solvent with the corn bran is achieved and, which mostly represents the exterior layers, the hull of the corn kernels, responsible also for the purple colour and known for the great content of the phenolic compounds (Razgonova et al., 2022; Li et al., 2017).

The samples obtained after 60 minutes of aqueous extraction were frozen (-18°C) and then lyophilized. For the two MC and SMC samples, the obtained dry matter for the extracts was, as follows: 3.3% (MC, magnetic stirring), 2.3% (MC, ultrasounds), 2.7% (SMC, magnetic stirring), 2.5% (SMC, ultrasounds).

The assessment of the optimal extraction time based on TPC

Once established the optimal extraction solvent, the experimental procedure passes in the second step, identifying the optimal extraction time, with respect to low energy consumption. For this, TPC was determined for the purple corn extracts, obtained in DW-EtOH, for both MC and SMC products, employing the two magnetic stirring and ultrasound treatment methods, at different extraction times, according to Table 3.

Table 3. TPC of the purple corn extracts at different extraction time (solvent, DW-EtOH)

Extraction time (min.)	Magnetic stirring		Ultrasound treatment	
	TPC (mg GAE/100 g DM)		TPC (mg GAE/100 g DM)	
	MC	SMC	MC	SMC
1	23.93 ± 4.77	31.06 ± 6.26	37.17 ± 10.51	43.79 ± 7.12
5	53.97 ± 10.32	58.56 ± 12.24	70.78 ± 12.27	70.78 ± 7.87
10	76.38 ± 17.57	85.04 ± 17.47	104.89 ± 9.11	98.27 ± 10.42
15	98.78 ± 23.28	108.97 ± 15.39	119.66 ± 21.74	143.08 ± 14.19
20	116.10 ± 18.36	129.34 ± 20.22	176.18 ± 31.14	204.19 ± 26.35
30	128.32 ± 22.28	169.05 ± 19.75	208.26 ± 14.05	220.48 ± 22.06
45	144.61 ± 26.88	178.22 ± 20.29	230.66 ± 19.73	238.81 ± 24.17
60	202.98 ± 27.08	216.07 ± 38.85	290.25 ± 25.74	275.71 ± 49.01

In accordance with the TPC values calculated for the obtained purple corn extracts, it may be assumed that 20 minutes represents the optimal time for an efficient extraction process of the phenolics from the purple corn. The phenolics content ranges from almost 416% (for SMC, magnetic stirring) to 485% (for MC with magnetic stirring), while for the products extracted by ultrasounds the increase of the TPC is 466% (for SMC) and 474% (for MC), all comparisons being made against the TPC values at 1 min of extraction. The results show that after 20 minutes of extraction (either with magnetic stirring or by ultrasound treatment), the increase of the TPC values is lower, probably correlated with the instability of these compounds exposed to oxygen and light. Usually, longer extraction times increase the extraction yield, but stability of the phenolic compounds has to be considered too because they are responsible for the biological effects.

In this experimental phase, the registered TPC values were greater when sifted corn brans were used as starting material. Probably the elimination of the fine part (as flour, which mainly comes from grinding of the corn kernels pulp) leads to a very good contact of the solvent with the brans, rich in phenolic derivatives (Razgonova et al., 2022). Also, ultrasound treatment resulted in higher amount of TPC, magnetic-stirring treatment being an adequate alternative to obtain corn extracts with high content of phenolics.

The microwave-assisted extraction

A non-conventional method was tested in the current study to extract the phenolic compounds from the selected corn variety, for both MC and SMC raw materials. Thus, microwave assisted extraction was performed, using two different power levels: 350W and 700W respectively, the process being performed at different extraction time (5, 10, 15 and 20 seconds) of contact of the samples with the optimal solvent DW-EtOH. The obtained purple corn extracts were evaluated regarding their TPC (mg GAE/ 100g DM), experimental results being reported in the graphs below (Figure 1).

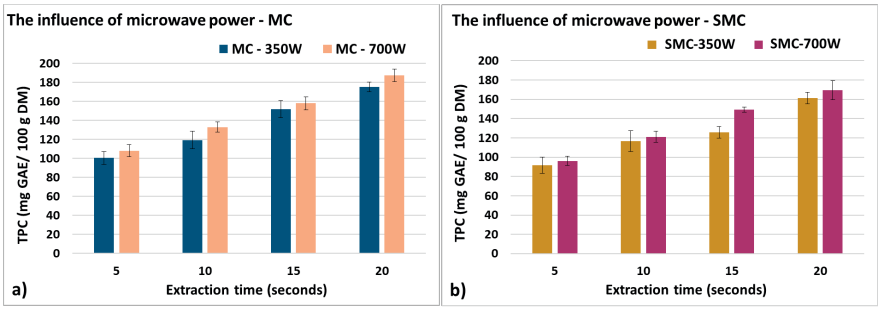


Figure 1. TPC of the corn extracts obtained using microwave assisted extraction: a) MC extract, b) SMC extract

According to the obtained results, the mean TPC values (mg GAE/100 g DM) increase with the increase of the extraction time, the higher phenolics level being noted for those MC and SMC extract resulted after 20 seconds of microwaves exposure. The experiment revealed that higher power level of the microwaves (700W) leads to an increase in the content of phenolic compounds in the MC and SMC extracts, at each extraction time (5-20 seconds). When comparing the MC and SMC from which the extracts were obtained, it may be assumed that the microwave treatment is more effective for the unsifted purple corn bran. Like ultrasound treatment, the microwave mediates good contact of the solvent with the samples, leading to extracts richer in phenolics, which are probably extracted both from the outer shell and from the ground core of the corn kernels. Additionally, probably higher temperature developed for short period during the process contributes to higher extraction yield due to the increased solubility and diffusion coefficient.

Comparing the tested methods, with respect to solvent and time optimized parameters for the extraction of the phenolic compounds from the MC and SMC products, it is obvious that ultrasound assisted extraction and microwave assisted extraction conducted to higher content of the phenolic moieties, while magnetic stirring

is associated with the lowest levels for the studied extracts (Figure 2).

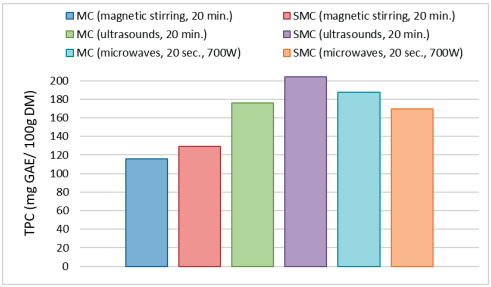


Figure 2. Comparison of the TPC levels for MC or SMC extracts through different extraction methods

Also, low energy consumption method using microwaves conducted to great TPC values, especially when the extraction was performed for the MC material, while for SMC material the highest extraction yield was obtained when performing ultrasound procedure.

Antioxidant activity

The total antioxidant activity was assessed for some MC or SMC selected extracts, obtained through the different extraction methods, using different experimental parameters, as shown in Table 4.

Table 4. Total antioxidant activity evaluated for selected corn extracts

Total antioxidant capacity (mM TE/100 g DM)											
Magnetic stirring				**Ultrasound treatment**				**Microwaves**			
MC	**SMC**	**MC**	**SMC**	**MC**	**SMC**	**MC**	**SMC**	**MC**	**SMC**	**MC**	**SMC**
20 min	20 min	60 min	60 min	20 min	20 min	60 min	60 min	350W	350W	700W	700 W
150.45	214.61	339.29	451.71	296.29	345.11	402.73	430.71	275.00	289.85	385.34	396.67

The total antioxidant activity of the studied corn extract (mM TE/ 100g DM) is well correlated with the total polyphenolic content discussed before, for low extraction period (20 min). The highest antioxidant activity resulted for SMC after 60 minutes of magnetic stirring extraction (451.71 mM TE/100 g DM). For the samples subjected to microwave treatment, the antioxidant activity is higher, particularly when SMC material is used to obtain the extracts, with values around 290 mM TE/100 g DM (350W power level) and 397 mM TE/100 g DM (700W power level). The modified ranking of the effectiveness of the extraction methods may be explained by taking into consideration that the antioxidant potential of the phenolic compounds is correlated with their molecular structure, associated with the total number of -OH groups and also to the position of this functionalities on the chemical structure (Heim et al., 2002; Zheng et al., 2019). Probably longer exposer to ultrasounds (60 min) and higher temperature developed during the process, may affect this chemical structure resulting lower antioxidant capacity for the extracted samples comparing with magnetic stirring ones. When microwaves are used, it is possible to obtain corn extract rich in bioactive compounds with high antioxidant capacity, such as anthocyanins and phenolic acids (Razgonova et al., 2022; Heim et al., 2002). Also, the aqueous ethanol solvent used for the extractive procedures may promote the extraction of the cyanidins, with strong antioxidant potential due to their -OH groups position within the molecule (Hao et al., 2022; Tan et al., 2019). On the other hand, obtaining the best results when magnetic steering was applied may suggest that other factors than the total amount of polyphenols in the extracts could play a significant role in determination of the antioxidant capacity.

CONCLUSIONS

In this study, several extraction experiments were carried out in order to establish the best parameters for extracting phenolics from Bloody Butcher red corn variety cultivated in Romania. Therefore, the water-ethanol (1/1) solvent lead to higher total phenolics content after 60 minutes at room temperature, using ultrasound treatment. Good results were also obtained with only 20 minutes of extraction with both magnetic stirring and ultrasound treatments, for both sifted and un-sifted milled corn brans. Regarding microwave assisted extraction, higher power, and longer exposure lead to higher total phenolics contents. Thus,

700 W power level and 20 seconds extraction time lead to similar results such as ultrasound assisted extractions.

The total antioxidant activity lead to better results for sifted milled corn for either type of extraction, the highest being obtained after 60 minutes of magnetic stirring extraction (451.71 mM TE/100 g DM).

Based on the obtained experimental results, it was concluded that this variety of purple corn can be considered in future experiments for the isolation of phenolic compounds. Also, the profile of phenolic compounds deserves to be studied and these extracts to be tested in food, pharmaceutical or cosmetic applications, as potential additives with antioxidant activity. This may be considered due to the selected extraction parameters, such as cell-friendly solvents (water and ethanol), an aspect closely related to sustainability considerations.

REFERENCES

Ballus, C. A., Meinhart, A. D., de Souza Campos, F. A., & Godoy, H. T. (2015). Total phenolics of virgin olive oils highly correlate with the hydrogen atom transfer mechanism of antioxidant capacity. Journal of the American Oil Chemists' Society, 92, 843-851.

Bangar, S. P., Maqsood, S., & Siroha, A. K. (Eds.). (2023). Pigmented Cereals and Millets: Bioactive Profile and Food Applications. Royal Society of Chemistry.

Cristianini, M., & Guillén Sánchez, J. S. (2020). Extraction of bioactive compounds from purple corn using emerging technologies: A review. Journal of food science, 85(4), 862-869.

De Nisi, P., Borlini, G., Parizad, P. A., Scarafoni, A., Sandroni, P., Cassani, E., ... & Pilu, R. (2021). Biorefinery approach applied to the valorization of purple corn cobs. ACS Sustainable Chemistry & Engineering, 9(10), 3781-3791.

Drăgan, M., Spulber, R., Livadariu, O., & Jurcoane, Ș. (2022). The Antioxidant Effect Of Raw Bee Pollen Collected From Ecological Crops-Minireview. Scientific Bulletin Series F. Biotechnologies, 26(2).

Francavilla, A., & Joye, I. J. (2020). Anthocyanins in whole grain cereals and their potential effect on health. Nutrients, 12(10), 2922.

Hao, R., Zhang, H., Feng, Y., Yang, D., Zhao, Z., & Zhao, S. (2022). The effect of different solvents and acidifying reagents on the anthocyanin profiles and antioxidant capacity of purple corn. Chemical Papers, 76(8), 4691-4704.

Heim, K. E., Tagliaferro, A. R., & Bobilya, D. J. (2002). Flavonoid antioxidants: chemistry, metabolism and structure-activity relationships. The Journal of nutritional biochemistry, 13(10), 572-584.

Ivanovic, J., Tadic, V., Dimitrijevic, S., Stamenic, M., Petrovic, S., & Zizovic, I. (2014). Antioxidant properties of the anthocyanin-containing ultrasonic extract from blackberry cultivar "Čačanska Bestrna". Industrial Crops and Products, 53, 274-281.

Kim, H. Y., Lee, K. Y., Kim, M., Hong, M., Deepa, P., & Kim, S. (2023). A Review of the Biological Properties of Purple Corn (Zea mays L.). Scientia Pharmaceutica, 91(1), 6;

Kim, J. T., Chung, I. M., Kim, M. J., Lee, J. S., Son, B. Y., Bae, H. H., ... & Yi, G. (2022). Comparison of antioxidant activity assays in fresh purple waxy corn (Zea mays L.) during grain filling. Applied Biological Chemistry, 65(1), 1.

Lao, F., & Giusti, M. M. (2018). Extraction of purple corn (Zea mays L.) cob pigments and phenolic compounds using food-friendly solvents. Journal of Cereal Science, 80, 87-93.

Lau, T., Harbourne, N., & Oruña-Concha, M. J. (2019). Valorisation of sweet corn (Zea mays) cob by extraction of valuable compounds. International Journal of Food Science & Technology, 54(4), 1240-1246.

Li, Q., Somavat, P., Singh, V., Chatham, L., & de Mejia, E. G. (2017). A comparative study of anthocyanin distribution in purple and blue corn coproducts from three conventional fractionation processes. Food chemistry, 231, 332-339.

Lungu, L., Savoiu, M. R., Manolescu, B. N., Farcasanu, I. C., & Popa, C. V. (2016). Phytotoxic and Antioxidant activities of leaf extracts of Ailanthus altissima swingle. Rev. Chim, 67, 1928-1931.

Magni, G., Marinelli, A., Riccio, D., Lecca, D., Tonelli, C., Abbracchio, M. P., ... & Ceruti, S. (2018). Purple corn extract as anti-allodynic treatment for trigeminal pain: role of microglia. Frontiers in Cellular Neuroscience, 12, 378.

Miu, D., Shaat, F., & Pavaloiu, R. D. (2023). Methods To Improve Bioavailability Of Natural Senolytics-A Mini Review. Scientific Bulletin Series F. Biotechnologies, 27(1).

Muangrat, R., Pongsirikul, I., & Blanco, P. H. (2018). Ultrasound assisted extraction of anthocyanins and total phenolic compounds from dried cob of purple waxy corn using response surface methodology. Journal of food processing and preservation, 42(2), e13447.

Munteanu, I. G., & Apetrei, C. (2021). Analytical methods used in determining antioxidant activity: A review. International Journal of Molecular Sciences, 22(7), 3380.

Nignpense, B., Latif, S., Francis, N., Blanchard, C., & Santhakumar, A. B. (2022). Bioaccessibility and Antioxidant Activity of Polyphenols from Pigmented Barley and Wheat. Foods, 11(22), 3697;

Odumosu, P., Ojerinde, S., & Egbuchiem, M. (2015). Polyphenolic contents of some instant tea brands and their anti-oxidant activities. Journal of Applied Pharmaceutical Science, 5(9), 100-105.

Piyapanrungrueang, W., Chantrapornchai, W., Haruthaithanasan, V., Sukatta, U., & Aekatasanawan, C. (2016). Comparison of anthocyanin extraction methods from high anthocyanin purple corn cob

hybrid: KPSC 901, and quality of the extract powder. Journal of Food Processing and Preservation, 40(5), 1125-1133.

Ramos-Escudero, F., Muñoz, A. M., Alvarado-Ortíz, C., Alvarado, A., & Yáñez, J. A. (2012). Purple corn (Zea mays L.) phenolic compounds profile and its assessment as an agent against oxidative stress in isolated mouse organs. Journal of medicinal Food, 15(2), 206-215.

Razgonova, M., Zinchenko, Y., Pikula, K., Tekutyeva, L., Son, O., Zakharenko, A., ... & Golokhvast, K. (2022). Spatial distribution of polyphenolic compounds in corn grains (Zea mays L. var. Pioneer) studied by laser confocal microscopy and high-resolution mass spectrometry. Plants, 11(5), 630.

Romano, G., Tufariello, M., Calabriso, N., Del Coco, L., Fanizzi, F. P., Blanco, A., ... & Laddomada, B. (2023). Pigmented cereals and legume grains as healthier alternatives for brewing beers. Food Bioscience, 102463.

Tan, J., Li, Y., Hou, D. X., & Wu, S. (2019). The effects and mechanisms of cyanidin-3-glucoside and its phenolic metabolites in maintaining intestinal integrity. Antioxidants, 8(10), 479.

Tociu, M., Oprea, O., Stănescu, M. D., & Stanescu, M. D. (2019). Preliminary investigations for the valorization of grape seeds after the oil extraction. Bull. Ser. B, 81, 47-56.

Ursu, M. G. S., Milea, Ș. A., Păcularu-Burada, B., Dumitrașcu, L., Râpeanu, G., Stanciu, S., & Stănciuc, N. (2023). Optimizing of the extraction conditions for anthocyanin's from purple corn flour (Zea mays L): Evidences on selected properties of optimized extract. Food Chemistry: X, 17, 100521.

Zheng, Y. Z., Deng, G., Guo, R., Fu, Z. M., & Chen, D. F. (2019). The influence of the H5⋯ OC4 intramolecular hydrogen-bond (IHB) on the antioxidative activity of flavonoid. Phytochemistry, 160, 19-24.

Scientific Bulletin. Series F. Biotechnologies, Vol. XXVII, No. 2, 2023
ISSN 2285-1364, CD-ROM ISSN 2285-5521, ISSN Online 2285-1372, ISSN-L 2285-1364

METHODS FOR IDENTIFYING LIGNIN DEGRADING MICROORGANISMS

Alexandra BURLACU (GRIGORAȘ), Aglaia POPA, Florentina ISRAEL-ROMING

University of Agronomic Sciences and Veterinary Medicine from Bucharest, 59 Mărăști Blvd, District 1, Bucharest, Romania

Corresponding author email: aglaia_popa@yahoo.com

Abstract

Lignocellulosic biomass has a tremendous potential for obtaining value-added products useful in applications in medicine, food and feed, textile, biofuels, carbon fibres etc. Amongst the three main components, lignin is the most abundant natural aromatic polymer that is the least valorised. The main enzymes involved in lignin degradation are mainly oxidases (laccases and peroxidases), but several esterases (hydroxycinnamic acid esterases) are also important for their activity on releasing lignin from hemicellulose and cellulose. This study is focused on describing the most used compounds that can mimic the structure of lignin components and presents the available methods used for identifying microorganisms capable of degrading lignin. Also, it provides an array of industrial applications for depolymerised lignin.

Key words*: lignin, lignocellulose, oxidase, peroxidase.*

INTRODUCTION

With the increase of demand for agricultural production due to rapid growth of world population, there is also an increase in agro-waste, mainly called lignocellulose biomass. This biomass is comprised of three main components: cellulose, hemicellulose and lignin, which are interconnected and interspersed, making it difficult to depolymerise. Although, the biomass derives mainly from agricultural waste, it can also result from forest residues, first generation feedstock, paper/pulp waste, or recycling stations (Usmani et al., 2021). Recent reports suggest that the annual production of lignocellulosic biomass is approximately 181.5 billion tons (Mujtaba et al., 2023), out of which 173.7 billion tons are not used properly and usually are discarded or burned to produce energy or steam. Advancements in this area are made for producing biofuels, environmentally friendly bioproducts, bioplastics, bio-composites (substrates for medical applications, environment remediation or inks for additive manufacturing) etc. (Coulibaly et al., 2023; Rusu et al., 2022; Mujtabai et al., 2023; Usmani et al., 2021).

Lignin is the most abundant natural aromatic polymer with a heterogenous structure and a recalcitrance to depolymerisation. The importance of degrading lignin derives from the increased degradation yield of lignocellulose materials and the possibility to obtain chemical compounds that could replace petroleum products (Ohta et al., 2012).

The valorisation of lignin from lignocellulose is one of the primary barriers to its recovery and utilization. The following issues should be considered: reducing the environmental impact of burning; preventing carbon loss and completely utilizing the lignin from waste sources (Sharma et al., 2023).

Lignin is comprised of phenylpropanoid units: coniferyl, sinapyl and p-coumaryl alcohol groups (Figure 1) which form an integral part of the cell walls of softwood (25-35%), hardwood (18-25%) or grass (15-25%) (Sharma et al., 2023). The most common linkages found in lignin are: β-O-4, α-O-4, 4-O-5, β-5, β-1, 5-5 and β- β (Figure 1).

Currently, the microorganisms used for degrading lignin are white and brown-rot fungi from *Basidiomycetes* division. Some bacteria can also degrade lignin, but their pathways are not fully elucidated. The microorganisms are producing oxidases such as laccase, manganese peroxidase, lignin peroxidase, or versatile peroxidase. Other enzymes are also known as helpful with lignin degradation and are known as auxiliary enzymes: ferulic acid esterase, p-coumaric acid esterase or acetyl xylan esterase.

These enzymes are used in the cleavage of linkages that connect lignin to hemicellulose. Although they are considered secondary enzymes, they are important in releasing lignin from the lignocellulosic materials and enabling the access to lignin structures of oxidases.

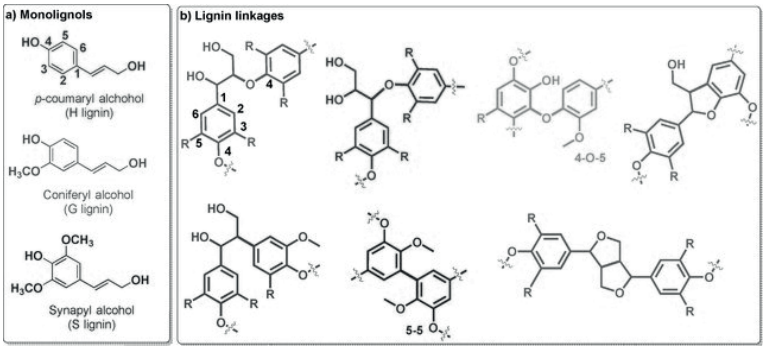


Figure 1. Structural units (a) and linkages (b) found in lignin (Garedew et al., 2020)

This study is focused on describing the most used compounds that have similar structure to lignin components and presents the available methods used for identifying microorganisms able to degrade lignin.

MOLECULAR STRUCTURES OF AROMATIC COMPOUNDS USED AS LIGNIN MODELS

In order to identify microbial strains with the ability to degrade lignin from lignocellulosic materials, the first step would be selecting substrates that can mimic structural components of lignin. According to Gonçalves (2020), the substrates used more often are either chromogenic ones, precursors of lignin or aromatic dyes. With chromogenic substrates, it is possible to quantify the oxidases enzymatic activity and therefore to develop high-throughput screening methods.

The most used chromogenic substrates suitable for oxidases (laccases or peroxidases) involved in lignin degradation are: ABTS (2,2′-Azinobis-(3-Ethylbenzthiazolin-6-Sulfonic Acid)), 2,6-DMP (2,6-dymethylphenol), guaiacol, o-dianisidine, L-DOPA (l-3,4-dihydroxyphenylalanine), veratryl alcohol, 2,4-DCP (2,4-dichlorophenol), phenol red, TMPD (N,N,N',N'-Tetramethyl-p-phenylenediamine dihydrochloride), vanillylacetone (Figure 2).

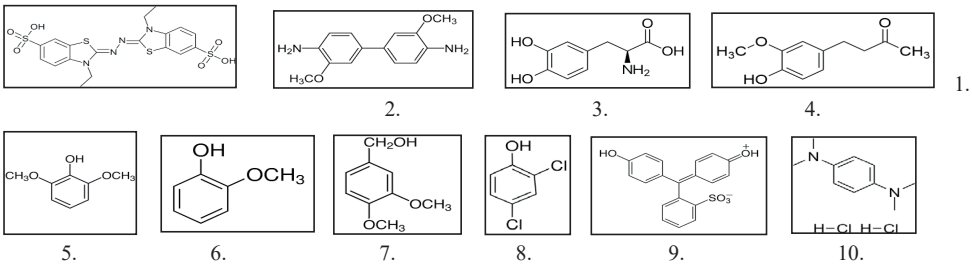


Figure 2. Substrates used for investigating lignin degradation: ABTS (1), o-dianisidine (2), L-DOPA (3), vanillylacetone (4), 2,6-DMP (5), guaiacol (6), veratryl alcohol (7), 2,4-DCP (8), phenol red (9), TMPD (10)

The aromatic dyes used are: Azure B, Remazol Brilliant Blue R, toluidine blue, Remazol Marine Blue, malachite green, induline, methylene blue (Figure 3). The use of dyes in liquid media (malachite green, methylene blue, toluidine blue) allow the evaluation of lignin degradation by measuring the optical density at 620 nm (Bandounas et al., 2011; Melo-Nascimento et al, 2018).

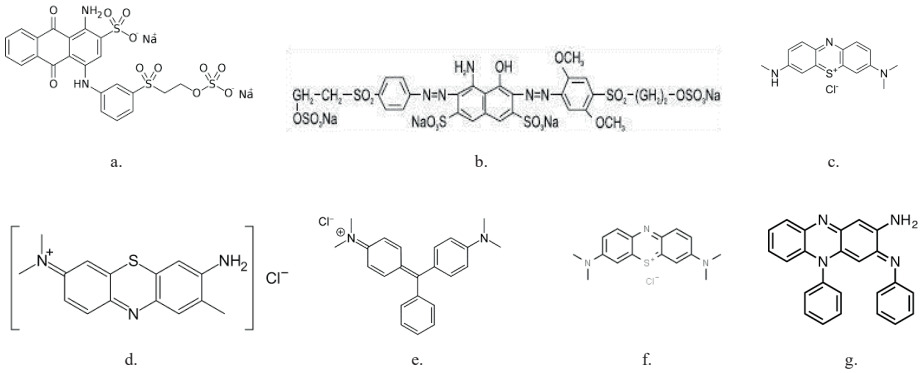


Figure 3. Aromatic dyes used for identifying microorganisms capable of degrading lignin
a. Remazol Brilliant Blue R, b. Remazol Marine Blue, c. Azure B, d. toluidine blue, e. malachite green, f. methylene blue, g. induline

Other studies (Ohta et al., 2012) suggest to use compounds with similar structures as lignin precursors: p-coumaryl alcohol (p-coumaric acid, 4-vinyl phenol, protocatechuic acid, p-hydroxybenzoic acid), coniferyl alcohol (ferulic acid, 4-vinyl guaiacol, vanillin, veratryl alcohol) or sinapyl alcohol (sinapic acid, 4-vinyl syringol, syringaldehyde) (Figure 4).

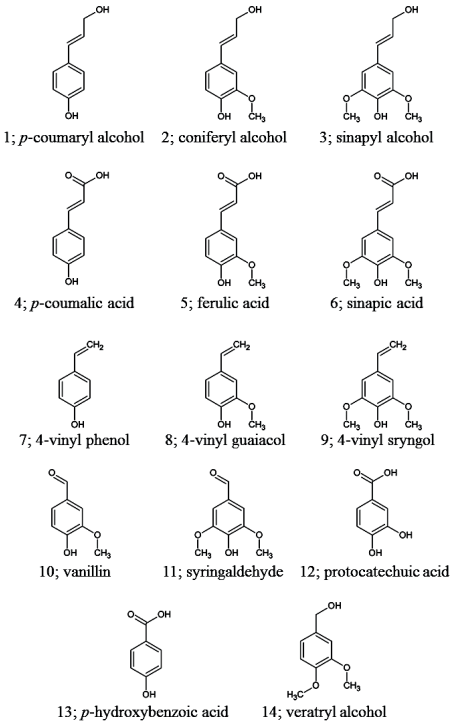


Figure 4. Aromatic compounds with similar structure to lignin precursors (Ohta et al., 2012)

For auxiliary enzymes mentioned before, the most used substrates for identifying microbial producers are: α- or β-naphtyl acetate, p-methylumbelliferyl acetate, p-nitrophenyl acetate or even chemically acetylated xylan (for acetyl xylan esterase); methyl ferulate, ethyl ferulate, vinyl ferulate, prenyl ferulate, L-arabinose ferulate, p-nitrophenyl ferulate, feruloyl glucose (for ferulic acid esterase); methyl p-coumarate, ethyl p-coumarate, p-coumaroyl glucose (for p-coumaric acid esterase) (Popa et al., 2020a; Popa et al., 2020b; Antonopoulou et al., 2019; Li et al., 2019; Ramírez-Velasco et al., 2016; Collombel et al., 2019).

METHODS FOR DETECTING LIGNIN DEGRADING ACTIVITIES

Lignocellulose biorefineries could convert lignocellulose biomass to high value-added bioproducts. Due to lack of high producers of multiple enzymes that could degrade lignin, there is a need for screening novel microorganisms for their ability to depolymerise lignin. Therefore, screening methods for lignin degradation have to be optimised or developed in order to detect lignin degrading activities rapidly and at lower costs.

The most widely used methods for isolating microorganisms that produce ligninases are cultivation-based methods, some of them being well established.

Usually, the qualitative screening of lignin degrading fungi or bacteria is performed using

natural substrates that resembles the structure of lignin.

There are many methods employed for evaluating lignin degradation that vary in detection limit and sensitivity, but usually the most frequently used are colorimetric and enzymatic assays.

Growth-based assays

An easy and efficient method for evaluating lignin-degrading activities is described by Ravi et al. (2017). In this study, an enriched vegetal compost is used to isolate three *Pseudomonas* strains with high ligninases activities and are compared to the activity of *Pseudomonas putida* KT2440. Several lignin model compounds are used in order to characterise the growth of the isolates: vanillin, vanillate, ferulate, 4-hydroxybenzoate, p-coumarate and benzoate. The results indicate that specific growth rates were considerably higher on p-coumarate, benzoate and 4-hydroxybenzoate than those on ferulate and vanillate. There is no growth on vanillin, mainly due to the fact that the substrate is rapidly converted to vanillate. The results also highlight the fact that vanillin, benzoate and 4-hydroxybenzoate are preferentially consumed first and ferulate last. With this research, the scientists are able to understand better the metabolic pathways of microorganisms and how they include aromatic compounds. However, the method can only be used for identifying microbial strains that are easy to work with (Ravi et al., 2017).

Colorimetric assays

Bandounas et al. (2011) isolates soil bacteria (*Pandoraea norimbergensis* LD001, *Pseudomonas* sp. LD002, *Bacillus* sp. LD003) by enrichment of Kraft lignin and use them for comparing their lignin degrading activities. The capacity to degrade lignin is determined based on three assessments: ability to grow on high and low-molecular weight lignin fractions, ability to use lignin aromatic monomers and ability to decolourise several dyes (Azure B, toluidine blue O, Congo red, methylene blue). The results indicate that the strains have a slow and small growth on the lignin fractions, the ability to use lignin monomers is also limited for the isolates and the best dye-decolourizing activity is noted for *Bacillus* sp. LD003. The advantage of this method was that it resulted in an accurate evaluation of degradation of lignin monomers. The disadvantage was that the activities of ligninases can vary with different types of lignin.

Another screening method (Jeong et al., 2012) is based on identifying enzymes capable of degrading synthetic organophosphates (based on fluorescence). The advantage of this method is that it is possible to improve the enzymatic activity by using phenolics responsive transcription activator. The disadvantage is that the enzymatic activities registered with synthetic molecules may not be similar to those in natural substrates with high lignin content.

In the method proposed by Melo-Nascimento et al. (2018), 21 ligninolytic strains (*Klebsiella* spp.) are screened based on their ability to grow on culture media containing lignin as the main carbon source. In media supplemented with dye (methylene blue, toluidine blue, Congo red, malachite green), the best decolourization (98% after 48 hours) is observed with *Klebsiella* P3TM1 in the presence of methylene blue. The advantage of this method is that it provides a better understanding of the mechanism of depolymerisation of lignin, since the study concluded that the breakdown of lignin is based mostly on the extracellular activities of peroxidases produced by *Klebsiella* spp. The disadvantage of this method is that results may vary based on using different lignin models.

A method for isolating lignin degrading microorganisms is presented by Taylor et al. (2012). In this research, microbial isolates (*Microbacterium* sp., *Ochrobactrum* sp., *Micrococcus* sp., *Thermobifida fusca*, *Rhodococcus erythropolis*, *Sphingobacterium* sp., *Rhizobiales* sp.,) are cultivated on high and low molecular Kraft lignin. Lignin degradation is measured with the products obtained (protocatechuic acid and oxalic acid), the best activity being registered with *Sphingobacterium* isolate. Although the method is successful in detecting new microbial strains able to produce ligninases, it is only applicable to samples with nitrated lignin.

A method proposed by Zhou et al. (2017) uses Azure B as a target molecule for identifying microorganisms capable of degrading lignin. The microorganisms tested are isolated from termite gut (*Enterobacter hormaechei*, *Bacillus licheniformis*). The method is also used to

identify fungi capable of producing lignin peroxidase. The advantage of this technique was that it is low cost, but it is however time consuming and requires high amounts of enzymes.

Enzymatic assays

An easy and accessible method for evaluating degradation of ABTS with *Trametes versicolor* laccase can be performed spectrophotometrically, as suggested by Alcade et al. (2005). The presence of enzymatic activity is observed when a green colour is developed suggesting the formation of ABTS radical. Although the substrate used is soluble, stable and available, this screening method is less sensitive, therefore not accurate enough and time consuming (Gonçalves et al., 2020).

In a research lead by Huang et al. (2013), 140 bacterial strains are isolated from soil and screened based on their ability to oxidise ABTS (known as a substrate for laccase). The two selected strains (*Bacillus pumilus* and *Bacillus atropheus*) are able to degrade guaiacylglycerol-b-guaiacylether (the most abundant linkage in lignin) and also fragments of kraft lignin. Although the method allows the identification of ligninolytic strains, it is also time consuming and only limited to laccases activities (Gonçalves et al., 2020).

Laccase activity can also be identified using guaiacol supplemented PDA plates, the enzymatic activity being observed as reddish-brown zones around microbial colonies (Abd El Monssef et al., 2016).

Spectrophotometric assays with nitrated lignin

Ahmad et al. (2011) describes a scalable and semiquantitative method for identifying microorganisms able to degrade lignin using nitrated milled wood lignin. The breakdown of chemically nitrated lignin produces nitrated phenol products which lead to changes in absorbance at 430 nm. With this assay, the researchers identified several bacterial strains (*Rhodococcus jostii* and *Pseudomonas putida*) that produce peroxidases that can degrade lignin to low molecular weight phenolic byproducts. Unfortunately, the method is growth dependant and time consuming (Gonçalves et al., 2020).

The research proposed by Taylor et al. (2012) involves spraying a nitrated lignin solution on inoculated agar plates. The bacterial strains are identified based on a signal (fluorescent yellow) produced which is measured spectrophotometrically at 430nm.

Chromatographic methods

Reverse phase HPLC can be an useful method to detect the products of lignin depolymerisation. In a study (Taylor et al., 2012), *Microbacterium* sp., *Rhodococcus erythropolis* and *Sphingobacterium* sp. are cultivated on Luria-Bertani broth supplemented with wheat straw lignocellulose in order to establish the amount of oxalic acid produced (formed by oxidation of glycolaldehyde generated). Additionaly, *Microbacterium* sp. produces protocatechuic acid, an intermediate molecule in aromatic degradation pathways. The disadvantage of this analytical method is that same mass to charge ratio (m/z) in various compounds may give deceiving results (Gonçalves et al., 2020).

Other studies (Ohta et al., 2012) use LC/MS or GC/MS to identify and quantify products obtained from lignin degradation such as: ferulic acid and p-coumaric acid. The substrates related to lignin structure are: vanillin, p-hydroxybenzoic acid, protocatechuic acid, sinapic acid, p-hydroxybenzaldehyde, ferulic acid, p-coumaric acid and veratryl alcohol. Supplementation with ferulic acid and p-coumaric acid is justified, since research shows that they can boost enzymatic activity of ligninases (especially laccases) (Kuhar and Papinutti, 2014). Although this method is able to highlight the biotransformation pathways of lignin depolymerisation, compounds with similar m/z ratio can lead to deceiving results (Gonçalves et al., 2020).

FTIR

Fourier-transform infrared spectroscopy is used as a technique for evaluating the changes in the chemical composition of lignin (from lignocellulosic materials) during degradation with enzymes produced by *Penicillium simplicissimum* (Liu et al., 2014). The analysis shows cleavage of ether linkages, demethylation and oxidation, lignin being transformed to pseudo-lignin.

Native gel electrophoresis

Native gel electrophoresis was employed for detection and quantification of lignin degradation since 1989 (Adhi et al., 1989), and

since then several researches optimised this method (Kumar et al., 2017; Sun et al., 2004). The polyacrylamide gels contain different substrates in order to detect enzymatic activities: O-tolidine (laccase activity) or L-DOPA (lignin peroxidase activity). The microorganisms tested with this technique are: *Streptomyces badius*, *Trametes gallica* and *Ganoderma lucidum*. Although these methods are accessible, rapid and can work with crude enzymes, they require a substantial amount of enzyme since they are less sensitive (Gonçalves et al., 2020).

Capillary electrophoresis

Kudo et al. (2017) describes a capillary electrophoresis method used for evaluating the activities of manganese peroxidase and lignin peroxidase (produced by *Phanerochaete chrysosporium*). The substrates required are veratryl alcohol and Mn(II) malonate. Since lignin peroxidase activity is overestimated in the presence of manganese peroxidase and vice versa, with this method the researchers are able to distinguish between the activity of both enzymes in a mixture without separation. However, with this method there are several disadvantages such as protein adsorption to capillary walls and small injectable sample size.

Chemiluminescent method

The base for several diagnostic immunoassays is peroxide-dependent oxidation of luminol by horseradish peroxidase, but peroxidases from other sources can also catalyse luminol oxidation.

Mercer et al. (1996) presents a chemiluminescent assay with an Amerlite analyser used to examine the peroxidase activity of a taxonomic range of actinomycetes (*Saccharomonospora* sp., *Thermomonospora* sp., *Streptomyces* sp., *Actinomadura* sp.). With this method, the researchers are able to identify producers with high extracellular peroxidase activity. Although the chemiluminescent method is sensitive to peroxidase and permits the use of the crude enzyme, it has short luminescence, weak signals, is sensitive to external factors and the apparatus is not widely available.

To address the issues with this method, Zhang et al. (2018) suggests the importance of high chemiluminescent intensity in providing low detection limit for analytes and high sensitivity. This is obtained with the use of enhancers such as derivatives of: lophine, p-phenol, substituted boronic acid or combination of N-alkyl phenothiazines and nucleophilic acylation catalysts as co-enhancer. Also, the use of amplification techniques can lead to higher sensitivity of chemiluminescence detection.

$^{14}CO_2$-autoradiography

Temp et al. (1998) develops a method for screening lignin degrading microorganisms using synthetic lignin (DHP-^{14}C ring labelled dehydrogenation polymerizate). Although the use of this method can easily, rapidly and accurately detect microbial strains with the ability to depolymerise lignin, is sensitive to culture conditions, it requires specific expertise and is time consuming and laborious.

Sequence-based methods

DNA-based methods can allow the ability to evaluate lignin degrading activity by searching and selecting genes encoding for key enzymes involved in lignin depolymerisation. Several databases (NCBI, PATRIC, KEGG, UniProt, Pfam, ExPASy) are useful for this approach, however eLignin is considered to contain the most published research regarding aspects such as: microbial strains, enzymatic systems, metabolic pathways, metabolites etc. (Gonçalves et al., 2020). Also, several annotation tools offer the possibility to study the molecular functional diversity at different levels: metabolic context subsystems, protein families and individual.

Biosensor-based methods

Biosensor-based methods are expected to overcome the shortcomings of conventional screening methods, including identifying novel enzymes and/or aromatic molecules involved in lignin degradation (Gonçalves et al., 2020).

Several studies (Eggeling et al., 2015; Rinaldi et al., 2016; Fritzsch et al., 2012; Ho et al., 2018; Siedler et al., 2017) are focused on whole-cell biosensors used to measure aromatic lignin derivatives such as p-coumaric acid, vanillin and syringaldehyde. These methods have limited sensitivity and accuracy but they are able to detect extracellular products and can have a broad range of applications (Gonçalves et al., 2020).

Other biosensors are employed to detect genes encoding for enzymes involved in lignin

depolymerisation (Uchiyama and Miyazaki, 2013; Alvarez-Gonzalez and Dixon, 2019; Mannan et al., 2017). Genetic Enzyme Screening System (GESS) is a biosensor that targets the phenol and cresol molecules. It is a quantitative enzyme biosensor and has a high sensitivity to targeted molecules. However, it only measures intracellular concentrations of compounds (Choi et al., 2014).

Substrate Induced Gene Expression (SIGEX) is a biosensor that measures aromatic compounds such as: chlorohydroquinone, 4-chlorocatechol, 3-methyl catechol and salicylate (Uchiyama and Miyazaki, 2013). This biosensor can be used to identify targeted genes, but it only measures intracellular concentrations. Also, most of the transcription regulator functions are still unknown (Gonçalves et al., 2020).

APPLICATIONS OF DEPOLYMERISED LIGNIN

Lignin degradation can generate various value-added products that can be tough, water and heat resistant, durable, friendly to the environment (Sharma et al., 2023). The bio-products obtained with lignin depolymerisation are: acids (ferulic acid, muconic acid, p-coumaric acid, acetic acid, acylic acid, lactic acid hydroxybenzoic acid, 3-hydroxypropinoic acid, adipic acid), benzene, pyrogallol, xylene, guaiacol, toluene, polyhydroxyalkanoates etc. These compounds are useful to various biotechnological applications: biofuels (biogas, bio-oils), bioremediation, pharmaceutics, cosmetics, bioplastics, textiles, biosensors, agrochemicals (biofertilisers, regulators for plant growth), nanocomposites, carbon fibres, dyes, flavoring compounds, sustainable construction materials etc. (Burlacu et al., 2018; Sharma et al., 2023; Ahmad et al., 2021; Xia et al., 2021; Chauhan et al., 2021; Sivagurunathan et al., 2021; Jędrzejczak et al., 2021).

Biofuels

Although bioethanol production from cellulose is environmentally friendly, the cost of the process is more expensive than that of fossil fuels. Therefore, lignin valorisation to obtain biogas could enhance the economics of lignocellulose biomass refineries. Biogas can be obtained through anaerobic digestion from hydrolysed lignin produced through steam explosion pretreated biomass. With this process, a high yield of volatile compounds is produced (even higher with microbial fermentation) facilitating the complete valorisation of lignocellulosic biomass (Sharma et al., 2023).

Lignin derived compounds can be converted by microorganisms into lipids through aromatic catabolism and later the lipids can be used for biodiesel production (Saini et al., 2022).

Pharmaceutics and biomedicine

Lignin has low cytotoxicity, biocompatibility, UV absorption capacity, and antioxidant and antibacterial qualities. Furthermore, many processed biomaterials can have their mechanical strength increased by lignin. As a result, lignin is a potential aromatic raw resource for the biomedical and pharmaceutical industries. The recent developments in the valorization of lignin involves creation of wound dressings, tissue engineering, medication and gene delivery systems, and sunscreen actives (Domínguez-Robles et al., 2020).

Bioplastics

By creating biodegradable and environmentally friendly bioplastics from renewable lignocellulosic biomass, the petrochemical industries may become less dependent on them. Using lignocellulosic wastes as substrates, a range of microorganisms can create polyhydroxyalkanoates (PHAs), or biopolyesters. PHAs have the potential to replace non-renewable petrochemical plastics because of their superior thermomechanical properties, biodegradability, UV stability, and biocompatibility. The development of synthetic biology and metabolic engineering methods creates new avenues for the affordable synthesis of PHAs from bacteria utilizing lignin as the substrate (Xia et al., 2021). Brown et al. (2022) research involves the overproduction of poly(3-hydroxybutyrate-co-3-hydroxyvalerate) (PHBV) from the breakdown product of lignin, p-coumarate.

Biosensors

Due to the tremendous rise of portable point-of-care testing devices based on disposable electrodes, it is necessary the production of disposable electrodes in large quantities in an economical and environmentally friendly way.

Meng et al. (2022) provides a method for converting lignin biomass, patterning, reducing the lignin derived graphene electrodes, water lift-off and sequential laser lithography to obtain electrochemical lactate biosensors. These disposable electrodes made from lignin may help ensure the sustainable production of biosensors for point-of-care and point-of-use applications by combining low-energy manufacture and patterning with renewable resource utilization.

Agrochemicals

In the recent years, there is a growing trend in producing biodegradable agrochemicals that can replace toxic chemicals and fertilizers. Lignin degradation of lignocellulosic waste can be a solution for this task. With a growing interest in research and development on lignin-derived agrochemicals, several value-added products could be used for biofertilizers, plant growth regulators, insecticides and soil improvers (Sharma et al., 2023).

Nanocomposites

Two types of uniform lignin nanoparticles (LNPs) are prepared by self-assembling of deep eutectic solvent (DES) and ethanol-organosolv extracted technical lignins derived from a two-stage fractionation pretreatment approach, respectively. In contrast to the DES-LNPs, which displays a more homogeneous particle size distribution, both LPNs display sphere shape and a distinct core-shell nanostructure. These LPN products show great potential to form a transparent nanocomposite film with additional UV-shielding efficacy and antioxidant properties when incorporated into the traditional polymeric matrix such as poly(vinyl alcohol) (Tian et al., 2017).

Carbon fibres

Out of all the possible applications of lignin depolymerisation, obtaining carbon fibres has attracted a great interest during these last years. Lignin-based carbon fibres can lower the production cost of this fiber by more than 35% (Souto et al., 2018). Since final properties of lignin based carbon fibers are not satisfactory (mechanical properties), several attempts are made in this direction including optimization of processing parameters (Wang et al., 2022).

Flavoring compounds

Aromatics like guaiacol, syringol, catechol and vanillin could be obtained through enzymatic conversion of lignin. These aromatic compounds are in demand due to their usefulness as flavouring components.

CONCLUSIONS

Since lignin's chemical composition is complicated and varies with the source, the substrates are limited in the full structural resemblance with native lignin. Therefore, the next step would be to screen the selected microorganisms with natural substrates that have a high lignin content.

The methods presented in this paper are important for providing several ways to identify microbial strains with lignin degrading capabilities, while broadening the understanding of metabolic pathways of lignin depolymerisation. With these methods it is also possible to identify new biocatalysts, enzymes or even genes involved in lignin degradation, which can be useful for future biotechnological approaches for lignin valorisation.

REFERENCES

Abd El Monssef, R. A., Hassan, E. A., & Ramadan, E. M. (2016). Production of laccase enzyme for their potential application to decolorize fungal pigments on aging paper and parchment. *Annals of Agricultural Sciences*, *61*(1), 145-154.

Adhi, T. P., Korus, R. A., & Crawford, D. L. (1989). Production of major extracellular enzymes during lignocellulose degradation by two Streptomycetes in agitated submerged culture. *Applied and Environmental Microbiology*, 55(5), 1165-1168.

Ahmad, M., Roberts, J. N., Hardiman, E. M., Singh, R., Eltis, L. D., & Bugg, T. D. (2011). Identification of DypB from Rhodococcus jostii *RHA1 as a lignin peroxidase. Biochemistry*, 50(23), 5096-5107.

Ahmad, U. M., Ji, N., Li, H., Wu, Q., Song, C., Liu, Q., ... & Lu, X. (2021). Can lignin be transformed into agrochemicals? Recent advances in the agricultural applications of lignin. *Industrial Crops and Products*, *170*, 113646.

Alcalde, M., Bulter, T., Zumárraga, M., García-Arellano, H., Mencía, M., Plou, F. J., & Ballesteros, A. (2005). Screening mutant libraries of fungal laccases in the presence of organic solvents. *Journal of Biomolecular Screening*, *10*(6), 624-631.

Alvarez-Gonzalez, G., & Dixon, N. (2019). Genetically encoded biosensors for lignocellulose valorization. *Biotechnology for biofuels*, *12*, 1-14.

Antonopoulou, I., Iancu, L., Jütten, P., Piechot, A., Rova, U., & Christakopoulos, P. (2019). Screening of novel feruloyl esterases from Talaromyces wortmannii for the development of efficient and sustainable

syntheses of feruloyl derivatives. *Enzyme and microbial technology*, *120*, 124-135.

Bandounas, L., Wierckx, N. J., de Winde, J. H., & Ruijssenaars, H. J. (2011). Isolation and characterization of novel bacterial strains exhibiting ligninolytic potential. *Bmc Biotechnology*, 11(1), 1-11.

Brown, B., Immethun, C., Alsiyabi, A., Long, D., Wilkins, M., & Saha, R. (2022). Heterologous phasin expression in Rhodopseudomonas palustris CGA009 for bioplastic production from lignocellulosic biomass. *Metabolic Engineering Communications*, 14, e00191.

Burlacu, A., Israel-Roming, F., & Cornea, C. P. (2018). Depolymerization of kraft lignin with laccase and peroxidase: A review. *Sci. Bull. Ser. F Biotechnol*, *22*, 172-179.

Chauhan, P. S., Agrawal, R., Kumar, R., Gupta, R. P., & Ramakumar, S. S. V. (2022). Next generation applications of lignin derived commodity products, their life cycle, techno-economics and societal analysis. *International Journal of Biological Macromolecules*, *197*, 179-200.

Choi, S. L., Rha, E., Lee, S. J., Kim, H., Kwon, K., Jeong, Y. S., ... & Lee, S. G. (2014). Toward a generalized and high-throughput enzyme screening system based on artificial genetic circuits. *ACS synthetic biology*, 3(3), 163-171.

Collombel, I., Melkonian, C., Molenaar, D., Campos, F. M., & Hogg, T. (2019). New insights into cinnamoyl esterase activity of Oenococcus oeni. *Frontiers in Microbiology*, *10*, 2597.

Coulibaly, W. H., Mian, T. M. A. S., Camara, F., Beugré, G. A. M., Diguță, C. F., & Matei, F. (2023). Bioethanol production through *Saccharomyces* and Non-*Saccharomyces* yeast strains isolated from cocoa mucilage juice fermented. *Scientific Bulletin Series F. Biotechnologies*, *27*(1).

Domínguez-Robles, J., Cárcamo-Martínez, Á., Stewart, S. A., Donnelly, R. F., Larrañeta, E., & Borrega, M. (2020). Lignin for pharmaceutical and biomedical applications–Could this become a reality?. *Sustainable Chemistry and Pharmacy*, 18, 100320.

Eggeling, L., Bott, M., & Marienhagen, J. (2015). Novel screening methods—biosensors. *Current opinion in biotechnology*, 35, 30-36.

Fritzsch, F. S., Dusny, C., Frick, O., & Schmid, A. (2012). Single-cell analysis in biotechnology, systems biology, and biocatalysis. *Annual review of chemical and biomolecular engineering*, 3, 129-155.

Garedew, M., Lin, F., Song, B., DeWinter, T. M., Jackson, J. E., Saffron, C. M., ... & Anastas, P. T. (2020). Greener routes to biomass waste valorization: lignin transformation through electrocatalysis for renewable chemicals and fuels production. *ChemSusChem*, 13(17), 4214-4237.

Gonçalves, C. C., Bruce, T., Silva, C. D. O. G., Fillho, E. X. F., Noronha, E. F., Carlquist, M., & Parachin, N. S. (2020). Bioprospecting microbial diversity for lignin valorization: dry and wet screening methods. *Frontiers in Microbiology*, 11, 1081.

Ho, J. C., Pawar, S. V., Hallam, S. J., & Yadav, V. G. (2018). An improved whole-cell biosensor for the discovery of lignin-transforming enzymes in functional metagenomic screens. *ACS synthetic biology*, 7(2), 392-398.

Huang, X. F., Santhanam, N., Badri, D. V., Hunter, W. J., Manter, D. K., Decker, S. R., ... & Reardon, K. F. (2013). Isolation and characterization of lignin-degrading bacteria from rainforest soils. *Biotechnology and bioengineering*, *110*(6), 1616-1626.

Jędrzejczak, P., Collins, M. N., Jesionowski, T., & Klapiszewski, Ł. (2021). The role of lignin and lignin-based materials in sustainable construction–a comprehensive review. *International Journal of Biological Macromolecules*, *187*, 624-650. for sustainable development. *Chemosphere*, *268*, 129326.

Jeong, Y. S., Choi, S. L., Kyeong, H. H., Kim, J. H., Kim, E. J., Pan, J. G., ... & Kim, H. S. (2012). High-throughput screening system based on phenolics-responsive transcription activator for directed evolution of organophosphate-degrading enzymes. *Protein Engineering, Design & Selection*, 25(11), 725-731.

Kudo, S., Harada, A., Kubota, H., Sasaki, K., & Kaneta, T. (2017). Simultaneous determination of manganese peroxidase and lignin peroxidase by capillary electrophoresis enzyme assays. *ACS omega*, 2(10), 7329-7333.

Kuhar, F., & Papinutti, L. (2014). Optimization of laccase production by two strains of Ganoderma lucidum using phenolic and metallic inducers. *Revista argentina de microbiología*, *46*(2), 144-149.

Kumar, A., Singh, D., Sharma, K. K., Arora, S., Singh, A. K., Gill, S. S., & Singhal, B. (2017). Gel-based purification and biochemical study of laccase isozymes from Ganoderma sp. and its role in enhanced cotton callogenesis. *Frontiers in microbiology*, 8, 674.

Li, X., Guo, J., Hu, Y., Yang, Y., Jiang, J., Nan, F., ... & Xin, Z. (2019). Identification of a novel feruloyl esterase by functional screening of a soil metagenomic library. *Applied biochemistry and biotechnology*, *187*, 424-437.

Liu, Y., Hu, T., Wu, Z., Zeng, G., Huang, D., Shen, Y., ... & He, Y. (2014). Study on biodegradation process of lignin by FTIR and DSC. *Environmental Science and Pollution Research*, 21, 14004-14013.

Mannan, A. A., Liu, D., Zhang, F., & Oyarzún, D. A. (2017). Fundamental design principles for transcription-factor-based metabolite biosensors. *ACS synthetic biology*, 6(10), 1851-1859.

Melo-Nascimento, A. O. D. S., Treumann, C., Neves, C., Andrade, E., Andrade, A. C., Edwards, R., ... & Bruce, T. (2018). Functional characterization of ligninolytic Klebsiella spp. strains associated with soil and freshwater. *Archives of microbiology*, 200, 1267-1278.

Meng, L., Chirtes, S., Liu, X., Eriksson, M., & Mak, W. C. (2022). A green route for lignin-derived graphene electrodes: A disposable platform for electrochemical biosensors. Biosensors and *Bioelectronics*, 218, 114742.

Mercer, D. K., Iqbal, M., Miller, P., & McCarthy, A. J. (1996). Screening actinomycetes for extracellular peroxidase activity. *Applied and Environmental Microbiology*, 62(6), 2186-2190.

Mujtaba, M., Fraceto, L., Fazeli, M., Mukherjee, S., Savassa, S. M., de Medeiros, G. A., ... & Vilaplana, F. (2023). Lignocellulosic biomass from agricultural waste to the circular economy: A review with focus on biofuels, biocomposites and bioplastics. *Journal of Cleaner Production*, 136815.

Ohta, Y., Nishi, S., Haga, T., Tsubouchi, T., Hasegawa, R., Konishi, M., ... & Hatada, Y. (2012). Screening and phylogenetic analysis of deep-sea bacteria capable of metabolizing lignin-derived aromatic compounds. *Open Journal of Marine Science*, 2012.

Popa, A., Israel-Roming, F., Cornea, C. P., & Zugravu, M. M. (2020). A short review on acetyl xylan esterases. *Scientific Bulletin. Series F. Biotechnologies*, *24*(2), 173-178.

Popa, A., Israel-Roming, F., Cornea, C. P., & Zugravu, M. M. (2020). Biotechnological aspects of feruloyl esterase. *Scientific Bulletin. Series F. Biotechnologies*, *24*, 187-192.

Ramírez-Velasco, L., Armendáriz-Ruiz, M., Alberto Rodríguez-González, J., Müller-Santos, M., Asaff-Torres, A., & Carlos Mateos-Díaz, J. (2016). From classical to high throughput screening methods for feruloyl esterases: a review. *Combinatorial Chemistry & High Throughput Screening*, *19*(8), 616-626.

Ravi, K., García-Hidalgo, J., Gorwa-Grauslund, M. F., & Lidén, G. (2017). Conversion of lignin model compounds by Pseudomonas putida KT2440 and isolates from compost. *Applied Microbiology and Biotechnology*, 101, 5059-5070.

Rinaldi, R., Jastrzebski, R., Clough, M. T., Ralph, J., Kennema, M., Bruijnincx, P. C., & Weckhuysen, B. M. (2016). Paving the way for lignin valorisation: recent advances in bioengineering, biorefining and catalysis. *Angewandte Chemie International Edition*, 55(29), 8164-8215.

Rusu, I. C., Zăgrean, A. V., & Israel-Roming, F. (2022). Research on bioconversion of lignocellulosic waste for the cultivation of biocompounds producing macromycetes. *Scientific Bulletin Series F. Biotechnologies*, *26*(2).

Saini, R., Kaur, A., Saini, J. K., Patel, A. K., Varjani, S., Chen, C. W., ... & Dong, C. D. (2023). Trends in lignin biotransformations for bio-based products and energy applications. *BioEnergy Research*, 16(1), 88-104.

Sharma, V., Tsai, M. L., Nargotra, P., Chen, C. W., Sun, P. P., Singhania, R. R., ... & Dong, C. D. (2023). Journey of lignin from a roadblock to bridge for lignocellulose biorefineries: A comprehensive review. *Science of The Total Environment*, *861*, 160560.

Siedler, S., Khatri, N. K., Zsohár, A., Kjærbølling, I., Vogt, M., Hammar, P., ... & Joensson, H. N. (2017). Development of a bacterial biosensor for rapid screening of yeast p-coumaric acid production. *ACS synthetic biology*, 6(10), 1860-1869.

Sivagurunathan, P., Raj, T., Mohanta, C. S., Semwal, S., Satlewal, A., Gupta, R. P., ... & Kumar, R. (2021). 2G waste lignin to fuel and high value-added chemicals: Approaches, challenges and future outlook

Souto, F., Calado, V., & Pereira, N. (2018). Lignin-based carbon fiber: a current overview. *Materials Research Express*, 5(7), 072001.

Sun, X., Zhang, R., & Zhang, Y. (2004). Production of lignocellulolytic enzymes by Trametes gallica and detection of polysaccharide hydrolase and laccase activities in polyacrylamide gels. *Journal of Basic Microbiology: An International Journal on Biochemistry, Physiology, Genetics, Morphology, and Ecology of Microorganisms*, *44*(3), 220-231.

Taylor, C. R., Hardiman, E. M., Ahmad, M., Sainsbury, P. D., Norris, P. R., & Bugg, T. D. H. (2012). Isolation of bacterial strains able to metabolize lignin from screening of environmental samples. *Journal of Applied Microbiology*, 113(3), 521-530.

Temp, U., Eggert, C., & Eriksson, K. E. L. (1998). A small-scale method for screening of lignin-degrading microorganisms. *Applied and environmental microbiology*, 64(4), 1548-1549.

Tian, D., Hu, J., Bao, J., Chandra, R. P., Saddler, J. N., & Lu, C. (2017). Lignin valorization: Lignin nanoparticles as high-value bio-additive for multifunctional nanocomposites. *Biotechnology for biofuels*, 10(1), 1-11.

Uchiyama, T., & Miyazaki, K. (2013). Metagenomic screening for aromatic compound-responsive transcriptional regulators. *PLoS One*, 8(9), e75795.

Usmani, Z., Sharma, M., Awasthi, A. K., Lukk, T., Tuohy, M. G., Gong, L., ... & Gupta, V. K. (2021). Lignocellulosic biorefineries: The current state of challenges and strategies for efficient commercialization. *Renewable and sustainable energy reviews*, 148, 111258.

Wang, S., Bai, J., Innocent, M. T., Wang, Q., Xiang, H., Tang, J., & Zhu, M. (2022). Lignin-based carbon fibers: Formation, modification and potential applications. *Green Energy & Environment*, 7(4), 578-605.

Wang, Z., & Deuss, P. J. (2023). The isolation of lignin with native-like structure. *Biotechnology Advances*, *68*, 108230.

Xia, Q., Chen, C., Yao, Y., Li, J., He, S., Zhou, Y., ... & Hu, L. (2021). A strong, biodegradable and recyclable lignocellulosic bioplastic. *Nature Sustainability*, *4*(7), 627-635.

Zhang, Z., Lai, J., Wu, K., Huang, X., Guo, S., Zhang, L., & Liu, J. (2018). Peroxidase-catalyzed chemiluminescence system and its application in immunoassay. *Talanta*, 180, 260-270.

Zhou, H., Guo, W., Xu, B., Teng, Z., Tao, D., Lou, Y., & Gao, Y. (2017). Screening and identification of lignin-degrading bacteria in termite gut and the construction of LiP-expressing recombinant Lactococcus lactis. *Microbial pathogenesis*, 112, 63-69.

Scientific Bulletin. Series F. Biotechnologies, Vol. XXVII, No. 2, 2023
ISSN 2285-1364, CD-ROM ISSN 2285-5521, ISSN Online 2285-1372, ISSN-L 2285-1364

HOW GHI MAY BE SUCCESSFUL IN IMPROVING FOOD SECURITY AND SAFETY

Huub LELIEVELD

Global Harmonization Initiative (GHI), Florianigasse 63/14, Vienna, Austria

Corresponding author email: huub.lelieveld@globalharmonization.net

Abstract

GHI can be successful if GHI produces useful material that indeed reduce food safety incidents and improve food security. GHI does this without any influence of industries or governments: GHI is impartial, with 1400 scientists. No government is going to change regulations because of GHI. GHI must show that changes in regulations may be good. With about 20 working groups, GHI attempts to show how food safety and security can be improved by effectively addressing actual serious problems. Examples are: • A proposal for a legal requirement for food companies to employ a certified food safety professional (CP), without one the company cannot operate. The CP cannot be dismissed. • Cassava, kills 100,000`s because often prepared in the wrong way. GHI develops a training programme for people who cannot read. • GHI developed an anonymous whistleblowing webpage, in 40 languages. Even GHI cannot find out who reported a food safety incident. GHI alerts local food safety authorities of real and serious incidents.

Key words*: food security, food safety, cassava, whistleblowing, certified food safety professionals.*

INTRODUCTION

The The goal of the Global Harmonization Initiative (GHI) is Achieving consensus on the science of food regulations and legislations to ensure the global availability of safe and wholesome food products for all consumers.

Establishing GHI was triggered by global news, in 2004, that people in northern Africa were starving to death - in its literal meaning - while there was food in the harbours but governments refused the import because the authorities did not trust the safety of the food. Then, discussing among colleague food scientists, it was concluded that this was not unique to Africa or to 2004, but in many places in the world frequently food is not allowed to cross borders because of differences in food safety regulations, such as the maximum acceptable level of certain substances, which could be antibiotics, pesticides, preservatives, basically everything. These differences were also convenient for governments when they needed "legal" reasons to stop import of certain types of food for economic reasons and the measures, however, were masqueraded as caring for "food safety". There appeared to be many of such examples.

GHI would try to harmonise food safety regulations globally so that food can no longer be blocked at borders for false reasons. If food safety regulations would be the same everywhere, authorities would have to admit that they do not want the food from the delivering country for other reasons than food safety and they may be bad for their image if the people need the food.

GHI doesn't want to be perceived in any way as working in the interest of a particular (group of) countries or for a particular food company or industrial federation. Therefore, GHI is completely impartial, GHI doesn't accept funding from industries or governments. Stakeholders of food safety must be sure that opinions of GHI are based on science and nothing else. That is the reason that only food scientists and scientists in topics related to food science, such as microbiologists, toxicologists, food technologies and food engineers, can join GHI. Thus, membership of GHI is individual, members do never represent their employer, be it a company, a government or a governmental organisation. The Membership Director is responsible for carefully checking if an applicant complies with this requirement and whether the applicant indeed want to join to help GHI progressing with their goal. To avoid financial hurdles to membership, there is no

membership fee. Members, however, can make voluntary donations.

GHI can be successful if it is demonstrated that GHI produces useful material that indeed reduce food safety incidents and improve food security. At the same time, it must be evident that GHI does this without influence of industries or governments. GHI is impartial, consists of about 1400 scientists and everything GHI does is based on science. This is the reason that GHI can be trusted by governments and the public.

No government is going to change regulations because GHI wants it. Therefore, GHI needs to show that changes in regulations are needed, are possible and will be beneficial, for the people and for the government.

Hence, with about 20 working groups, GHI attempts to show how food safety and security can be improved by effectively addressing actual serious problems. A rapidly increasing number of scientists seem to agree with GHI's goal, as shown by the growing number of members, from 192 in 2012 to 447 in 2017, to 1103 in 2021 and more that 1400 early 2023. These members live in 113 countries.

Activities of a number of working groups are discussed below.

CERTIFIED FOOD SAFETY PROFESSIONALS

Worldwide each year unsafe food causes 600 million cases of foodborne diseases and 420,000 deaths of which 30% are children under 5 years of age (WHO). This happens despite the many efforts by food safety professionals in the industry and by food safety authorities.

To achieve higher standards in food safety practices, the WG Ethics in Food Safety Practices of GHI has developed a proposal that all countries make “Food Safety Professional” a regulated profession, similar to medical or legal professions, setting formal requirements for education, registration and the establishment of professional bodies.

The key-features of the proposal are:

1. Global recognition of Food Safety Professional (FSPro) as a legally regulated profession in production, retail, logistics, auditing and as a consultant.
2. Establishing educational and professional requirements and responsibilities for those in such positions in the food industry.
3. To promote ethical behaviour in food safety practices.
4. The legal requirement for food producers to have a certified FSPro in charge of the design and operation of their Food Safety system. The legal consequence will be that a company is not allowed to produce if there is no certified FSPro and hence, upon dismissing their FSPro will be closed down.

The benefit is that a company owner cannot fire the FSPro because of the decision made with respect to food safety. Currently it happens too often that companies continue with malpractices against professional objections of the responsible staff member and if the latter insists, dismissal is the consequence.

A FSPro must always act professionally, i.e., in accordance with the best available scientific standards of food safety, similar to the medical oath or the certification of a notary. It might require the establishment of a professional society. As for medical doctors and notaries, education requirements need to be established, ensuring that the FSPro has sufficient awareness of what food safety is and how food safety can be ensured.

ANONYMOUS WHISTLEBLOWING REPORTING

The Companies that disregard food safety have done enormous harm to people, well known serious cases are the addition of melamine to milk and and protein concentrates, affecting 294 000 infants, of which more than 50 000 were hospitalized and at least six died. Another case is continuation of marketing of peanut butter known to be contaminated with Salmonella, causing 9 death and more than 700 people ill (McCoy, 2015). There have been cases of addition of lead oxide to paprika powder, lead chromate to turmeric, diethylene glycol to wines, mineral oil to sunflower oil and so on. These are known cases where the criminals had been identified and punished, but that did not help the victims.

In all cases such as above, there have been persons who knew what was happening. When

they objected internally and their voices were ignored, very rarely these people dared to inform authorities, because of fear of retaliation or dismissal, while after all, they had a family to feed. There have been attempts to stimulate whistleblowing by promising that the reporting a of a case would be anonymous. In reality the anonymity appeared to be just pretence, because eventually the employer found out from the authority that warned them.

Until recently, in most countries the government protected the companies and not the employees. In the EU and North America this is gradually changing and hopefully it will be common sense in the future to protect the whistleblower and hence the consumer in stead of a dishonest company management.

The GHI working group Global Incident Alert Network developed a really anonymous whistleblowing opportunity. It is now possible to use a website in such a way that GHI is alarmed but GHI cannot know who the whistleblower (WB) is and so can literally nobody else. Nevertheless, by using the site, GHI can assess the information provided and if found to be serious by an international team of food safety experts and potentially very harmful, GHI will alert the local food safety authorities, asking to investigate the case. To be able to judge whether the reported case is truthful and serious, the webpage has a form that must be completed as accurate as possible, without revealing the identity of the WB.

Thanks to GHI's ambassadors, the webpage is available in about 40 languages. In all languages the first question is: "Would you let your family eat the food that you make at work?". The forms can be accessed by going to https://whistle.globalharmonization.net and there is also a frequently asked questions (FAQ) page that explains in detail how anonymity is ensured, also is all of those languages.

PREVENTING PARALYSIS IN CHILDREN FROM CONSUMPTION OF WRONGLY PREPARED CASSAVA

Cassava is one of the most drought-tolerant crops, capable of growing on marginal soils. It is the staple food of about a billion people, mostly in Africa and Asia. Although there are two types of cassava, a sweet and a bitter variety, the bitter variety is the mostly consumed one, because it is the one with by far has the highest yield. The bitterness, however, is caused by cyanides in the plant, which protect the plant against insects, but is also toxic to humans. Linamarin is a cyanogenic glucoside in cassava that, when ingested, releases HCN that is very toxic and may cause severe disease and even death. Therefore, before consumption, cassava must be processed to break down the linamarin and remove the cyanide. Linamarin can be removed by the enzymatic activity of linamarase, present in the leaves of the cassava plant. If not sufficiently processed, linamarin will remain in cassava and release cyanide in the body. It may kill people, in particular children and if ingested in small amounts for a long time, it causes the illness konzo: The child will wake up one morning, limping or only able to crawl, a condition that will remain unchanged throughout the child's life (Scutti, 2022).

There are various processes that are used to remove the Linamarin from cassava, but they take a long time. Often the link between eating cassava and the illness it is not clear to the people preparing the food, because the effect is not immediate, may take months to develop - suddenly.

A GHI project team is testing a relatively fast method to remove Linamarin, using the enzyme Linamarase, which is abundant in the leave of the plant. The idea is that using the leaves in preparing the food, it can be made safe in a couple of hours in stead of several days or weeks. Once confirmed, the working group Education and Training of Food Handlers, led by Obadina Adewale, will produce pictorial and verbal training material to train trainers, who will in turn train more trainers, who then again will train trainers, etc., all the way to the food handlers at home. It has to be done this way because many of those who prepare food in the villages in Africa do not read and would otherwise not learn about the cause of the disease and how to prepare cassava food in such a way that the illness is prevented.

For more information, see the working group website,
https://www.globalharmonization.net/wg-food-safety-training-and-education.

CONCLUSIONS

The Global Harmonization Initiative (GHI) will be successful in improving food security and safety by showing that it is possible to do so, by initiating and then completing necessary actions. Anybody interested in contributing to such actions may contact the chairs of the relevant working groups (see https://www.globalharmonization.net/working-groups).

REFERENCES

McCoy, K. (2015). Peanut exec in salmonella case gets 28 years. USA Today 2015/09/21. https://eu.usatoday.com/story/money/business/2015/09/21/peanut-executive-salmonella-sentencing/72549166/, accessed 23 July 2023.

Scutti, S. (2022). The children of Kahemba and the researchers hoping to save them. Global Health Matters Nov/Dec 2022. https://www.fic.nih.gov/News/GlobalHealthMatters/november-december-2022/Pages/konzo-and-the-children-of-kahemba.aspx, accessed 23 Juli 2023.

CHARACTERIZATION OF HEAVY METALS RESISTANT *Rhizobium* sp. ISOLATES FROM DIFFERENT REVEGETATION PLOTS ON MANDENA MINING SITE (FORT DAUPHIN-MADAGASCAR)

Irinah RATSIZAFY[1], Cătălina VOAIDEŞ[2], Oana-Alina BOIU-SICUIA[2], Voarisoa Sandrine RANDRIANANTENAINA[1, 3], Mendrika RAKOTONINDRINA[3], Martial Doret ANDRIANANDRASANA[1], Rondro Harinisainana BAOHANTA[1], Adamson Tsoushima Ernest RAZAKATIANA[1], Heriniaina RAMANANKIERANA[1]

[1]National Centre for Environmental Research, BP 1739 Rasamimanana Road, Fiadanana, Antananarivo, Madagascar
[2]University of Agronomic Sciences and Veterinary Medicine of Bucharest, Faculty of Biotechnologies, 59 Marasti Blvd, District 1, Bucharest, Romania
[3]University of Antananarivo, Faculty of Sciences, BP 566 101 Ankatso, Antananarivo, Madagascar

Corresponding author email: catalina.voaides@biotehnologii.usamv.ro

Abstract

This study was focused on nine rhizobial strains associated with Mimosa latispinosa, a fast-growing pioneer species native to the Mandena mining site and able to thrive on post-mining soils. The objectives were to: (i) evaluate the heavy metals tolerance of these isolates by comparing their growth on medium supplemented with different concentrations of heavy metals (Ni, Cd, Cu, and Pb); and (ii) screen the presence of the heavy metal resistance genes on the isolated DNA. While all the strains tested showed varying degrees of tolerance to the metals tested, significantly greater tolerance was observed for isolates RMN 4, RMN 5, RR 11 and RR 14. Regarding the presence of genes responsible for heavy metal resistance, only strains RMN 4, RMN 6 and RR 13 presented the specific amplicons in the case of pcoR gene amplification. The amplification of heavy metals resistance genes for other samples did not yield any specific PCR amplicons, probably due to the use of inappropriate primers or the presence of different mechanisms that allow them to survive under stress conditions.

Key words*: heavy metals, rhizobia, mining sites, restoration, Mimosa latispinosa.*

INTRODUCTION

Heavy metals released by mining and the use of chemicals in agricultural practices can have adverse effects on various ecosystems, including plants (Wani et al., 2008). In the specific case of post-mining sites, soil contamination by these metals, combined with poor soil structure, low water retention capacity, lack of organic matter, and associated nutrients such as nitrogen (N) and phosphorus (P), can hinder the reintroduction of plant species during revegetation and/or site restoration programs. In this regard, exploiting the symbiotic relationship between legumes and rhizobia is presented as an attractive and cost-effective alternative to improve nitrogen supply in the plant-soil system (Gómez-Sagasti & Daniel, 2015).

Rhizobia are Gram-negative soil bacteria that can form symbiotic associations with the *Fabaceae* family, during which they reduce atmospheric nitrogen to ammonia. The symbiosis between rhizobia and legumes has a fundamental contribution to the nitrogen cycle in natural and agricultural ecosystems and is a major reason why legumes are able to colonize marginal lands and nitrogen-deficient soils (De Hoff & Hirsch, 2003). However, several studies have shown that increasing concentrations of heavy metals such as Cu, Zn, and Pb, lead to a reduction in the bacterial count of *Rhizobium* sp. and a change in nodal gene expression (Stan et al., 2011; Chaudri et al., 2008), other authors have suggested that Rhizobium can tolerate high concentrations of heavy metals and play an important role in the remediation of contaminated soils (Carrasco et al. 2005; Teng et al., 2015; Mohamad et al., 2017).

Studies have proposed applying this symbiotic interaction to metal-contaminated soils to improve soil fertility and extract or stabilize

metals simultaneously (Ike et al., 2007; Dary et al., 2010). Indeed, rhizobia heavy metal tolerance mechanisms may include: (i) adsorption and accumulation of heavy metals; and (ii) microbial secretion of enzymes and bioactive metabolites to increase their bioavailability and sequester their toxicity. These mechanisms can directly enhance phytoremediation through nitrogen fixation and growth factor production, or adsorb and accumulate metals, indirectly contributing to phyto-stabilization (Hao et al., 2014).

So, in order to take full advantage of rhizobia-assisted legumes for metal phytoremediation, the first essential step would be to isolate and characterize metal-tolerant rhizobial strains. Many symbiotic rhizobial strains showing resistance to heavy metals have been found in legumes growing in polluted regions, such as mine deposits and serpentine soils (Vidal et al., 2009; Grison et al., 2015).

This study focused on rhizobial strains associated with *Mimosa latispinosa*, a fast-growing pioneer species native to the Mandena mining site (Madagscar) and able to thrive on post-mining soils. The objectives were to: (i) evaluate the heavy metals tolerance of these isolates by comparing their growth on medium supplemented with different concentrations of heavy metals (Ni, Cd, Cu, and Pb); and (ii) screen the presence of the heavy metal resistance genes on the isolated DNA.

MATERIALS AND METHODS

Isolation of Rhizobia

Nine (09) isolates of Rhizobia were collected from rhizospheres' soils of *Mimosa latispinosa* from revegetation plots of different ages (6 months, 1 year, and 3 years, respectively) at Mandena mining site (Fort-Dauphin, Madagascar) (Figure 1) according to the methods described by Vincent, 1970. Bacterial colonies were grown on YEMA (yeast extract mannitol agar) (HiMedia Laboratories Pvt. Mumbai-400086, India) supplemented with Congo red and incubated at 28°C for 3 days. The Congo red was used to check the colony colour that represents *Rhizobium* sp. One (01) strain from the 6-month plot, three (03) strains from the 1-year plot, and five (05) for the 3-year plot were used in further experiments (Table 1).

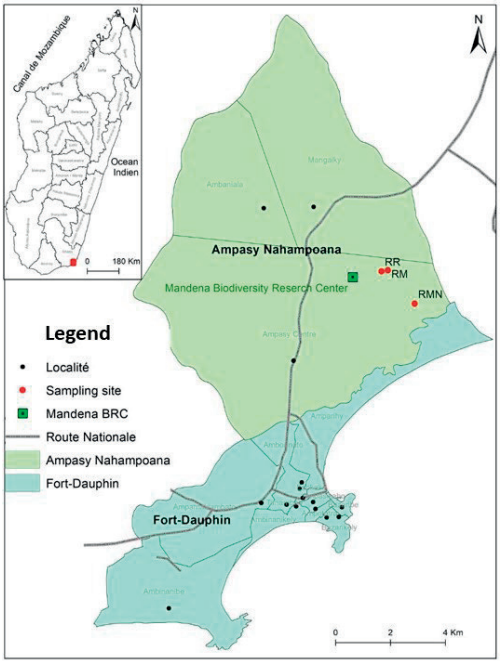


Figure 1. Origin of samples used in experiments.

Table 1. Origin of *Rhizobium* isolates

Isolates cods	Age of plot	Localisation
RM4	6 months	S 24°57'819'' E 047°00'443"
RR 11 **RR 13** **RR 14**	1 year	S 24°57'960" E 047°00'367"
RMN 4 **RMN 5** **RMN 6** **RMN 11** **RMN 12**	3 years	S 24°57'476" E 047°01'159"

Heavy metal resistance test by agar plate method

Heavy-metal-resistance of the isolates was tested by growing in YEMA media supplemented with one heavy metal at one concentration per plate. Three (03) concentrations of heavy metals (0.5 mM, 1 mM and 2 mM, respectively) were added to the medium using stock solutions of each heavy metal. The resistance against four different heavy metals (Ni, Cu, Cd, and Pb) was tested. The stock solutions of heavy metals were added to sterile agar as follows: Ni, Cd, Cu, and Pb were used respectively in the form of nickel chloride ($NiCl_2$), cadmium chloride ($CdCl_2$),

copper sulphate ($CuSO_4$) and lead acetate [Pb $(C_2H_3O_2)_2$]. The agar plates were inoculated with bacterial cells and incubated at 28°C for 3 days. Isolates were considered resistant if growth was observed or sensitive if otherwise.

Screening for enzymatic activity

Arginine- and ornithine-decarboxylase, activities were performed by inoculating the rhizobia strains on specific substrates. Thus, the enzymatic activity was performed on solid media containing 0.2% L-arginine hydrochloride, or 0.2% L-ornithine hydrochloride, respectively, using phenol red as an indicator dye (Sicuia et al, 2015). After one day of incubation, the result (enzymatic activity) was evaluated due to the colour change of the medium from yellow to pink.

In order to evaluate phytate solubilization, TS agar medium (5 g/L glucose, 10 g/L bacto-peptone, 5 g/L yeast extract, 1 g/L magnesium sulfate, 1 g/L calcium chloride, and 2 g/L phytic acid sodium salt, final pH 7.0) (Demirkan et al, 2014) was used. The bacterial strains that developed a clear halo around their colonies were phytase producers.

DNA extraction

The pellet of the centrifuged liquid culture of bacteria was mixed with 400 µl distilled sterilized water. The samples were incubated on thermo-block (Dry Block Heating Thermostat Bio TDB-100, Biosan Ltd, UE) at 98ºC for 10 min. and then incubated instantly on ice, for 5 min. The supernatant was used directly in PCR reactions.

PCR for heavy metal genes

For PCR reactions DreamTaq green PCR master mix (Thermo Scientific™, Vilnius, Lithuania) was used. The total volume of the PCR reaction was 25 µl, containing: 12.5 µl buffer, specific forward and reverse primer 10 µM (Table 2), DNA 10 ng, and water. PCR program (Eppendorf Mastercycler gradient, Hamburg, Germany) was as follows: 95ºC – 3 min, 35 cycles of - 94ºC – 1 min, annealing temperature – 1 min (Table 2), 72ºC – 2 min, and final extension 72ºC – 7 min. Amplicons were visualized in 1.2% agarose gel, in UV light using the BioDoc-It Imaging System (Ultra-Violet Products Ltd., Upland, CA). Specific primers were used as follows: pcoR, pbrA, nccA, czcD, chrB, chrA (Table 2).

Table 2. Heavy metal resistance genes used in PCR

Heavy metal	Resistance gene	Annealing temp.	Reference
Cu	*pcoR*	54ºC	Fan et al. (2011), Abdel-Lateif (2017)
Pb	*pbrA*	57 ºC	Wei et al. (2009), Abdel-Lateif (2017), Rahal et al (2023)
Cd/Ni	*nccA*	57 ºC	Renitta et al (2015), Amer et al (2015), Rahal et al (2023)
Zn	*czcD*	57 ºC	Wei et al (2009), Abdel-Lateif et al (2017)
Cr	*chrB*	52ºC	Ndeddy Aka &Babalola (2017)

RESULTS AND DISCUSSIONS

The nine (09) *Rhizobium* sp. isolates were tested for resistance to heavy metals using concentrations of 0.5, 1 and 2 mM, respectively, of Ni, Cd, Cu, and Pb (Table 3). At the lowest concentration (0.5 mM), all isolates were able to grow on media containing Ni, Cu and Pb, while for Cd, most isolates proved resistance except for RMN 12 and RM 4 isolates.

At 1 mM concentration, no growth was observed for isolates RMN 5 and RM 4 in the presence of Ni, RMN 12 and RM 4 in the presence of Cd and RMN 11 and RMN 12 in the presence of Cu. However, at the same concentration, all isolates tested showed resistance to Pb (Figure 2).

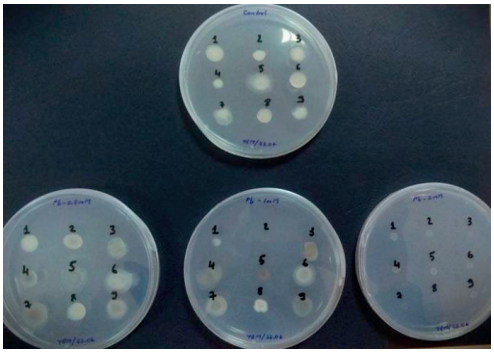


Figure 2. Growth of tested strains on YEMA medium with increasing concentrations of Pb: above: control; bottom, from left to right: 0.5 mM, 1 mM, 2 mM

At the highest concentration (2 mM), no isolate was able to grow in the presence of Pb. No growth was observed for isolates RMN 12 and RM 4 in the presence of Ni, Cd and Cu, RMN 4 in the presence of Ni and Cu, RMN 5 and

RMN 6 in the presence of Ni and RR 13 in the presence of Cd.

In general, the isolates' ability to resist the heavy metals decreased with increasing concentrations. However, strong tolerance was observed for almost all isolates from 3 years old plot: RMN 4, RMN5 and RMN 11 for Cd and Pb and RMN 6 for Cu. Isolates from one year plot also showed high tolerance for Ni and Cd (RR11 and RR 14) and Pb (RR 14) (Table 3).

Table 3. Effects of different concentrations of heavy metals on the growth of rhizobial isolates on YEMA plates

Strain	C	Ni (mM)			Cd (mM)			Cu (mM)			Pb (mM)		
		0.5	1	2	0.5	1	2	0.5	1	2	0.5	1	2
RM4	+++	++	-	-	-	-	-	+	+	-	+++	++	-
RR 11	+++	+++	++	+	+++	+++	++	++	++	-	+++	+	-
RR 13	+++	++	++	+	+	-	-	++	++	+	+++	+	-
RR 14	+++	+++	++	+	+++	+++	++	++	++	+	+++	++	-
RMN 4	+++	++	+	-	+++	+++	+++	++	++	+	+++	+++	-
RMN 5	+++	++	-	-	+++	+++	+++	++	++	+	+++	+++	-
RMN 6	+++	++	++	-	+++	++	++	++	++	+	+++	+	-
RMN 11	+++	++	+	+	+++	+++	+++	+	-	-	+++	+++	-
RMN 12	+++	+	+	-	-	-	-	++	-	-	+++	+++	-

(+++): very good growth; (++): moderate growth; (+): poor growth; (−): no growth.
C: Control; Ni: Nickel, Cd: Cadmium, Cu: Copper, Pb: Lead

The ability of *Mimosa latispinosa* to grow and develop in Mandena's post-mining soils is largely due to their association with rhizobia. These rhizobia are important ecological players, responsible for the entry of biologically fixed nitrogen into metal-contaminated areas and facilitating the activation of the restoration of the Mandena mining site (Mohamad et al., 2017).

The present study has shown that Rhizobium sp. isolates associated with Mimosa latispinosa grown on the post-mining soils of Mandena show varying degrees of tolerance to the tested heavy metals. This tolerance could be linked to the acquisition of metal resistance mechanisms, such as metal efflux transporters (Lakzian et al., 2002). However, it has also been shown that this tolerance tends to decrease with increasing metal concentration, in line with the results of previous studies which have shown that high concentrations of heavy metals can affect the growth, morphology, and nitrogen-fixing activities of microorganisms (Shi et al., 2002; Pereira et al., 2006).

The capacity of all nine (09) strains to decarboxylate arginine and ornithine was evaluated. The results showed that all strains have the capacity to decarboxylate the two amino acids tested, emphasizing the change of colour of the culture media, from orange to pink (Figure 3).

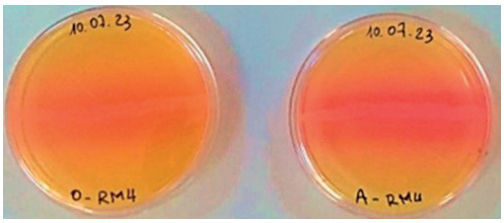


Figure 3. Enzymatic activity for arginine- and ornithine-decarboxylase for RM4 strain

The capacity of the strains to metabolise the phytate through organic phosphorus solubilization was qualitatively evaluated by the formation of halos around the colonies cultured on specific medium and incubated at 28°C for 10 days. Positive results (phytase producers) were obtained only for RMN 6, RMN 12 and RR 14 strains.

In these experiments, amplification based on heavy metal specific primers was used to screen and identify some heavy metal resistance genes (*nccA, pbrA, pcoR, chrB*, and *czcD*) in the tested samples. The *pbrA* gene encodes a P-type Pb(II) efflux ATPase in the lead resistance operon that is involved in the uptake, efflux, and accumulation of Pb(II) (Rahal et al, 2023). The czcD gene is a cation diffusion facilitator protein family transporter located in the cytoplasmic membrane and reduce ions accumulation (Cd^{2+}, Zn^{2+} and Co^{2+}) in the cytoplasm to the periplasm. The nccA gene provides resistance to nickel, cadmium, and cobalt using a similar mechanism (Rahal et al, 2023).

In our experiments, only three strains (RMN 4, RMN 6, and RR 13) yielded specific amplicons (650 pb) after *pcoR* gene amplification. These results are in accordance with the microbiological analysis. Also, in the case of *nccA* gene amplification, the primers used in PCR reaction yielded a band with approximately 300 bp. This result differs from those mentioned in the literature (Ndeddy Aka & Babalola, 2017; Rahal et al, 2023).

The amplification of heavy metals resistance genes for other samples did not yield any specific PCR amplicons, probably due to the use of inappropriate primers or the presence of different mechanisms that allow them to survive under stress conditions. That includes transporting heavy metals out of the cell, metal precipitation outside or inside the cell, or heavy metal binding through exopolysaccharides (Wei et al. 2009; Rahal et al, 2023). Due to the poor amplification and reproducibility of results of most tested isolates with the nccA, pcoR, pbrA, czcD and chrB primers, sequencing and nucleotide translation for these genes were not followed up further.

CONCLUSIONS

The isolation and selection of rhizobia strains resistant to stresses such as heavy metals are an important part of efficient nitrogen fixation and improved plant productivity, particularly in contaminated and severely depleted areas such as post-mining soils.

The absence of amplicons in bacterial isolates suggests that the tolerance/resistance to heavy metals could be due to other heavy metal resistance mechanisms.

The use of these strains in revegetation trials could not only increase the level of nitrogen fixation by *Mimosa latispinosa*, but also improve the efficiency of phytoremediation.

ACKNOWLEDGEMENTS

This research work was carried out with the support of the Romanian government and the Agence Universitaire de la Francophonie (AUF), which granted a mobility scholarship as part of the "Eugen Ionescu" program 2023, which enabled to carry out this work.

REFERENCES

Abdel-lateif, K.S. (2017). Isolation and characterization of heavy metals resistant *Rhizobium* isolates from different governorates in Egypt. *Afr. J. Biotechnol* 16(13), pp. 643-647.

Amer, R.A., Mapelli, F., El Gendi, H.M., Barbato, M., Goda, D.A., Corsini, A., Cavalca, L., Fusi, M., Borin, S., Daffonchio, D., Abdel-Fattah, Y.R. (2015). Bacterial Diversity and Bioremediation Potential of the Highly Contaminated Marine Sediments at El-Max District (Egypt, Mediterranean Sea). *BioMed Research International,* article ID 981829.

Carrasco, J.A., Armario, P., Pajuelo, E., Burgos, A. (2005). Isolation and characterisation of symbiotically effective *Rhizobium* resistant to arsenic and heavy metals after the toxic spill at the Aznalcollar pyrite mine. *Soil Biol. Biochem*. 37:1131-1140.

Chaudri, A., McGrath, S., Gibbs, P., Chambers, B., Carlton-Smith, C., Bacon, J., Campbell, C., Aitken, M. (2008). Population size of indigenous *Rhizobium leguminosarum* biovar *trifolii* in long-term field experiments with sewage sludge cake, metal amended liquid sludge or metal salts: Effects of zinc, copper, and cadmium. *Soil Biol. Biochem*. 40:1670-1680.

Dary, M., Chamber-Perez, M., Palomares, A., Pajuelo, E. (2010). "In situ" phytostabilisation of heavy metal ´ polluted soils using *Lupinus luteus* inoculated with metal resistant plant-growth promoting rhizobacteria. *J Hazard Mater* 177:323–330.

De Hoff, P. & Hirsch, A.M. (2003). Nitrogen comes down to earth: report from the 5th European Nitrogen Fixation Conference. *Mol Plant Microbe Interact* 16:371–375.

Demirkan, E., Baygin, E., Ustam, A. (2014). Screening of phytate hydrolysis *Bacillus sp.* isolated from soil and optimization of the certain nutritional and physical parameters on the production of phytase. *Turk. J. Biochemistry. Scimago Press.* 39:206–214.

Fan, L.M., Maa, Z.Q., Liang, J.Q., Li, H.F., Wangc, E.T., Wei, G.H. (2011). Characterization of a copper-resistant symbiotic bacterium isolated from *Medicago lupulina* growing in mine tailings. *Biores. Technol.* 102:703–709.

Gómez-Sagasti, M.T. & Daniel, M. (2015). PGPRs and nitrogen-fixing legumes: a perfect team for efficient Cd phytoremediation?. *Frontiers in Plant Science* 6: 81.

Grison, C.M., Jackson, S., Merlot, S., Dobson, A., Grison, C. (2015). *Rhizobium metallidurans sp. nov*., a symbiotic heavy metal resistant bacterium isolated from the *Anthyllis vulneraria* Zn-hyperaccumulator. *Int. J. Syst. Evol*. 65 :1525-1530.

Hao, X., Taghavi, S., Xie, P., Orbach, M.J., Alwathnani, H.A., Rensing, C., Wei, G. (2014). Phytoremediation of heavy and transition metals aided by legume-rhizobia symbiosis. *Int. J. Phytoremediat*. 16:179-202.

Ike, A., Sriprang, R., Ono, H., Murooka, Y., Yamashita, M. (2007). Bioremediation of cadmium contaminated soil using symbiosis between leguminous plant and recombinant rhizobia with the MTL4 and the PCS genes. *Chemosphere*, 66(9), 1670-1676.

Lakzian, A., Murphy, P., Turner, A., Beynon, J.L., Giller, K.E. (2002). *Rhizobium leguminosarum bv. viciae* populations in soils with increasing heavy metal contamination: abundance, plasmid profiles, diversity, and metal tolerance. *Soil Biol Biochem* 34:519 –529.

Mohamad, R., Maynaud, G., Le Quéré, A., Vidal, C., Klonowska, A., Yashiro, E., Cleyet-Marel, J.C., Brunel, B. (2017). Ancient heavy metal contamination in soils as a driver of tolerant *Anthyllis*

vulneraria rhizobial communities. *Appl Environ Microbiol* 83:e01735-16.

Ndeddy Aka, R.Jr. & Babalola, O.O. (2017). Identification and characterization of Cr-, Cd-, and Ni-tolerant bacteria isolated from mine tailings. *Bioremed. J.,* DOI: 10.1080/10889868.2017.1282933

Pereira, S.I., Lima, A.I., Figueira, E.M. (2006). Screening possible mechanisms mediating cadmium resistance in *Rhizobium leguminosarum bv. viciae* isolated from contaminated Portuguese soils. *Microb Ecol* 52:176–186.

Rahal, S., Menaa, B., Chekireb, D. (2023). Characterization of heavy metal-resistant rhizobia and non-rhizobia isolated from root nodules of *Trifolium sp.* in a lead and zinc mining area. *Res. Square*, preprint, https://doi.org/10.21203/rs.3.rs-2692408/v1

Renitta, J., Jha, P., Desai, N. (2015). *Sinorhizobium*, a potential organism for bioremediation of nickel. *Int J Adv Res* 3:706-717.

Shi, W., Bischoff, M., Turco, R., Konopka, A. (2002). Long-term effects of chromium and lead upon the activity of soil microbial communities. *Appl. Soil Ecol.* 21:169-177.

Sicuia, O.A., Grosu, I., Constantinescu, F., Voaides, C., Cornea, P.C. (2015). Enzymatic and genetic variability in *Bacillus spp*. strains with plant beneficial qualities. *AgroLife Sci. J*. IV:124–131.

Stan, V., Gament, E., Cornea, C.P., Voaides, C., Dusa, M., Plopeanu, G. (2011). Effects of heavy metal from polluted soils on the *Rhizobium* diversity. *Not. Bot. Horti. Agrobot*. Cluj. Napoca 39:88-95.

Teng, Y., Wang, X., Li, L., Li, Z., Luo, Y. (2015). Rhizobia and their bio-partners as novel drivers for functional remediation in contaminated soils. *Front. Plant Sci.* 6:32

Vidal, C., Chantreuil, C., Berge, O., Maure, L., Escarre, J., Bena, G., Brunel, B., Cleyet-Marel, J.C. (2009). *Mesorhizobium metallidurans sp. nov*., a metal-resistant symbiont of *Anthyllis vulneraria* growing on metallicolous soil in Languedoc, France. *Int J Syst Evol Microbiol* 59:850–855.

Wani, P.A., Khan, M.S., Zaidi, A. (2008). Effect of metal-tolerant plant growth-promoting *Rhizobium* on the performance of pea grown in metal-amended soil. *Arch Environ Contam Toxicol* 55:33–42

Wei, G., Fan, L., Zhu, W., Fu, Y., Yu, J., Tang, M. (2009). Isolation and characterization of the heavy metal resistant bacteria CCNWRS33-2 isolated from root nodule of *Lespedeza cuneata* in gold mine tailings in China. *J. of Hazard. Mat.* 162:50–56.

Scientific Bulletin. Series F. Biotechnologies, Vol. XXVII, No. 2, 2023
ISSN 2285-1364, CD-ROM ISSN 2285-5521, ISSN Online 2285-1372, ISSN-L 2285-1364

INFLUENCE OF MANUFACTURING RECIPE AND BAKING CONDITIONS ON ACRYLAMIDE CONTENT IN BREAD

Adriana Laura MIHAI, Mioara NEGOIȚĂ, Alina Cristina ADASCĂLULUI, Laurențiu Mihai PALADE, Nastasia BELC

National Research & Development Institute for Food Bioresources, IBA Bucharest, Chromatography Department, 6 Dinu Vintilă Street, District 2, 021102, Bucharest, Romania

Corresponding author email: mioaranegoita@yahoo.com

Abstract

The aim of the study was to assess and correlate the acrylamide content, determined by GC-MS/MS, and colour parameters of bread samples prepared using different wheat flours, dough preparation process and baking conditions (230, 240°C/40, 50 min). Our results showed that, regardless of the preparation process and baking conditions, the acrylamide content in bread varied under the effect of flour type. Refined wheat flour bread showed lower acrylamide levels (6.42-12.02 µg/kg) compared to whole-wheat flour bread (9.57-33.04 µg/kg). Acrylamide formation varied in response to dough preparation, ranging between 6.42-15.80 µg/kg for indirect process (IP), and 10.22-33.04 µg/kg for direct process (DP). With respect to the influence of baking conditions, a 10°C increase in baking temperature elevated the acrylamide content by up to 2.87-fold. Similarly, a 10 min extension of baking time resulted in higher acrylamide content (up to 1.30-fold). Moreover, strong negative correlations were observed between the acrylamide content and the L colour parameter of breads, with r= -0.7522 ÷ -0.8428 for DP, and -0.9796 ÷ -0.9724 for IP. Our findings revealed the lowest acrylamide content achieved in bread prepared using wheat flour, indirect process, and lower baking time and temperature.*

Key words*: acrylamide, bread, flour type, manufacturing recipe, sourdough.*

INTRODUCTION

Bread is known to be one of the most consumed food products, being a basic food resource all over the world. Flour, water, yeast and salt are the basic ingredients in the manufacturing recipe of this food product. For breadmaking the following steps are followed: dough mixing, dough fermentation and baking.

During baking, the Maillard reaction also known as non-enzymatic browning reaction takes place between asparagine and reducing sugars, such as glucose and fructose. This reaction contributes to obtaining the organoleptic characteristics of food products but it is also responsible for acrylamide formation. Acrylamide is a chemical contaminant, formed in starchy products under conditions employing temperatures above 120°C and low moisture (EFSA, 2015). This compound was classified by the International Agency for Research for Cancer (IARC, 1994) as a "probable human carcinogen".

Bread usually contains low levels of acrylamide (Andačić et al., 2020; Mihai et al., 2020). However, given its high consumption, bread is an important contributor to acrylamide dietary intake. As such, acrylamide mitigation in bread has received increased consideration in both industrial and domestic processing.

In this context, reports involving acrylamide content mitigation studies on bread samples included factors such as: flour selection (Capuano et al., 2009; Przygodzka et al., 2015), asparaginase addition (Capuano et al., 2009; Mohan Kumar et al., 2014; Ghorbani et al., 2019), amino acids addition (Capuano et al., 2009), using sourdough fermentation (Codină et al., 2021; Zhou et al., 2022), baking parameters modulation (Onacik-Gür et al., 2022), baking to a lighter colour endpoint of final product (Abdulazeez et al., 2023; Gunduz, 2023), antioxidants addition such as green tea extract (Onacik-Gür et al., 2022). The content can be reduced by recipe reformulation or by modifying the baking conditions.

Sourdough utilization to obtain bakery products is an old technique that can improve the physical, nutritional and sensorial characteristics of bread, also prolonging the

product shelf life (Codină et al., 2021; Susman et al., 2021; Kezer, 2022).

This technique started to be reused lately due to the high interest for traditional products. Sourdough is obtained my mixing cereal flour and water which are fermented through lactic acid bacteria and yeasts (Lutter, Jõudu and Andreson, 2023). Sourdough confers to bread a special flavour as a result of volatile and non-volatile compounds formed during fermentation (Warburton et al., 2022). Through fermentation, the acidity increases as organic acids are formed, while the pH decreases, this mechanism being related to the acrylamide reduction in bread (Zhou et al., 2022). Also, during this fermentation process, the protein content is reduced, asparagine being consumed by yeast fermentation (Wang et al., 2017; Zhou et al., 2022), while sucrose, is transformed to glucose and fructose due to the invertase enzyme present in yeast (Nachi et al., 2018).

The aim of this study was to investigate the influence of processing conditions such as wheat flour (refined and whole-wheat), dough preparation method (direct and indirect processes), and baking parameters (temperature, time) on the acrylamide content and colour parameters of bread samples. Moreover, we evaluated the corresponding relationships between the resulting acrylamide content and colour parameters.

MATERIALS AND METHODS

Chemicals and solvents

A standard of native acrylamide of min. 97.7% purity, purchased from Dr. Ehrenstorfer (LGC Group, North Charleston, USA), and an internal standard of acrylamide of ≥ 98% purity, with labelled carbon atoms (1,2,3-13C), containing 100 ppm hydroquinone, purchased from Cambridge Isotope Laboratories (Andover, MA, USA), were used for acrylamide determination.

All chemicals, solvents and reagents used in experiments were of chromatographic purity. Solvents used (n-hexane, ethyl acetate, and methanol) in experiments were picograde for residue analysis and were purchased from LGC Promochem GmbH (Wesel, Germany).

For the solid-phase extraction (SPE), two cartridges from Biotage (Upsala, Sweden) were used: Isolute Multimode (1.000 mg, 6 ml), and Isolute ENV+ (500 mg, 6 ml).

Ingredients

For bread formulation, the main ingredient comprised 2 kg of either refined wheat flour (type 650) or whole-wheat flour (type 2200). The following raw materials were also used: *Saccharomyces cerevisiae* yeast (0.05/0.06 kg), salt (0.03 kg), and water (1.2 l).

Bread preparation

The breads assessed in the present study were prepared using two types of flour (wheat flour and whole-wheat flour), and two fermentation processes, direct and indirect.

Bread prepared through DP (natural fermentation)

Through the natural fermentation, without sourdough, all raw materials were mixed and kneaded in one step.

The dough preparation included the following main operations: dosing of raw materials, kneading and fermentation. When preparing the dough, a larger amount of yeast (0.06 kg) was used compared to sourdough fermentation, and the duration of the dough preparation cycle was shorter. Flour was mixed for 10 min in a small capacity blender (Diosna, DM 08 - 4/6) with 300 ml of 10% sodium chloride solution (w/v), 300 ml of 15% fresh yeast emulsion (w/v) and 600 ml tap water. Subsequently, the resulting dough was subjected to fermentation (Gima Forni fermenter, Mod. Forno Rotor 60-80 Elett, 50 kW) for 90 min at 30°C. The dough was then divided into pieces of 600 g, which were properly shaped, placed in rectangular trays (20 x 100 mm) and left for final proofing (steam proofers) at 30°C and 85% relative humidity for 45 min. The loaves of bread were baked in a rotary kiln oven with controlled temperature and baking time (Mondial Forni, Italy), at either 230°C or 240°C, for 40 min or 50 min.

Bread prepared through IP (sourdough fermentation)

In the case of IP, the sourdough was prepared prior to obtaining the dough. The scope of using sourdough fermentation is to ensure a proper environment for yeast cells multiplication, which will in turn loosen the dough during fermentation, as well as to achieve the formation of fermentation compounds, especially lactic

acid; this will improve the properties of the dough and further contribute to the sensorial properties of the bread. Sourdough was prepared from flour, water and yeast. To obtain the sourdough, 50% of the total quantity of flour was used for the preparation of the dough. The ratio of the amount of flour to water was about 2:1. The amount of yeast used to obtain the sourdough was 100% of the total amount of yeast used (0.05 kg). The resulting sourdough was left to ferment for 2 h at 30°C. After fermentation, the remaining flour, water and salt were added to the sourdough and kneaded for about 10 min. The obtained dough underwent fermentation for 45 min at 30°C. Similar to the case of DP, the dough followed the same steps throughout preparation and baking (230°C/ 240°C; 40 min/ 50 min).

Sample preparation for acrylamide and colour determination

Subsequent to drying (3 h; 50°C), samples were finely ground (B-400, BÜCHI Labortechnik AG, Switzerland), transferred in centrifuge tubes, and stored at -21°C until analysis.

Acrylamide determination by GC-MS/MS

Preparation of stock, working and calibration solutions for acrylamide determination were realized as previously described by Negoiță et al. (2022).

For the acrylamide analysis, the method presented by Negoiță et al. (2022) with modification was used. Briefly, 1.5 g of homogenized sample was weighted into 50 ml centrifuge tubes, to which 440 µl internal standard solution (1 mg/l) and 30 ml water were added. Samples were vortexed for 30 min at ambient temperature and then centrifuged at 5°C, 10.000 x g for 15 min (5804R Eppendorf centrifuge with cooling, Eppendorf, Germany). The aqueous extract was collected (10 ml) and cleaned-up using the HyperSep Universal Vacuum Solid Phase Extraction Manifold (Thermo Fisher Scientific, USA). The Isolute Multimode SPE cartridge was conditioned with methanol (3 ml) and water (12 ml). The acrylamide extract was loaded onto the cartridge and the resulting eluate was collected. The Isolute ENV$^+$ cartridge was conditioned with methanol (5 ml) and water (5 ml), followed by the extract loading. The cartridge was rinsed with 4 ml water which was discarded with the eluate. A volume of 5 ml of 60% MeOH in water was loaded on the cartridge and the acrylamide extract was collected.

The resulting extract was derivatized with bromine compounds and was subsequently extracted and concentrated as described by Negoiță et al. (2022). The final extract was injected on GC-MS/MS (Thermo Fisher Scientific, USA).

The method was validated internally based on the following parameters: linearity, linearity range, limit of detection (LOD), limit of quantification (LOQ), selectivity, precision, accuracy, recovery and measurements uncertainty, as described by Negoiță & Culeţu (2016). Two calibration curves were plotted, with both correlation coefficients above 0.998, within the ranges of 0.05–0.5 mg/L, and 0.4–3 mg/L, respectively. The linearity domain was achieved within the range of 6.20- 116.34 µg/kg, accompanied by a correlation coefficient (R^2) higher than 0.998. The LOD and LOQ were set at 2.06 and 6.20 µg/kg, respectively. To verify the selectivity, the SRM detection mode and internal standard method were used. The method's accuracy was expressed as relative standard deviation (RSD) under repeatability (0.82-3.57%) and reproducibility (4.54-12.94%) conditions. The recovery of the method was ≤ 110%. The method uncertainty was set at ± 17.5% of concentration. To demonstrate the method's precision and accuracy, the laboratory participated in a proficiency test launched by Food Analysis Performance Assessment Scheme (FAPAS) (Sand Hutton, UK) on cereal-based products (biscuits- PT 30114) and obtained a z-score of -0.3. The validated parameters fulfilled the criteria set by European Commission (2017/2158).

Colour determination

For colour analysis a Konica Minolta colorimeter (Universal Software V4.01 Miniscan XE Plus) was used. The CIELAB´76 colour parameters were assessed on ground bread. Measurements were realized in 10 different areas of the ground bread samples, followed by the calculation of the colour parameters (L^*, a^* and b^*), expressed as mean ± standard deviation (SD).

Statistical analysis

The analyses were performed in duplicate, all data are expressed as mean ± standard deviation (SD). Results were submitted to Minitab statistical software version 20. One-way analysis of variance (ANOVA) was performed to examine the statistical differences between samples, followed by Tukey's test using a significance level of $p < 0.05$. Pearson correlation was used to assess the relationship between acrylamide content and colour parameters of the tested bread samples.

RESULTS AND DISCUSSIONS

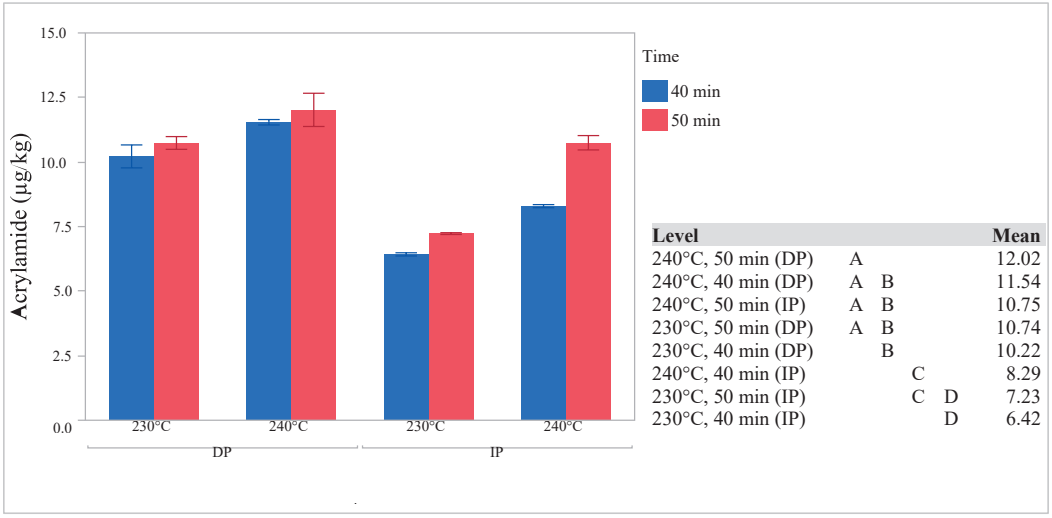
Influence of manufacturing recipe on the acrylamide content of bread

The main raw materials used in the bread manufacturing recipe were flour, water, salt and yeast. For this study, bread samples were prepared using either refined wheat flour and whole-wheat flour. The later in abundant in vitamins, minerals, and dietary fibers, and represents a rich source of antioxidants, carotenoids, flavonoids and phenolic acids (Ma et al., 2021; Sun et al., 2023). Recently it was shown that a higher intake of whole-wheat flour in consumed products helps reduce type 2 diabetes, cardiovascular, intestinal or chronic diseases (Tebben et al., 2018; Adams et al., 2020; Gomez et al., 2020; Hu et al., 2020; Reynolds et al., 2020). However, products made with this kind of flour are darker, have a harder texture, bitter taste compared with the products made using refined flour (Gómez et al., 2020).

When investigating the effect of manufacturing recipe on the acrylamide content of bread samples, our results showed that bread formulated with whole-wheat flour had a higher content than that of bread made with refined wheat flour (Figures 1 and 2). Similarly, in the study realized by Capuano et al. (2009) in a bread crisp model system, it was shown that whole-wheat flour produces more acrylamide than wheat flour.

The acrylamide content of bread samples made with wheat flour and DP ranged between 10.22-12.02 µg/kg (Figure 1), while using the whole-wheat flour and the same process, the content ranged between 11.01-33.04 µg/kg (Figure 2).

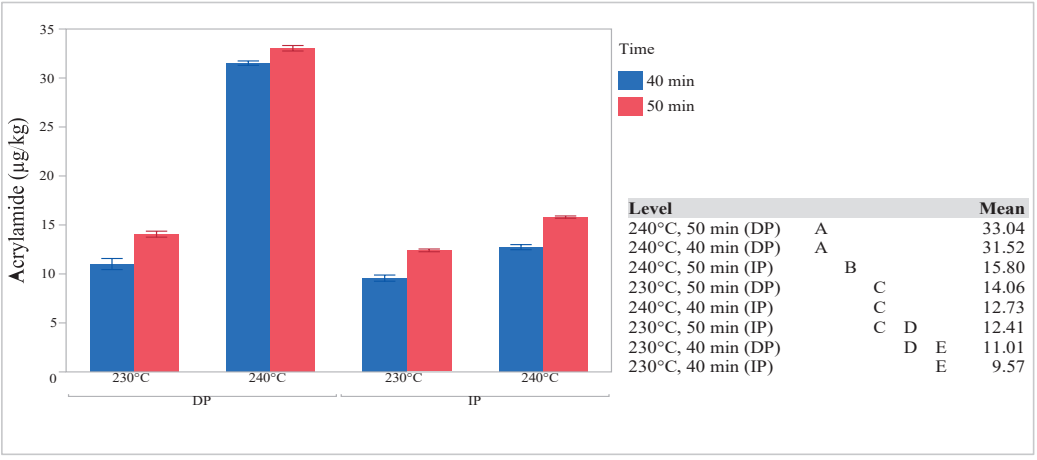
In the case of bread obtained using wheat flour through sourdough fermentation (IP), the acrylamide content varied between 6.42-10.75 µg/kg (Figure 1). By employing the same process for the whole-wheat flour, the acrylamide content registered higher levels, ranging between 9.57-15.80 µg/kg (figure 2).



Level					Mean
240°C, 50 min (DP)	A				12.02
240°C, 40 min (DP)	A	B			11.54
240°C, 50 min (IP)	A	B			10.75
230°C, 50 min (DP)	A	B			10.74
230°C, 40 min (DP)		B			10.22
240°C, 40 min (IP)			C		8.29
230°C, 50 min (IP)			C	D	7.23
230°C, 40 min (IP)				D	6.42

Figure 1. Changes of acrylamide content in wheat flour bread obtained by different dough preparation processes (DP and IP), under different baking conditions (230/240°C; 40/50 min). Means not connected by same letter are significantly different

By contrast to the case of natural sourdough fermentation, the acrylamide content in bread samples prepared through the IP decreased significantly irrespective of the flour type. Similar results were reported by Wang et al. (2017), Nachi et al. (2018) and Codină et al. (2021) who determined a lower acrylamide content in bread formulated with leaven/ sourdough compared with the one unleavened.
Zhou et al. (2022) evaluated the effect of some *Lactobacilli* and *Saccharomyces cerevisiae* strains on sourdough in comparison with the natural fermentation of sourdough, rendering viable the utilization of these microorganisms as they enabled a considerable decrease in acrylamide content by 24.38-58.83%. During fermentation, the pH of bread samples decreased, and the acidity increased, resulting a reduction of acrylamide content.
The acrylamide content of all bread samples evaluated in the present study are lower than the ones reported by Roszko et al. (2019) of 3.6-163 µg/kg for soft bread, by Dessev et al. (2020) of 4-77 µg/kg in bread, and by Basaran et al. (2022) of 60.7-130 µg/kg, respectively.
Most importantly, the samples evaluated herein did not exceed the benchmark level of 50 µg/kg regulated by the European Commission (2017/2158) when considering wheat-based bread.



Level						Mean
240°C, 50 min (DP)	A					33.04
240°C, 40 min (DP)	A					31.52
240°C, 50 min (IP)		B				15.80
230°C, 50 min (DP)			C			14.06
240°C, 40 min (IP)			C			12.73
230°C, 50 min (IP)			C	D		12.41
230°C, 40 min (DP)				D	E	11.01
230°C, 40 min (IP)					E	9.57

Figure 2. Changes of acrylamide content in whole-wheat flour bread obtained by different dough preparation processes (DP and IP), under different baking conditions (230/240°C; 40/50 min). Means not connected by same letter are significantly different

Influence of baking conditions on the acrylamide content of bread

In the present study, we employed 4 different baking conditions comprising combinations of 230°C and 240°C with 40 min and 50 min, in order to investigate the exerted effect on the acrylamide content achieved in the formulated bread.
In this context, our findings revealed that the baking parameters influenced the acrylamide content in bread. As such, the lowest acrylamide content was measured in bread baked at the lowest temperature and shortest baking time, respectively (230°C, 40 min).
In addition, the results showed that by increasing the baking temperature with 10°C (from 230 to 240°C) for both flour types irrespective of the baking time (40/50 min), an increase in acrylamide levels was generated (Figures 1 and 2). This increase ranged between 11.9-13.0% for bread made from wheat flour using DP of dough preparation, and much higher for bread obtained using IP of dough preparation ranging between 29.1-48.7%. For bread formulated with refined wheat flour, the acrylamide content was higher for bread baked at 240°C for 50 min (12.02 µg/kg), compared with that of bread baked at 230°C for 50 min (10.74 µg/kg). Similarly, in the case of whole-wheat flour, a 10°C increase in the baking temperature resulted in a considerably high increase in acrylamide formation when DP was used (135.0-188.3%),

whereas a much lower elevation was registered for IP, ranging between 27.4-33.0%.

The maximum level of acrylamide (33.04 µg/kg) was achieved for whole-wheat bread baked at 240°C for 50 min using the DP, 2.35-fold higher than the one of the breads baked at 230°C for the same time (14.06 µg/kg).

Similarly, the increase in baking time by 10 min (from 40 to 50 min) generated higher acrylamide formation. For bread made from wheat flour baked at 230/240°C using the DP of dough preparation, the increase of acrylamide content ranged between 4.2-5.2%, while for the IP of dough preparation it ranged between 12.6-29.7%. Comparably, for bread made from whole-wheat flour baked at 230/240°C using DP, the increase in acrylamide levels ranged between 4.8-27.7%, whereas the IP preparation registered an increase in acrylamide content in the range of 24.1-29.6%. Overall, our findings showed that by increasing the baking temperature, a higher increase of acrylamide level was caused compared to the increase in baking time.

The impact of the baking parameters was investigated in several studies. Surdyk et al. (2004) reported the proportion of crust in the dry breads increased with temperature and time of baking, from 30% (170°C/17 min) to 45% (270°C/32 min). By increasing the baking parameters, the content of acrylamide in the crust increased. When bread was baked at 290°C for 25 min, the acrylamide content of dry crust was very high (1800 µg/kg).

In the study conducted by Haase et al. (2003) it was shown that flour milling intensity as well as baking temperature have a high impact on acrylamide content in bread. Additionally, Claus et al. (2018) reported that acrylamide in wheat bread increased linearly with baking parameters. Breads baked at a lower temperature and a longer baking time (200°C/70 min) compared to those presented in our study, had an acrylamide content of 124.1 mg/kg. When bread was baked at similar conditions like the ones from our study (240°C/50 min), the acrylamide content was 92.4 mg/kg, much higher than the one obtained in our study.

Colour parameters of bread samples

Colour is an important parameter that can be correlated with the acrylamide content of food products. When food products have a lighter colour, a lower acrylamide content is found in product (Figure 3).

Food and Drug Administration (FDA, 2022) recommended to modify the baking parameters and to cook cereal-based products to a lighter colour endpoint in order to reduce acrylamide content in this category of products.

Figure 3. Bread samples obtained in experiments

The colour parameters (L^*, a^*, and b^*) of bread samples were calculated and results are presented in Table 1.

The more intense the baking treatment was, the more evident the Maillard reaction occurred. Hence, while the darkest colour was observed in bread baked at the highest temperature (240°C) and time (50 min), the lightest colour was noted for bread baked at the lowest settings (230°C; 40 min). It can be said that by increasing the baking temperature, bread lightness decreased. Also, by increasing the baking time, the bread samples became darker.

In comparison with the L^* parameter, the a^* parameter showed overall lower values for refined wheat bread than those of whole-wheat bread, whereas the b^* parameter reflected the opposite outcome.

No significant patterns were observed among the two colour parameters, with regards to the effect of baking conditions or fermentation process.

Table 1. Colour parameters of bread samples

Experimental variant			Colour parameters, mean ± sd		
			*L**	*a**	*b**
Wheat flour	DP	230°C, 40 min	76.44 ± 0.03^{a}	3.06 ± 0.03^{d}	17.43 ± 0.02^{d}
		230°C, 50 min	74.55 ± 0.05^{b}	3.95 ± 0.02^{a}	18.95 ± 0.04^{c}
		240°C, 40 min	74.41 ± 0.01^{c}	3.84 ± 0.01^{b}	19.37 ± 0.02^{a}
		240°C, 50 min	73.99 ± 0.19^{d}	3.69 ± 0.03^{c}	19.19 ± 0.04^{b}
	IP	230°C, 40 min	77.33 ± 0.04^{a}	3.14 ± 0.02^{c}	17.71 ± 0.02^{c}
		230°C, 50 min	77.21 ± 0.04^{b}	3.21 ± 0.01^{b}	18.25 ± 0.03^{b}
		240°C, 40 min	76.78 ± 0.05^{c}	2.96 ± 0.03^{d}	17.38 ± 0.10^{d}
		240°C, 50 min	75.41 ± 0.06^{d}	3.26 ± 0.03^{a}	18.53 ± 0.04^{a}
Whole-wheat flour	DP	230°C, 40 min	70.87 ± 0.01^{a}	4.28 ± 0.02^{b}	14.83 ± 0.03^{a}
		230°C, 50 min	68.27 ± 0.05^{b}	4.11 ± 0.02^{d}	12.58 ± 0.02^{d}
		240°C, 40 min	67.78 ± 0.04^{c}	4.21 ± 0.03^{c}	12.81 ± 0.08^{c}
		240°C, 50 min	66.16 ± 0.09^{d}	4.94 ± 0.04^{a}	14.33 ± 0.12^{b}
	IP	230°C, 40 min	65.89 ± 0.03^{a}	5.15 ± 0.01^{b}	14.99 ± 0.03^{a}
		230°C, 50 min	65.36 ± 0.04^{b}	5.16 ± 0.02^{b}	13.90 ± 0.05^{b}
		240°C, 40 min	65.12 ± 0.03^{c}	5.11 ± 0.03^{c}	13.46 ± 0.05^{c}
		240°C, 50 min	63.92 ± 0.06^{d}	5.27 ± 0.04^{a}	13.94 ± 0.09^{b}

*L** (lightness, 0= black to 100= white), *a** (redness, +*a**= redness, –*a**= greenness), and *b** (yellowness, +*b**= blueness, -*b**= yellowness). Values followed by different letters in the same column for each manufacturing recipe are significantly different ($p < 0.05$)

Variation of colour parameters based on the acrylamide content of bread samples

The changes in the *L** colour parameter in relation to the acrylamide content of bread samples is presented in Figure 4 and Figure 5.

By increasing the temperature and baking time, the acrylamide content increased, while the colour parameter *L** decreased irrespective of the flour type or baking process.

Correspondingly, the extent to which the acrylamide content and *L** colour parameter change concomitantly at the evaluated rate of temperature change, reveals high linear relationships between the two parameters.

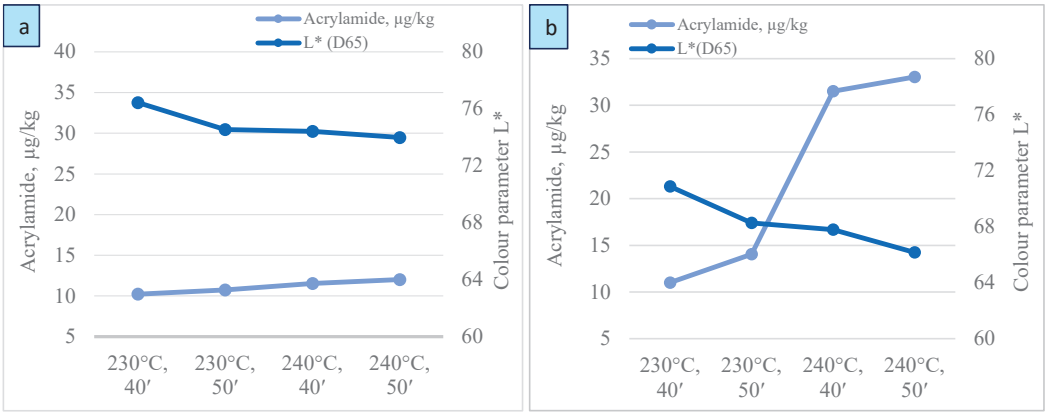


Figure 4. Changes of the colour parameter *L** based on the acrylamide content of bread made with wheat flour (a) and whole-wheat flour (b) through the direct process and baked under different conditions

Very strong negative correlations were observed for both DP ($r = -0.7522$; $p = 0.0313$) and IP ($r = -0.9796$; $p < 0.0001$) when using refined wheat flour. Similarly, in the case of whole-wheat flour, we found very strong negative correlations between the acrylamide content and the *L** colour parameter for both DP ($r = -0.8428$; $p = 0.0086$) and IP ($r = -0.9724$; $p < 0.0001$). By contrast, no significant correlations were determined between the acrylamide content and the *a** and *b** colour parameters of the evaluated bread samples.

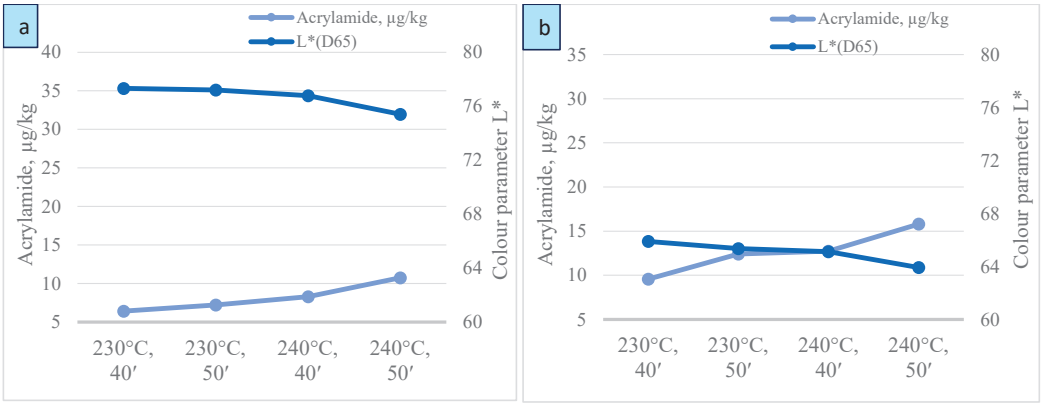


Figure 5. Changes of the colour parameter *L** based on the acrylamide content of bread made with wheat flour (a) and whole-wheat flour (b) through the indirect process and baked under different conditions

Regardless of the baking process, darker colours and higher acrylamide contents were determined for bread formulated with whole-wheat flours. Also, in the study realized by Abdulazeez et al. (2023) it was shown that the acrylamide content in cereal-based products is correlated with the colour intensity of the product, a lighter colour meaning a lower acrylamide content.

CONCLUSIONS

The acrylamide content of bread sample was influenced by the wheat flour used in the recipe, the fermentation type and the baking conditions. Even though whole-wheat flour is a rich source of bioactive compounds having beneficial effects for human health, when bread was formulated with this flour, the acrylamide content was higher.

When using the sourdough fermentation, the acrylamide content decreased compared with the DP.

The baking parameters exerted a significant influence on the acrylamide content measured in all bread samples. By increasing the temperature with 10°C, while maintaining the same baking time of 40/50 min, the acrylamide content was 1.13-2.87-fold/1.12-2.35-fold higher. By extending the baking time by 10 min and maintaining the same baking temperature of 230°C/240°C, the acrylamide content was 1.05-1.30-fold/1.05-1.30-fold higher. The highest acrylamide content was determined for the bread baked at the highest temperature (240°C), and the longest time (50 min) using the direct process of fermentation. The acrylamide content of bread formulated in this study didn't exceed the benchmark level set by EC 2017/2158.

The acrylamide content of bread was strongly correlated with the colour parameter *L**, lighter products having a lower acrylamide content.

In this context, it is important to prevent dark-colour products.

ACKNOWLEDGEMENTS

This paper was supported by a grant offered by the Romanian Minister of Research, Innovation and Digitalization, as Intermediate Body for the Competitiveness Operational Program 2014-2020, call POC/78/1/2/, project number SMIS2014 + 136213, acronym METROFOOD-RO, and the following projects: Metrofood-PP (Horizon 2020, Grant agreement no. 871083) and contract no. 17 PFE/2021.

REFERENCES

Abdulazeez, Z.M., Mustafa, A.M.I., Yazici, F. (2023). Correlation between acrylamide content and colour in some baked products. *Czech Journal of Food Sciences, 41*(2), 137-143.

Adams, J., Hofman, K., Moubarac, J. C., & Thow, A.M. (2020). Public health response to ultra-processed food and drinks. *BMJ*, *369*, m2391.

Andačić, I.M., Tot, A., Ivešić M., Krivohlavek, A., Thirumdas, R., Barba, F.J., Sabolović, M.B., Kljusurić J.G., & Brnčić, S.R. (2020). Exposure of the Croatian adult population to acrylamide through bread and bakery products. *Food Chemistry, 322*. 126771.

Basarn, B., Anlar, P., Oral, Z.F.Y., Polat, Z., Kaban, G. (2022). Risk assessment of acrylamide and 5-hydroxymethyl-2-furfural (5-HMF) exposure from bread consumption: Turkey. *Journal of Food Composition and Analysis, 107*. 104409.

Capuano, E., Ferrigno, A., Acampa, I., Serpen, A., Özge, A.Ç., Gökmen, V., & Fogliano V. (2009). Effect of flour type on Maillard reaction and acrylamide formation during toasting of bread crisp model systems and mitigation strategies. *Food Research International, 42*. 1295–1302.

Claus, A., Mongili, M.,Weisz, G., Schieber, A., & Carle, R. (2008). Impact of formulation and technological factors on the acrylamide content of wheat bread and bread rolls. *Journal of Cereal Science, 47*. 546-554.

Codină, G.G., Sarion, C., & Dabija A. (2021). Effects of Dry Sourdough on Bread-Making Quality and Acrylamide Content. *Agronomy, 11*. 1977.

Dessev, T., Lalanne, V., Keramat, J., Jury, V., Prost, C., & Le-Bail, A. (2020). Influence of baking conditions on bread characteristics and acrylamide concentration. *Journal of Food Science and Nutrition Research, 3*(4). 291-310.

European Commission (EC). (2017). Commission regulation (EU) 2017/2158 of 20 November 2017 establishing mitigation measures and benchmark levels for the reduction of the presence of acrylamide in food. *Official Journal of the European Union, 304*. 22-44.

Food and Drug Administration (FDA) (2022). Reducing Acrylamide in cereal-based foods: Fact sheet. *Retrieved from https://www.fda.gov/media/149435/download*

Ghorbani, N.S., Tehrani, M.M., Khodaparast, M.H.H., & Farhoosh, R. (2019). Effect of temperature, time, and asparaginase on acrylamide formation and physicochemical properties of bread. *Acta Alimentaria, 48*(2). 160-168.

Gómez, M, Gutkoski, LC, Bravo-Núñez, Á. (2020). Understanding whole-wheat flour and its effect in breads: A review. *Comprehensive Reviews in Food Science and Food Safety, 19*. 3241– 3265.

Gunduz, C.P.B. (2023). Formulation and processing strategies to reduce acrylamide in thermally processed cereal-based foods. *International Journal of Environmental Research and Public Health*, *20*. 6272

Haase, N.U., Matthaus, B., & Vosmann K. (2003). Acrylamide formation in foodstuffs - minimizing strategies for potato crisps. *Deutsche Lebensmittel-Rundschau, 99*. 87-90.

Hu, Y., Ding, M., Sampson, L., Willett, W. C., Manson, J. E., Wang M., & Sun Q. (2020). Intake of whole grain foods and risk of type 2 diabetes: Results from three prospective cohort studies. *BMJ, 370*, m2206.

IARC. (1994). IARC monographs on the evaluation for carcinogenic risk of chemicals to humans: Some industrial chemicals. Chapter 60, pp. 389–433, Lyon, France.

Kezer, G. (2022). Functional Perspective on sourdough bread. *Turkish Journal of Agriculture – Food Science and Technology, 10*(8). 1410-1414.

Lutter, L., Jõudu, I., & Andreson H. (2023). Volatile organic compounds and their generation in sourdough. *Agronomy Research, 21.*

Ma, S., Wang, Z., Guo, X., Wang, F., Huang, J., Sun, B., & Wang, X. (2021). Sourdough improves the quality of whole-wheat flour products: Mechanisms and challenges—A review. *Food Chemistry, 360*. 130038.

Mihai, A.L., Negoiță, M., Horneț, G.A. (2020). Assessment of the acrylamide level of cereal-based products from Romania market in accordance with Commission Regulation (EU) 2017/2158. *The Annals of the University Dunarea de Jos of Galati Fascicle VI – Food Technology, 44*(1). 104-117.

Mohan, Kumar, N.S., Shimray, C.A., Indrani, D., & Manonmani, H.K. (2014). Reduction of Acrylamide Formation in Sweet Bread with L-Asparaginase Treatment. *Food and Bioprocess Technology, 7*. 741–748.

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

Negoită, M., Mihai, A.L., & Hornet, G.A. (2022). Influence of Water, NaCl and Citric Acid Soaking Pre-Treatments on Acrylamide Content in French Fries Prepared in Domestic Conditions. *Foods, 11*. 1204.

Negoiță, M., & Culeţu, A. (2016). Application of an accurate and validated method for identification and quantification of acrylamide in bread, biscuits and other bakery products using GC-MS/MS System. *Journal of the Brazilian Chemical Society, 27*(10). 1782–1791.

Onacik-Gür, S., Szafrańska, A., Roszko, M., & Stępniewska, S. (2022). Interaction of dough preparation method, green tea extract and baking temperature on the quality of rye bread and acrylamide content. *LWT - Food Science and Technology, 154*. 112759.

Przygodska, M., Piskula, M.K., Kukurová, K., Ciesarová, Z., Bednarikova, A., & Zieliński H. (2015). Factors influencing acrylamide formation in rye, wheat and spelt breads. *Journal of Cereal Science, 65*. 96-102.

Reynolds, A. N., Akerman, A. P., & Mann J. (2020). Dietary fibre and whole grains in diabetes management: Systematic review and meta-analyses. *PLoS Medicine, 17*(3). Article e1003053.

Roszko, M. L., Szczepańska, M., Szymczyk, K., & Rzepkowska, M. (2019). Dietary risk evaluation of acrylamide intake with bread in Poland, determined by two comparable cleanup procedures. *Food Additives & Contaminants: Part B, 13*(1). 1-9.

Sun, X., Wu, S., Koksel, F., Xie, M., & Fang, Y. (2023). Effects of ingredient and processing conditions on the rheological properties of whole wheat flour dough during breadmaking - A review. *Food Hydrocolloids, 135*. 108123.

Surdyk, N., Rosén, J., Andersson, R., & Aman P. (2004). Effects of asparagines, fructose, and baking conditions on acrylamide content in yeast-leavened wheat bread. *Journal of Agricultural and Food Chemistry, 52*. 2047-2051.

Susman, I.-E., Schimbator, M., Stamatie, G., Culeţu, A., Dobre, A., Mulţescu, M., & Popa, M.E. (2021). Sourdough fermentation in gluten-free bread: A shelf-life improvement. *Scientific Bulletin. Series F. Biotechnologies, XXV*(2). 61-69.

Tebben, L., Shen, Y., & Li Y. (2018). Improvers and functional ingredients in whole wheat bread: A review

of their effects on dough properties and bread quality. *Trends in Food Science & Technology, 81*. 10–24.

Wang, S., Yu, J., Xin, Q., Wang, S., & Copeland, L. (2017). Effects of starch damage and yeast fermentation on acrylamide formation in bread. *Food Control, 73*. 230-236.

Warburton, A., Silcock, P., & Eyres, G.T. (2022). Impact of sourdough culture on the volatile compounds in wholemeal sourdough bread. *Food Research International, 161*. 111885.

Zhou, X., Duan, M., Gao, S., Wang, T., Wang, Y., Wang, X., & Zhou, Y. (2022). A strategy for reducing acrylamide content in wheat bread by combining acidification rate and prerequisite substance content of Lactobacillus and Saccharomyces cerevisiae. *Current Research in Food Science, 5*. 1054–1060.

Scientific Bulletin. Series F. Biotechnologies, Vol. XXVII, No. 2, 2023
ISSN 2285-1364, CD-ROM ISSN 2285-5521, ISSN Online 2285-1372, ISSN-L 2285-1364

CHARACTERIZING WATER KEFIR BEVERAGES WITH ANTIOXIDANT EFFECTS: PRELIMINARY ANALYSIS

Emanuel VAMANU[1], Dodji Brice DANGNON[2]

[1]University of Agronomic Sciences and Veterinary Medicine of Bucharest,
59 Marasti Blvd, District 1, Bucharest, Romania
[2]Laboratory of Biology and Molecular Typing in Microbiology,
University of Abomey-Calavi, Benin

Corresponding author email: emanuel.vamanu@gmail.com

Abstract

Water kefir is a beverage that undergoes natural fermentation by inoculating water kefir grains in a mixture of filtered water, cane sugar, and fruits. The beverage has a mild level of carbonation, making it appropriate for individuals adhering to a vegan diet. Moreover, it is a viable substitute for individuals experiencing nutritional imbalances stemming from dysbiosis of their microbiota. This study aimed to examine the characteristics of water kefir produced from different fruit juices and evaluate the bioactive properties of the resultant beverages. The methods used were the antioxidant activity in vitro, the determination of major bioactive compounds, BRIX values, and a microbiological quantification of lactic acid bacteria, yeasts and actinobacteria. Using fruit juice to obtain water kefir was a beneficial and advantageous addition to functional drinks. The presence of certain compounds (phenolic compounds, e.g.) determined the potent antioxidant effects, proved by the in vitro method. The microbiological pattern of the beverages was correlated with the fermentative profile, BRIX levels, and functional characteristics.

Key words*: juice; antioxidant; fermentation; vegan; brix; bacteria.*

INTRODUCTION

Water kefir beverage (WKb) is a fermented drink gaining popularity due to its numerous health benefits and refreshing taste. Obtaining water kefir involves fermenting water kefir grains in a sugar-water solution. Water kefir grains are composed of a symbiotic consortium of bacteria and yeast that engage in the metabolic process of sugar fermentation. Water kefir grains are minute, translucent, gelatinous structures composed of a symbiotic consortium of bacteria and yeast. Water kefir is produced by fermenting sugar water with grains, producing a softly carbonated beverage with a mildly acidic and sweet taste. Water kefir grains possess unique attributes in contrast to milk kefir grains since they undergo fermentation in a water medium rather than milk, resulting in a beverage characterized by a tangy and creamy taste profile (https://blog.soin-et-nature.com/) The bacteria and yeast in water kefir grains work together to break down and metabolize sugars, creating a nutrient-rich beverage packed with live probiotics and beneficial enzymes (https://www.kombuchakamp.com).

Water kefir grains can generally be propagated and reused for many batches, making them a sustainable and cost-effective way to produce a healthy probiotic drink (Mazhar, 2021).

The fermentation process produces lactic and acetic acid and a range of vitamins, minerals, and probiotics that benefit human health. Water kefir is a fermented drink gaining popularity amongst health-conscious individuals due to its numerous potential health benefits. This refreshing beverage is packed with live probiotics and beneficial enzymes that help to improve gut health and boost the immune system. The probiotics in water kefir, created through fermentation, support the growth of healthy bacteria in the gut and help maintain good digestive health. This can lead to various benefits, including increased nutrient absorption and improved bowel function. Water kefir is also a great source of vitamins and minerals, including vitamin B12, K, calcium, and magnesium (https://www.wildfoods.co). These essential nutrients help to support overall health and well-being, including maintaining strong bones and teeth, promoting healthy blood clotting, and improving nerve and muscle function.

Moreover, water kefir exhibits anti-inflammatory and antioxidant characteristics, potentially mitigating inflammation and oxidative stress inside the human body. Consequently, this may protect against chronic ailments, including cancer, cardiovascular disease, and Alzheimer's disease (FoodPorty, 2023). Regularly drinking water kefir has been suggested to enhance gut health, bolster the immune system, support optimal bone and dental health, and protect against the development of chronic diseases. Nevertheless, further investigation is required to substantiate these prospective advantages (https://www.elperroflaco.com). This article aims to analyze the procedure involved in acquiring beverages using the water kefir fermentation technique, along with the evaluation of the resultant product.

MATERIALS AND METHODS

Water kefir beverages obtaining

WKb was prepared in 50 g of cane sugar (bio product) and 500 mL of mineral water (Laureys, 2017). The mixture was inoculated with 25 g water kefir grains (Fairment GmbH, Berlin). The water kefir grains were subjected to activation in a solution containing 5% cane sugar, which lasted for 48 hours. The beverage was acquired inside a container made of borosilicate glass, and the fermentation process was conducted in two stages at a temperature of 220°C, as seen in Figure 1.

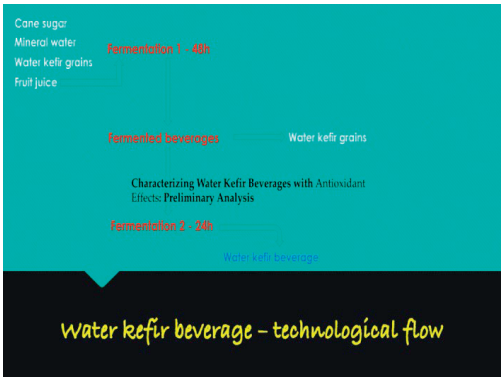


Figure 1. Water kefir obtaining - technological flow

Following the first fermentation stage, filtering isolated the water kefir grains from the liquid. Subsequently, they were immersed in water containing 5% cane sugar for at least 24 hours before their utilization in a subsequent fermentation process (Laureys, 2017).

Fermented drinks were obtained from the following mixtures:

1. WKR - Water and freshly squeezed and filtered pomegranate juice (ratio 4:1);
2. WKT - Cold peach tea (1 bag/250 mL water), 5% cane sugar;
3. WKC - 800 mL water and 200 mL fresh multi-fruit juice, 3% cane sugar;
4. WKPG - 600 mL water and 400 mL pomegranate nectar.

The fermentation process was conducted in a state of calmness, devoid of any agitation, and monitored regularly with the collection of samples every 12 hours (Cevik et al., 2019). Control 1 means the water kefir fermented at the end of the first fermentation phase with apple concentrate; Control 2 means water kefir fermented at the end of the second fermentation phase with apple concentrate; Control 3 means with apple concentrate water kefir fermented at the end of the second fermentation phase with lemon juice concentrates (Laureys & De Vuyst, 2014).

Microbiological analysis of the beverages

The viability of lactic acid bacteria was determined using MRS. The seeded plates were placed under anaerobic conditions at 300C. The yeasts were determined using YPG, and the plates were incubated at 300C. The presence of bacteria of the genus Acetobacter was highlighted by using the following culture medium (g/500 mL, Glucose - 10 g, Peptone - 2.5 g, Yeast extract - 2.5 g, Na2HPO4 - 1.35 g, Citric acid - 0.75 g, agar - 1g, pH 7.5). The plates were also incubated at 300C (https://2018.igem.org). Microbiological examination was conducted after the completion of each stage inside the technical procedure (Gulhan, 2023).

Physico-chemical analyses

The pH value was measured with a portable pH electrode, ATC.

The Brix value of the solution was measured using a portable refractometer (Okamura et al., 2022) and was correlated with the soluble sugar

contents in fermented beverages (https://www.vinolab.hr).

To quantify the overall phenolic content, the Folin-Ciocalteu test was used. The spectrophotometer was used to measure the absorbance of the combination at a wavelength of 760 nm. The obtained data were then quantified as micrograms of gallic acid equivalents per milligram of the sample (Periya et al., 2021). (µg GAE/mg) (Molole et al., 2022).

The DPPH scavenging activity assay measures the antioxidant capacity and reflects the specific antioxidant compounds present in the sample. The absorbance of the mixture was read at 517 nm using a spectrophotometer. The percentage of DPPH scavenging activity was determined by comparing the absorbance value of the sample to a control containing only DPPH solution and ethanol (Baliyan et al., 2022).

RESULTS AND DISCUSSIONS

The pH level is a crucial factor in the fermentation process of water kefir. The pH of a solution is a quantitative measure of its acidity or alkalinity, which significantly impacts the development and metabolic processes of microorganisms participating in fermentation (Laureys et al., 2017). The pH values directly depend on the formula used in the fermentation process. A typical behavior was presented by the WKR sample, determining a decrease after 24 hours of fermentation ($p \leq 0.001$). WKC showed an atypical behavior, with a sudden decrease, and then the value showed higher values by 0.4 units (Figure 2).

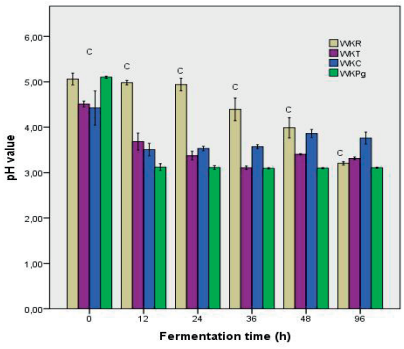


Figure 2. pH values of water kefir beverages conforming to technological flow

The rest of the samples had relatively constant evolutions that were assimilated to the fermentative capacity of the response of the inoculum to the carbon source.

A negative correlation was observed between the pH and Brix levels in the process of water kefir fermentation. During fermentation, the pH undergoes a decline as a result of the synthesis of organic acids, while the Brix value experiences a reduction owing to the use of sugars by microbes (Gulhan, 2023) (Figure 3).

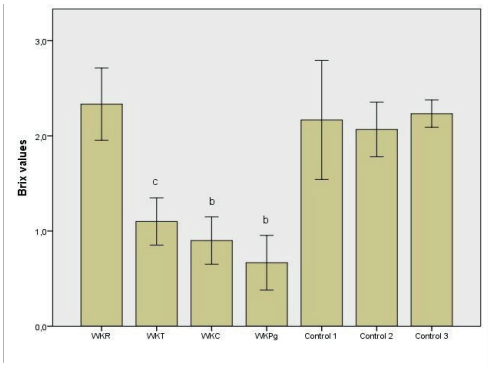


Figure 3. Brix values of water kefir beverages conforming to technological flow (Control 3 vs. samples; n = 3)

This means that as the water kefir becomes more acidic (lower pH), the beverage's sweetness decreases (lower Brix value). The correlation between pH and Brix value might exhibit variability contingent upon the distinct parameters of fermentation, the composition of microorganisms involved, and the length of the fermentation process (Safak et al., 2023). It was very clearly represented in Figure 4 in the case of Control 1 and Control 2 variations. The classic fermentation mixture (Control 3) was stable and influenced only by the initial quantity of cane sugar. The type of inoculum has no additional influence because it was the same in all samples.

The level of phenolic compounds in water kefir depends on several factors, including the ingredients used, fermentation conditions, and the specific strains of bacteria and yeast in the inoculum. The phenolic compounds in water kefir primarily come from the ingredients used during fermentation (Şafak et al., 2023). These compounds can be released into the beverage during fermentation due to enzymatic activity,

microbial metabolism, and other biochemical reactions (Figures 3 and 5).

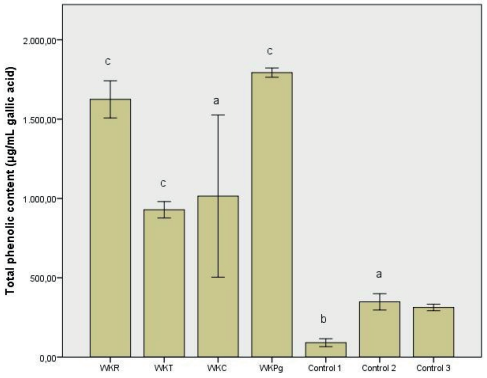


Figure 4. Total phenolic content of water kefir beverages conforming to technological flow
(Control 3 vs. samples; n = 3)

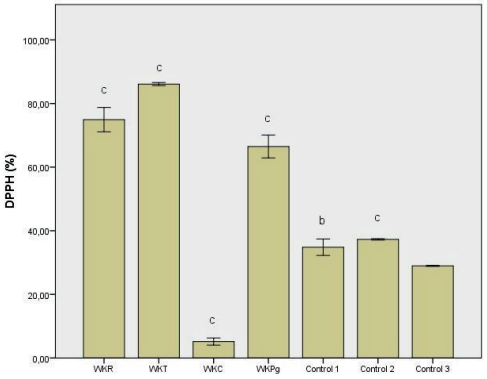


Figure 5. DPPH scavenging activity (%) of water kefir beverages conforming to technological flow
(Control 3 vs. samples; n = 3)

The key microbiological components in water kefir include water kefir grains, yeast, and bacteria. The yeast in water kefir grains plays a crucial role in fermentation. Yeasts consume sugars in the sweetened water (Figure 4) and produce alcohol and carbon dioxide (Laureys et al., 2014). As the fermentation process progresses, the alcohol produced by yeast is further metabolized by bacteria into various compounds, including lactic acid. The lactic acid bacteria (LAB) present in water kefir grains were primarily responsible for lactic acid production and values of the pH (Figure 2).
Figures 6-8 show a pattern specific to each formula compared to the control (Control 3). Controls 1 and 2 demonstrated a balanced ratio of the number of strains analyzed. WKT was shown to support a high value of acetic bacteria, demonstrating better options than this one. Pomegranate juice (Figure 6) determined a value similar to the control in the number of strains of Lactobacillus and WKC strains.

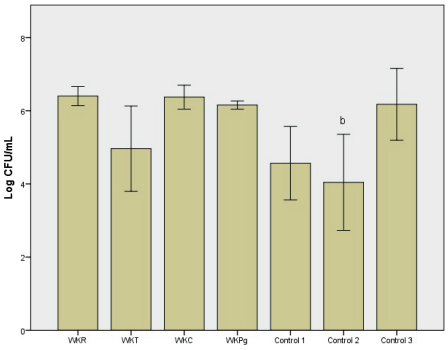


Figure 6. Log CFU/mL for lactic acid bacteria from water kefir beverages conforming to technological flow
(Control 3 vs. samples; n = 3)

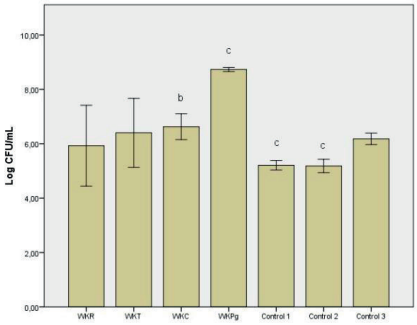


Figure 7. Log CFU/mL for yeast from water kefir beverages conforming to technological flow
(Control 3 vs. samples; n = 3)

Water kefir is a fermented drink gaining popularity due to its refreshing taste and potential health benefits. Our study variation of water kefir involves using fruit juice as the sugar solution. This method adds flavor and nutrients to the drink, making it a tasty and healthy alternative to sugary sodas or juices (Randazzo et al., 2016). During fermentation, the water kefir grains consume the sugar in the juice, producing a tangy, fizzy drink loaded with beneficial probiotics. This aspect was especially available for pomegranate nectar, especially. The longer the fermentation time,

the stronger the flavor could diminish, and the carbonation degrees the sweet taste (Sabokbar & Khodaiyan, 2016).

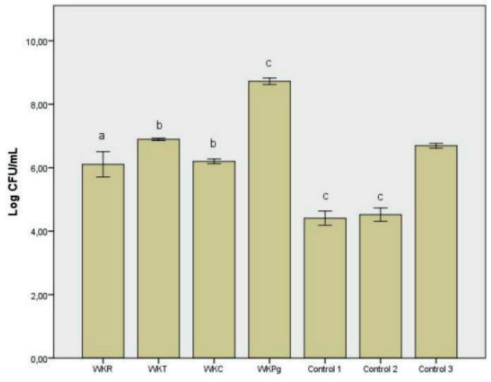


Figure 8. Log CFU/mL for *Acetobacter* from water kefir beverages conforming to technological flow (Control 3 vs. samples; n = 3)

During fermentation, the pH of the water kefir mixture gradually decreases due to the accumulation of organic acids. This decrease in pH is essential for creating an acidic environment that favors the growth of beneficial bacteria and yeasts while inhibiting the growth of potentially harmful microorganisms (Kim et al., 2016). The optimal pH range obtained for WKR and WKPG for water kefir fermentation typically exceeds 3.0.

The kefir grains and the microorganisms they contain exhibit optimal growth and demonstrate efficient fermentation processes within this specified range (Moretti et al., 2022). The acidic nature of water kefir, characterized by a low pH, acts as a growth inhibitor for spoilage bacteria and pathogens. This property plays a crucial role in preserving the quality and ensuring the safety of water kefir. (Pihurov et al., 2023).

Examining the correlation between the antioxidant content and the microbial composition of water kefir is a complex aspect of this study. The microbial makeup of water kefir is distinguished by a diverse array of microorganisms, including lactic acid bacteria (LAB), acetic acid bacteria, yeasts, and a variety of other microbial species (Peluzio et al., 2021). The composition and abundance of microorganisms in kefir might vary according to variables such as the specific kefir grains used and the fermenting conditions Laureys & De Vuyst, 2014).

The microbial processes occurring during fermentation can impact the synthesis and concentration of antioxidants (Vieira et al., 2021).

As an illustration, lactic acid bacteria (LAB) can generate specific antioxidant molecules through fermentation, augmenting the resultant product's overall antioxidant potential (Feng and Wang, 2020). The fluctuation in antioxidant concentrations across different batches is a significant obstacle in considering water kefir as a reliable and constant provider of antioxidants (da Silva Vale et al., 2023).

Although water kefir it should not be seen as a substitute for a properly balanced diet. Moreover, it should be noted that there might be variations in individual reactions to antioxidants (Moretti et al., 2022).

Furthermore, consuming excessive water, kefir, or other fermented drinks could have unfavorable consequences for some individuals, such as experiencing gastrointestinal pain due to probiotics (Peluzio et al., 2021).

Acknowledging that the possible health advantages associated with antioxidants in water kefir may exhibit a different prominence than those found in fruits and vegetables, which serve as abundant reservoirs of these molecules (Constantin et al., 2023).

CONCLUSIONS

Incorporating fruit juice in producing water kefir is a pleasant and beneficial inclusion to one's regular regimen. The preparation based on different fruit juices was accessible, while its consumption presents many benefits. In addition to providing a delightful and palatable experience, this product offers a substantial concentration of probiotics, for WKPg. Moreover, it should be noted that there might be variations in rest of the samples. The inclusion of water kefir in a complete nutritional strategy warrants careful consideration, as it has been reported to possess antioxidant benefits in the context of WKR, WKT, and WKPg. The final samples exhibit the strongest association between the benefits of probiotics and their antioxidant characteristics.

REFERENCES

Baliyan, S., Mukherjee, R., Priyadarshini, A., Vibhuti, A., Gupta, A., Pandey, R.P., & Chang, C.M. (2022). Determination of Antioxidants by DPPH Radical Scavenging Activity and Quantitative Phytochemical Analysis of *Ficus religiosa*. *Molecules*, *27*, 1326.

Çevik, T., Aydoğdu, N.S., Özdemir, N., & Taş, T.K. (2019). The Effect of Different Sugars on Water Kefir Grains. Turk. J. *Agr. - Food Sci. Technol., 7*, 40-45.

Constantin, E.A., Popa-Tudor, I., Matei, F., Constantinescu-Aruxandei, D., & Oancea, F. (2023). Evaluation of Polyphenol Content and Antioxidant Activity of Standard Water Kefir. *Chem. Proc.*, *13*, 7.

da Silva Vale, A., Venturim, B.C., da Silva Rocha, A.R.F., Martin, J.G.P., Maske, B. L., Balla, G., De Dea Lindner, J., Soccol, C. R., & de Melo Pereira, G.V. (2023). Exploring Microbial Diversity of Non-Dairy Fermented Beverages with a Focus on Functional Probiotic Microorganisms. *Fermentation*, *9*, 496.

Esatbeyoglu, T., Fischer, A., Legler, A.D.S., Oner, M.E., Wolken, H.F., Köpsel, M., Ozogul, Y., Özyurt, G., De Biase, D., & Ozogul, F. (2023). Physical, chemical, and sensory properties of water kefir produced from *Aronia melanocarpa* juice and pomace. *Food Chem X*., *18*, 100683.

Feng, T., & Wang, J. Oxidative stress tolerance and antioxidant capacity of lactic acid bacteria as probiotic: a systematic review. *Gut Microbes*. **2020**, *12*, 1801944.

FoodPorty (2023, June 16). Antioxidant Supplements: Unlocking the Secrets to Optimal Health and Wellness. https://foodporty.com/blog/post?ID=1333&Antioxidant-Supplements&lang=en

Gulhan, A. (2023). Physicochemical, microbiological and sensory analyses of functional detox juices fermented with water kefir grains. *GIDA Journal of food, 48*, 715-727

Kim, D. H., Jeong, D., Kim, H., Kang, I. B., Chon, J. W., Song, K. Y., & Seo, K. H. (2016). Antimicrobial Activity of Kefir against Various Food Pathogens and Spoilage Bacteria. *Korean J Food Sci Anim Resour*., *36*, 787-790.

Laureys D, & De Vuyst L. (2014). Microbial species diversity, community dynamics, and metabolite kinetics of water kefir fermentation. *Appl Environ Microbiol., 80*, 2564-72.

Laureys, D., & De Vuyst, L. (2014). Microbial species diversity, community dynamics, and metabolite kinetics of water kefir fermentation. *Appl. Environ. Microbiol*., *80*, 2564-72.

Laureys, D., De Vuyst, L., & Vandamme, P. (2017). Microbial species diversity, community dynamics, substrate consumption, and metabolite produciton during water kefir fermentation. 9789078810179

Mazhar, A. (2021, November 22). Wild Strawberry Aroma In Foods Comes From Fungus Growing On Fruit Waste. https://cropforlife.com/wild-strawberry-aroma-in-foods-comes-from-fungus-growing-on-fruit-waste/

Molole, G.J., Gure, A., & Abdissa, N. (2022). Determination of total phenolic content and antioxidant activity of *Commiphora mollis* (Oliv.) Engl. resin. *BMC Chemistry*, *16*, 48.

Moretti, A.F., Moure, M.C., Quiñoy, F., Esposito, F., Simonelli, N., Medrano, M., & León-Peláez, A. (2022). Water kefir, a fermented beverage containing probiotic microorganisms: From ancient and artisanal manufacture to industrialized and regulated commercialization. *Future Foods*, *5*, 100123.

Okamura, M., Hosoi, J., Nagata, K., Koba, K., Sugiura, D., Arai-Sanoh, Y., Kobayashi, N., & Kondo, M. (2022). Cross-locational experiments to reveal yield potential and yield-determining factors of the rice cultivar 'Hokuriku 193' and climatic factors to achieve high brown rice yield over 1.2kg m^{-2} at Nagano in central inland of Japan. *Plant Prod. Sci., 25*, 131-147.

Pariya, D., Valérie, O., & José _Luis, M. (2021). Process Optimization for Development of a Novel Water Kefir Drink with High Antioxidant Activity and Potential Probiotic Properties from Russian Olive Fruit (Elaeagnus angustifolia). *Food and Bioprocess Technology, 14*, 248–260.

Peluzio, M.D.C.G., Dias, M.M.E., Martinez, J.A., & Milagro, F.I. (2021). Kefir and Intestinal Microbiota Modulation: Implications in Human Health. *Front Nutr*. 2021, 8, 638740.

Pihurov, M., Păcularu-Burada, B., Cotârleț, M., Grigore-Gurgu, L., Borda, D., Stănciuc, N., Kluz, M., & Bahrim, G.E. (2023). Kombucha and Water Kefir Grains Microbiomes' Symbiotic Contribution to Postbiotics Enhancement. *Foods*, *12*, 2581.

Randazzo, W., Corona, O., Guarcello, R., Francesca, N., Germanà, M.A., Erten, H., Moschetti, G., & Settanni, L. (2016). Development of new non-dairy beverages from Mediterranean fruit juices fermented with water kefir microorganisms, *Food Microbiol*., *54*, 40-51.

Sabokbar, N., & Khodaiyan, F. (2016). Total phenolic content and antioxidant activities of pomegranate juice and whey based novel beverage fermented by kefir grains. *J Food Sci Technol*., *53*, 739-47.

Şafak, H., Gün, İ., Tudor Kalit, M., & Kalit, S. (2023). Physico-Chemical, Microbiological and Sensory Properties of Water Kefir Drinks Produced from Demineralized Whey and Dimrit and Shiraz Grape Varieties. *Foods*, *12*, 1851.

Vieira, C.P., Rosario, A.I.L.S., Lelis, C.A., Rekowsky, B.S.S., Carvalho, A.P.A., Rosário, D.K.A., Elias, T.A., Costa, M.P., Foguel, D., & Conte-Junior, C.A. (2021). Bioactive Compounds from Kefir and Their Potential Benefits on Health: A Systematic Review and Meta-Analysis. *Oxid Med Cell Longev*., *2021*, 9081738.

https://www.kombuchakamp.com/water-kefir-faq-frequently-asked-questions, access in May 12th, 2023.

https://www.wildfoods.co/blogs/wild-blog/recipe-coconut-water-kefir, access in May 12th, 2023.
https://www.elperroflaco.com/the-potential-therapeutic-benefits-of-delta-8-thc/, access in May 12th, 2023.
https://2018.igem.org/Team:SCAU-China/Experiments, access in May 12th, 2023.
https://www.vinolab.hr/calculator/gravity-density-sugar-conversions-en19, access from May 13th, 2023.

Scientific Bulletin. Series F. Biotechnologies, Vol. XXVII, No. 2, 2023
ISSN 2285-1364, CD-ROM ISSN 2285-5521, ISSN Online 2285-1372, ISSN-L 2285-1364

NEW DATA ON INSECTICIDAL ACTIVITY OF SOME NATIVE BACTERIAL AND FUNGAL STRAINS

Anna MOLDOVAN, Natalia MUNTEANU MOLOTIEVSKIY, Ion TODERAS

Moldova State University, Institute of Zoology, 1 Academiei Street, MD 2028, Chisinau, Republic of Moldova

Corresponding author email: anna.moldovan@yahoo.com

Abstract

A wide range of organisms, including insects, which can cause considerable losses, attacks tomato crop. Chemical insecticides continue to be used as the primary pest protection measure. The research aims to identify native strains of Bacillus thuringiensis and Beauveria bassiana to be used in the sustainable management of pests of tomato crops in the Republic of Moldova.

Based on conducted activities regarding isolating and characterizing bacterial strains, seven newly isolated strains were used in bioassay tests. Three other bacterial and ten fungal strains from the collection of the Institute of Zoology were tested against selected pests. Four experimental treatments were set up, two against adults and two against larvae of Leptinotarsa decemlineata. The bioassay tests against adults of Colorado potato beetle revealed two fungal and one bacterial strain with promising results for biological control. Low insecticidal activity was recorded against the larvae of potato beetle with the tested strains.

Key words*: Leptinotarsa decemlineata, Bacillus thuringiensis, Beauveria bassiana, native strains, insecticidal activity.*

INTRODUCTION

In the last centuries, *Leptinotarsa decemlineata* Say (Coleoptera: Chrysomelidae), known as the Colorado potato beetle, became a leading global pest, widely distributed through the Northern Hemisphere (Cingel et al., 2016). Feeding initially with a few wild hosts, the Colorado potato beetle is now causing severe attacks on a range of plants from the Solanaceae family. Although its' preferred host is potato (*Solanum tuberosum* L.), the tomato (*Solanum lycopersicum* L.) and eggplant (*Solanum melongena* L.) are also essential hosts (Weber, 2003; Vargas-Ortiz et al., 2018). Defoliation by this voracious pest can cause 40-80% of yield losses (Hare, 1980; Alyokhin et al., 2013; Cingel et al., 2016).

Despite all the efforts to implement different agricultural practices to control the Colorado potato beetle, chemical insecticides continue to be used as the primary pest protection measure. However, using chemical insecticides to control the pests is not the best plant protection strategy, especially when dealing with *L. decemlineata*. First of all, due to its diverse life cycle, phenotypic plasticity, high adaptability, and capability to detoxify or tolerate toxins, the Colorado potato beetle has successfully overcome chemical pesticides (Cingel et al., 2016). Moreover, using pesticides creates many problems related to human safety. Also, it leads to the emergence of secondary pest outbreaks, environmental pollution, biodiversity reduction, and pesticide-resistant insects (Damalas & Eleftherohorinos, 2011).

The need to identify an alternative to chemical insecticides stimulated the interest in developing inoffensive methods for humans and the environment, bringing traditional and biological pest control methods back to researchers' attention. Biological control represents a complex of techniques employing some organisms to reduce other harmful organisms below the economic threshold based on natural mechanisms such as predation, parasitism, competition, etc. These involve biological control agents such as parasites, predators, viruses, fungi, and bacteria. Natural enemies of *L. decemlineata* can become a valuable part in the pest management programs. Entomopathogenic organisms are harmless to the farmers, consumers, as well to the environment, and their specific action minimizes the impact on beneficial organisms and other non-targeted organisms (Lacey &

Siegel, 2000; Hokkanen & Hajek, 2003; Mudgal et al., 2013; Kumar et al., 2021). Moreover, entomopathogens favor biodiversity and the natural control of arthropods by parasitoids and predators (Lacey et al., 2015).

Numerous species of bacteria and fungi have been isolated from pests (Jurat-Fuentes & Jackson, 2012; Kumar *et al*., 2021), but few have been used for biopesticide development. Among microbial control agents, the bacterium *Bacillus thuringiensis* Berliner (*Bt*) and fungus *Beauveria bassiana* (Bals.-Criv.) Vuill. (*Bb*) (Kumar et al., 2021) are the most extensively used to control lepidopteran and coleopteran pests. Despite that, entomopathogens represent an underdeveloped and underutilized resource in arthropod pest management. Using more selective biopesticides supported by adequate habitat design can increase the enemies' impact on the Colorado potato beetle population (Cingel et al., 2016).

Considering the cost of biopesticides, the local production of biopesticides is significantly more affordable for farmers than importing plant protection products because the prices on the international market exceed their buying capacity (Sarwar, 2015). Also, using local strains as biological control agents can help avoid risks associated with decreased insecticidal activity due to environmental conditions. Thus, the research aimed to identify native strains of *Bt* and *Bb* for local production of biopesticides and promote sustainable management of *L. decemlineata.*

MATERIALS AND METHODS

Bacterial and fungal strains

Ten bacterial and ten fungal strains isolated from various hosts were used for pathogenicity tests against adult insects and larvae of *L. decemlineata* (Table 1). Some of the selected bacterial and fungal strains, *Bt* CNMN-BB-03, CNMN-BB-04, CNMN-BB-05, and *Bb* CNMN-FE-01, were previously described with high insecticidal activity against beetle pests *Neocoenorrhinus pauxillus* (Germ.), *Sitona lineatus* L., *Phyllobius oblongus* (L.), and *Tatianaerhynchites aequatus* L. (Munteanu et al., 2013; 2014a; 2014b). Some degree of activity against beetle species *Sitona lineatus* L. and *Hypera postica* (Gyll.) have shown the following fungal strains: Sl7, Sl9, Cg7, Cg10, Cg11, Cg12 (Moldovan et al., 2022), while others where not tested before (Table 1).

The *Bt* and *Bb* strains with high insecticidal activity are deposited in the Collection of Non-pathogenic Microorganisms of the Institute of Microbiology and Biotechnology, Technical University of Moldova. At the same time, the rest are preserved in the Collection of the Biological Invasions Research Center (BIRC), Institute of Zoology, Moldova State University.

Table 1. Data regarding bacterial and fungal strains used in the bioassay test

No.	Strain	Identity	Host	Reference
1.	CNMN-BB-03	*Bacillus thuringiensis*	*Neocoenorrhinus pauxillus* (Coleoptera)	Munteanu et al., 2013, 2014a, 2014b, Moldovan et al., 2017b
1.	CNMN-BB-04		*Phyllobius oblongus* (Coleoptera)	
2.	CNMN-BB-05		*Tatianaerhynchites aequatus* (Coleoptera)	
3.	S01	*Bacillus* spp.	*Helicoverpa armigera* (Lepidoptera)	Moldovan et al., 2018
4.	S02			
5.	S03			
6.	S04		*Spodoptera exigua* (Lepidoptera)	
7.	S05			
8.	S06			
9.	S07			
10.	CNMN-FE-01	*Beauveria bassiana*	*Sitona lineatus* (Coleoptera)	Moldovan et al. 2017a
11.	Sl7			
12.	Sl9			
13.	Cg7	*Beauveria* spp.		Moldovan et al., 2022
14.	Cg10			
15.	Cg11			
16.	Cg12			
17.	Hp2Cg	*Beauveria* spp.	*Hypera postica* (Coleoptera)	Moldovan et al., 2022
18.	Hp3Cg			
19.	Hp4Cg			

Culture media and growth conditions

For bioassay test bacterial strains were cultivated on solid T3 medium (Travers et al., 1987) at 30±0.2°C for 2-3 days in the darkness. Fungal strains were grown on PDA (Potato Dextrose Agar, Merck) at 25±0.2°C for 7-10 days, in the darkness, until complete sporulation. CFU and conidia were harvested using 10 µl sterile inoculation loop and transferred in tubes with sterile distilled water for inoculum preparation.

Insect rearing

For the bioassay purpose, a rearing colony was established from two wild populations of *L. decemlineata* collected in an infested tomato greenhouse (Orhei district) and a home garden (Causeni district) in the Republic of Moldova. No chemical or biological plant protection treatments were applied to control the wild Colorado beetle population. Sampled insects were placed in sterile cages with fresh tomato leaves and transported to the laboratory of BIRC, Institute of Zoology.

In the laboratory, insects were individually placed in new cages, fed with fresh tomato leaves, and monitored for three days to select only healthy specimens. By five healthy females and males were placed together in sterile insect-rearing cages for 24 hours for further insect rearing. After that, females were separated from males and kept individually for egg laying (Figure 1). Laid eggs were collected and stored in sterile Petri dishes until hatching. Healthy first-instar larvae and adults were taken for a bioassay test.

Bioassay test

Four bioassay experiments have been conducted to identify the most virulent bacterial or fungal strain. In the first and second experiments, eight bacterial strains and only one fungal strain (CNMN-FE-01, with known high insecticidal activity) were tested against both adults and larvae. In the third experiment, only fungal strains were tested; while in the fourth experiment, only bacterial strains were assessed. In the bioassay test, tomato leaves were used as a diet. At least ten insects and 2-5 leaves were used per repetition. Three repetitions were made for each tested strain. Sterile distilled water was used as a control. Insect mortality was recorded during ten days of incubation at 24°C, and a photoperiod of 14 h. Dead insects were removed from Petri dishes or cages every day.

1st experimental setup. Bacterial and fungal suspensions with a concentration of 10^8 CFU/ml were prepared. Fungal spore count has been conducted using a hemocytometer under 400X magnification of Meiji MT5000H microscope. The bacterial CFU count has been prepared by plate method. By 1 ml of each suspension was diluted with 4 ml of distilled sterile water, and the entire volume was sprayed over tomato leaves using a sterile hand-held sprayer. Leaves were left to air dry, and after that, ten adult insects (five males and five females) were transferred onto tomato leaves and left to feed for 24 h. After exposure, adults were transferred to sterile cages with fresh tomato leaf bouquets. Cages were inspected daily, fresh leaves were provided, and mortality data was recorded.

2nd experimental setup. Hatched larvae of *L. decemlineata* from collected eggs were transferred into sterile Petri dishes and fed with tomato leaves treated with bacterial and fungal suspensions as described earlier. After 24 h of exposure, insect larvae were transferred onto fresh leaves. Petri dishes were inspected and swapped daily. Also, fresh tomato leaves were provided. Mortality data was recorded daily.

3rd experimental setup. Following the performed activity of the fungal strain on adults of *L. decemlineata* species, in the 3rd experimental design, more fungal strains were tested. Fungal strains were applied topically by dipping each specimen in test tubes with 1 ml of conidial suspensions (10^7 conidia/ml) for 30 seconds and then transferring them to sterile cages with fresh tomato leaves.

In the *4th experimental setup*, the bacterial strains that performed the best and two additional *Bt* strains were tested against larvae of *L. decemlineata*. In this assay, bacterial suspensions (10^8 CFU/ml) were applied daily, leading to chronic larvae infection.

Statistical analysis

Obtained mortality data were corrected using the Abbott formula. The percentage of insects actually killed by the entomopathogenic bacteria or fungi was estimated by assessing the difference between the percentage of living insects in the control and the percentage of

insects living after treatment, divided by the percentage of insects living in the control (Abbott, 1925). Results are presented as mean ± standard error of the mean.

Figure 1. Insect rearing under laboratory conditions.

RESULTS AND DISCUSSIONS

In the first trial, the best results were for one fungal strain *Beauveria bassiana* CNMN-FE-01 causing 50% mortality and two bacterial strains *Bacillus* spp. S03 and *Bt* spp. *kurstaki* CNMN-BB-04, generating the highest mortality respectively, on day seven after treatment (Figure 2). Thus, data on mortality corrected according to the Abbott formula was 50.00 ± 3.21%, 42.59 ± 4.90%, and 28.52 ± 3.29% for best-performing strains, respectively. No significant data regarding insect mortality was recorded for the other tested strains.

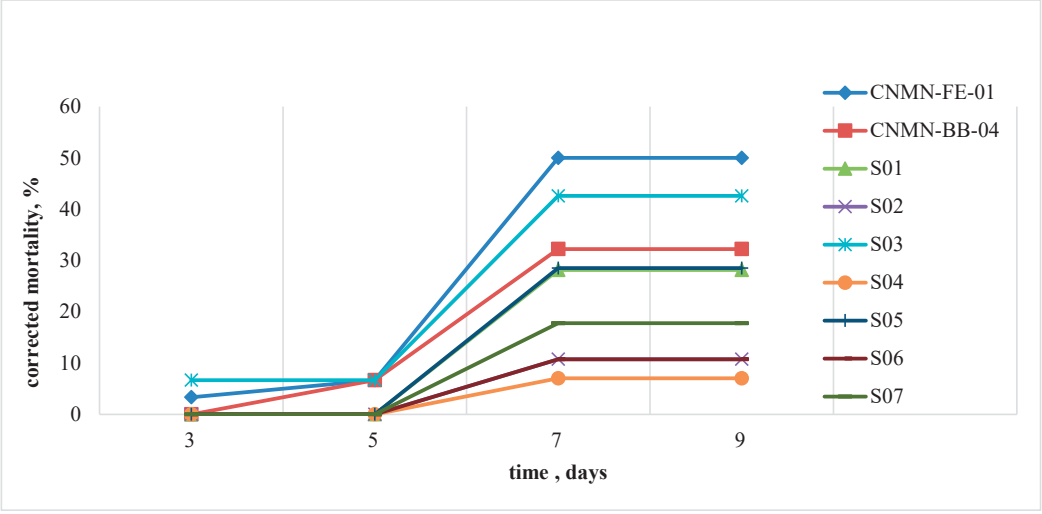


Figure 2. Cumulative mortality of *L. decemlineata* adults after exposure to bacterial and fungal strains. Data corrected according to Abbott formula

In the second experimental setup, larvae obtained from collected eggs were transferred to sterile Petri dishes and fed with tomato leaves treated with bacterial and fungal suspensions. No significant data regarding larvae mortality was recorded, the highest mortality being 33.33 ± 3.33% for *Bt* CNMN-BB-04 and *Bacillus* sp. S03 strains on day 9 after treatment (Figure 3).

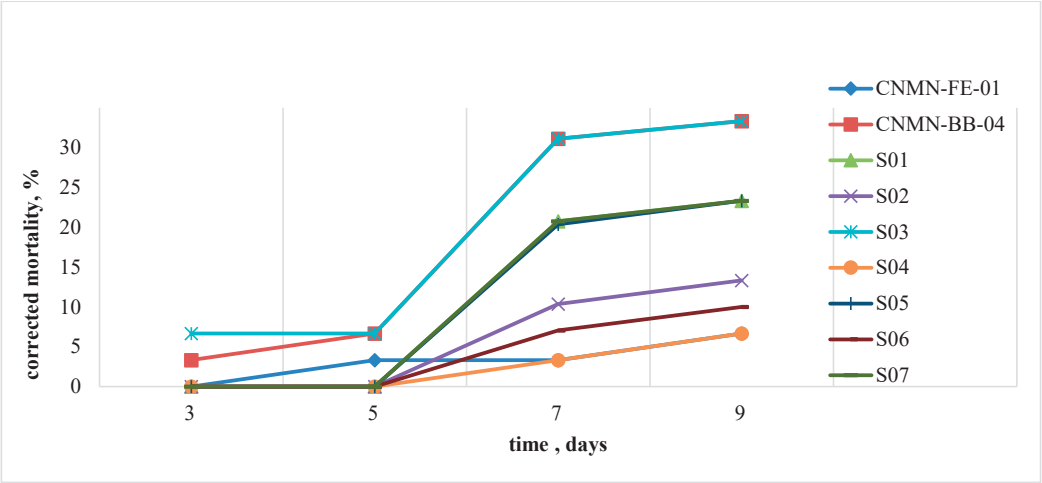


Figure 3. Cumulative mortality of *L. decemlineata* larvae after exposure to bacterial and fungal strains. Data corrected according to Abbott formula

In the third experimental setup, fungal strain Hp2Cg showed significantly high virulence, causing 53.33 ± 3.33% mortality on day 5 and 100% mortality on day seven after treatment (Figure 4). It was also noticed that insects stopped feeding from day two after treatment compared to the control variant, where insects were feeding actively and laying eggs. Fungal strain CNMN-FE-01 showed entomopathogenic activity with 53.33 ± 3.33% mortality on day seven after treatment. In comparison, the other eight strains have shown very low insect mortality, with values ranging from 33.33 ± 3.33% on day seven after treatment (Figure 4).

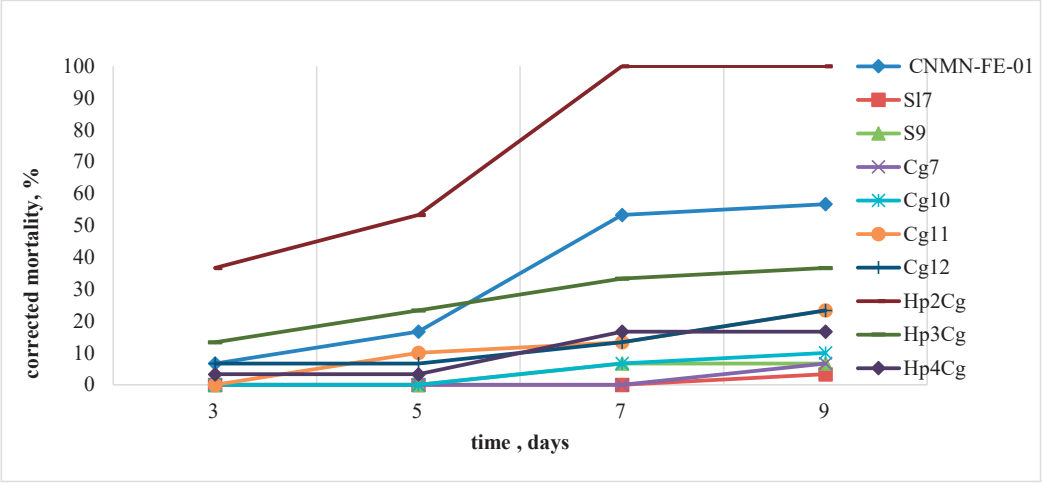


Figure 4. Cumulative mortality of *L. decemlineata* adults after exposure to fungal strains. Data corrected according to Abbott formula

In the fourth experimental setup, two bacterial strains, that performed the best in previous experiments, *Bt* CNMN-BB-04 and *Bacillus* sp. S03, and two additional *Bt* strains CNMN-BB-03 and CNMN-BB-05 were tested against larvae. Bacterial suspensions were applied daily, leading to chronic infection of larvae. Bacterial strains *Bt* CNMN-BB-05 and *Bacillus* sp. S03 has shown the best results, with 51.48 ± 4.55% and 41.11 ± 4.84% mortality recorded on day nine after treatment (Figure 5).

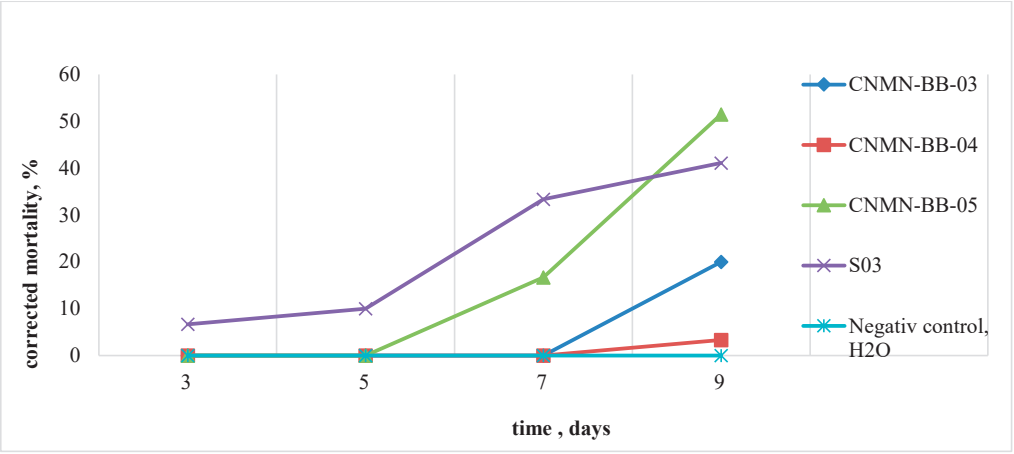


Figure 5. Cumulative mortality of *L. decemlineata* larvae after exposure to bacterial strains. Data corrected according to Abbott formula

In recent years, the scientific community has shown an increased interest in assessing entomopathogenic fungi against Colorado beetles. *Isaria fumosorosea* strain CCM 8367 caused high mortality against Colorado beetle larvae under laboratory conditions (Hussein *et al.*, 2016). Treatment of potato leaves with *Beauveria bassiana* significantly reduced Colorado beetle populations (Wraight & Ramos, 2015). LC50 values were equal to 10^5-10^6 spores/ml in larvae and 10^7-10^8 spores/ml in adults (Duan et al., 2018). New active strains of *B. bassiana* were recently reported from Turkey, causing mortality rates of 96.7 and 100% in larvae at a concentration of 1×10^7 conidia/ml (Baki et al., 2021). Also, highly active strains of *B. bassiana* were reported from the Czech Republic, and mortality of *L. decemlineata* adults caused by new native strains isolated reached up to 100% when beetles were treated with conidial suspensions at a concentration of 1×10^7 spores/ml. Authors recorded a LT50 equal to about seven days (Zemek et al., 2021). The present study reports similar results; the performing strain *Beauveria* sp. Hp2Cg has caused 100% mortality on day seven after treatment, at the same 1×10^7 conidia/ml concentration and the same application method by dipping the insect into conidial suspension. In their work, Zemek et al. (2021) focused on isolating novel native *Bb* strains from cadavers of Colorado beetle. Novel native strains had higher insecticidal activity than the GHA strain, the active ingredient of mycopesticide BotaniGard® WP. The present study was aimed at screening the local collection of *Beauveria* spp. and *Bacillus* spp. strains regarding their insecticidal potential. Strains that have shown some degree of activity were isolated from various hosts. *B. bassiana* strain CNMN-FE-01 has been isolated from *Sitona lineatus*. The strain has high insecticidal activity towards the host from which it was isolated, with LC50 equal to 1.127×10^4 conidia/ml on adults. Screened previously against *Hypera postica* and *Protapion apricans*, CNMN-FE-01 caused mortality rates of 70% and 40% on day seven after treatment (Moldovan et al., 2022). In the present study, this strain also had moderate insecticidal activity against adults and almost no activity against larvae, confirming the idea of host specificity expressed by Zemek et al. (2021). *Beauveria* sp. strain Hp2Cg, isolated from *Hypera postica* cadavers, was not previously assessed against pest beetles. Here, it had high insecticidal potential; thus, evaluating this strain against the host from which it was isolated will be interesting. This paper also reported results regarding assessing bacterial strains against adults and larvae. *Bacillus* sp. strain S03 exhibited some degree of activity against both adults and larvae of the Colorado beetle. Thus, future studies will be oriented toward assessing the synergistic potential of Hp2Cg and S03 strains against adults and larvae (Wraight & Ramos, 2005).

CONCLUSIONS

Nowadays agriculture faces numerous challenges, including those caused by the overuse of synthetic chemicals in pest control. The development of a sustainable farming system is a priority for the Republic of Moldova, the major goals being to provide food safety, environmental protection, biodiversity conservation, support farmers, and increase of international competitiveness. Microbial biopesticides show great potential in offering sustainable approaches towards efficient pest management.

The present paper reports on new data regarding the insecticidal activity of native bacterial and fungal strains against adults and larvae of Colorado beetle. Among screened strains, *Beauveria* sp. Hp2Cg has shown promising potential to be developed as a Colorado beetle biological control agent.

Future studies will address LC50 and LT50 of the selected strain and synergistic potential of *Beauveria* sp. Hp2Cg and *Bacillus* sp. S03 strains. Physiological characterization of *Beauveria* sp. Hp2Cg strain and field trials must be performed to advance toward local biopesticide production. Also, activities will be oriented towards the isolation of local entomopathogenic bacterial and fungal strains directly from *L. decemlineata*.

ACKNOWLEDGEMENTS

The research work was carried out with the support of STCU Project 6233, "Development and application of agro-ecological approaches in sustainable insect pest management for healthy tomato production," and partially of the project 20.80009.7007.12 DIVZOO "Diversity of hematophagous arthropods, zoo-, and phytohelminths, their vulnerability and tolerance strategies to climatic factors and elaboration of the innovative procedures for integrated control of species with socio-economic values".

REFERENCES

Abbott, W. S. (1925). A method of computing the effectiveness of an insecticide. *Journal of Economic Entomology*, *18*(2), 265–267.

Alyokhin, A., Vincent, C., & Giordanengo, P. (2013). *Insect Pests of Potato: Global Perspectives on Biology and Management*, 1st ed. Oxford, UK: Academic Press.

Baki, D., Tosun, H. S., & Erler, F. (2021). Efficacy of indigenous isolates of *Beauveria bassiana* (Balsamo) Vuillemin (Deuteromycota: Hyphomycetes) against the Colorado potato beetle, *Leptinotarsa decemlineata* (Say) (Coleoptera: Chrysomelidae). *Egyptian Journal of Biological Pest Control*, *31*, 1–11.

Cingel, A., Savić, J., Lazarević, J., Ćosić, T., Raspor, M., Smigocki, A., & Ninković, S. (2016). Extraordinary adaptive plasticity of Colorado potato beetle: "Ten-Striped Spearman" in the era of biotechnological warfare. *International Journal of Molecular Sciences*, *17*(9), 1538.

Damalas, C. A., & Eleftherohorinos, I. G. (2011). Pesticide exposure, safety issues, and risk assessment indicators. *International Journal of Environmental Research and Public Health*, *8*(5), 1402–1419.

Duan, Y., Wu, H., Ma, Z., Yang, L., Duan, X., & Ma, D. (2018). Identification and virulence of a fungal entomopathogen strain NDBJJ-BFG to Colorado potato beetle *Leptinotarsa decemlineata* (Say). *Journal of Plant Protection*, *45*(4), 751–758.

Hare, D. J. (1980). Impact of defoliation by the Colorado potato beetle on potato yields. *Journal of Economic Entomology*, *73*(3), 369–373.

Hokkanen, H.M.T., & Hajek, A.E. (Eds.). (2003). *Environmental impacts of microbial insecticides: need and methods for risk assessment*. Dordrecht, The Netherlands: Kluwer Academic Publishers.

Hussein, H. M., Skoková Habuštová, O., Půža, V., & Zemek, R. (2016). Laboratory evaluation of *Isaria fumosorosea* CCM 8367 and *Steinernema feltiae* Ustinov against immature stages of the Colorado potato beetle. *PLoS ONE*, *11*, e0152399.

Jurat-Fuentes, J. L., & Jackson, T. A. (2012). Bacterial entomopathogens. In Vega, F.E., Kaya, H.K. (Eds.), *Insect Pathology*, 2nd ed. (pp. 265–349). San Diego, USA: Academic Press.

Kumar, J., Ramlal, A., Mallick, D., & Mishra, V. (2021). An overview of some biopesticides and their importance in plant protection for commercial acceptance. *Plants*, *10*(6), 1185.

Lacey, L. A., & Siegel, J.P. (2000). Safety and ecotoxicology of entomopathogenic bacteria. In Charles, J.-F., Delecluse, A., Nielsen-LeRoux, C. (Eds.), *Entomopathogenic Bacteria: From Laboratory to Field Application* (pp. 253–273). Dordrecht, The Netherlands: Kluwer Academic Publishers.

Lacey, L.A, Grzywacz, D., Shapiro-Ilan, D.I., Frutos, R., Brownbridge, M., & Goettel M.S. (2015). Insect pathogens as biological control agents: Back to the future. *Journal of Invertebrate Pathology*, *132*, 1–41.

Moldovan, A., Toderas, I., Leclerque, A., & Munteanu-Molotievskiy, N. (2017a). Isolation and identification of fungal community of alfalfa pest weevils (Coleoptera: Curculionidae) in the Republic of Moldova. *IOBC WPRS Bulletin*, *129*, 70–73.

Moldovan, A., Toderas, I., & Munteanu-Molotievskiy, N. (2017b). Noi agenți bacterieni de control biologic al insectelor dăunătoare in Republica Moldova. In *Actual*

problems of zoology and parasitology: achievements and prospects (pp. 303–309).

Moldovan, A., Munteanu-Molotievskiy, N., & Toderas, I. (2018). Studii preliminare privind agenții de control biologic al dăunătorilor tomatelor în Republica Moldova. In *Functional Ecology of Animals* (pp. 287–289).

Moldovan, A., Munteanu-Molotievskiy, N., & Toderas, I. (2022). *Fungii entomopatogeni*. Chisinau, Republic of Moldova: F.E.-P. "Tipografia Centrala".

Mudgal, S., De Toni, A., Tostivint, C., Hokkanen, H., & Chandler, D. (2013). Scientific support, literature review and data collection and analysis for risk assessment on microbial organisms used as active substance in plant protection products–Lot 1 environmental risk characterisation. *EFSA Supporting Publications*, *10*(12), 518E.

Munteanu, N., Toderas, I., Moldovan, A., Malevanciuc, N., Toderaș, L., & Railean, N. (2013). Tulpina *Bacillus thuringiensis* subsp. *kurstaki* – insecticid biologic pentru combaterea coleopterelor curculionide. Patent No MD 4196. *The Official Bulletin of Industrial Property*, *2*, 19.

Munteanu, N., Toderas, I., Moldovan, A., Malevanciuc, N., Toderas, L., Bacal, S., & Railean, N. (2014a). Tulpina de bacterii *Bacillus thuringiensis* subsp. *kurstaki* – bioinsecticid pentru combaterea lepidopterelor din genul *Lymantria*. Patent No MD 4304. *The Official Bulletin of Industrial Property, 9,* 25.

Munteanu, N. V., Danismazoglu, M., Moldovan, A. I., Toderas, I. K., Nalcacioglu, R., & Demirbag, Z. (2014b). The first study on bacterial flora of pest beetles *Sciaphobus squalidus*, *Tatianaerhynchites aequatus* and *Bycticscus betulae* in the Republic of Moldova. *Biologia*, *69* (5), 681–690.

Sarwar, M. (2015). Biopesticides: an effective and environmental friendly insect-pests inhibitor line of action. *International Journal of Engineering and Advanced Research Technology*, *1*(2), 10–15.

Travers, R. S., Martin, P. A., & Reichelderfer, C. F. (1987). Selective process for efficient isolation of soil *Bacillus* spp. *Applied and Environmental Microbiology*, *53*(6), 1263–1266.

Vargas-Ortiz, E., Gonda, I., Smeda, J. R., Mutschler, M. A., Giovannoni, J. J., & Jander, G. (2018). Genetic mapping identifies loci that influence tomato resistance against Colorado potato beetles. *Scientific Reports*, *8*(1), 7429.

Weber, D. (2003). Colorado beetle: pest on the move. *Pesticide Outlook*, *14*, 256–259.

Wraight, S. P., & Ramos, M. E. (2005). Synergistic interaction between *Beauveria bassiana*- and *Bacillus thuringiensis tenebrionis*-based biopesticides applied against field populations of Colorado potato beetle larvae. *Journal of Invertebrate Pathology*, *90*(3), 139–150.

Wraight, S. P., & Ramos, M. E. (2015). Delayed efficacy of *Beauveria bassiana* foliar spray applications against Colorado potato beetle: impacts of number and timing of applications on larval and next-generation adult populations. *Biological Control*, *83*, 51–67.

Zemek, R., Konopická, J., Jozová, E., & Skoková Habuštová, O. (2021). Virulence of *Beauveria bassiana* strains isolated from cadavers of Colorado potato beetle, *Leptinotarsa decemlineata*. *Insects*, *12*(12), 1077.

Scientific Bulletin. Series F. Biotechnologies, Vol. XXVII, No. 2, 2023
ISSN 2285-1364, CD-ROM ISSN 2285-5521, ISSN Online 2285-1372, ISSN-L 2285-1364

ACCOUNTANCY IN BIOTECHNOLOGY FROM A CREATIVE POINT OF VIEW

Delia RĂDOI, Gabriela-Lucica MĂRGĂRIT

University of Agronomic Sciences and Veterinary Medicine of Bucharest,
59 Marasti Blvd, District 1, Bucharest, Romania

Corresponding author email: gabriela.margarit@biotehnologii.usamv.ro

Abstract

Creative accounting is a term used to describe the practice of manipulating financial information to present a more favorable picture of a company's financial performance and position than what might be an accurate representation under standard accounting principles. Creative accounting involves altering real accounting figures to convey specific messages desired by managers through exploiting accounting loopholes limitation. Nevertheless, in light of the changing accounting and legal context, researchers are increasingly preoccupied on the managerial outlook with regards to creative accounting and accounting manipulations. The influence of sustainable financial data governance tends to discourage creative accounting by promoting transparency, adherence to ethical principles, and stakeholder trust. On the other hand, political connections can have a more ambiguous impact, as they can either facilitate or discourage creative accounting, depending on the extent of influence and regulatory capture.

Key words: creative accountancy, ethical issues, fraud, management, audit

INTRODUCTION

Accountancy in biotechnology refers to the specialized financial and accounting practices and principles applied to businesses and organizations operating in the biotechnology industry. Biotechnology is a rapidly growing sector that involves the use of biological processes, organisms, or systems to develop or create products, technologies, and services. This can include pharmaceuticals, medical devices, genetic engineering, agriculture, and more. Effective accounting in biotechnology is essential for financial management, regulatory compliance, and decision-making. Here are some key aspects of accountancy in biotechnology:
Revenue Recognition: Biotech companies often have complex revenue recognition processes due to the long development timelines of products and services. Accountants must determine when and how to recognize revenue, taking into account factors like milestones, collaboration agreements, and licensing arrangements (Faragalla, W.A., 2015).
There are several important elements to consider such Research and Development Costs, Intellectual Property, Regulatory Compliance, Taxation, Funding Sources, Inventory Valuation, Financial Reporting, Risk Assessment and Cost Control.
Accountancy in biotechnology requires a deep understanding of the industry's unique challenges and financial intricacies. Biotech accountants work closely with scientists, researchers, and management to provide accurate financial information, support decision-making, and ensure compliance with industry regulations. They play a critical role in helping biotech companies achieve their financial goals and bring innovative products and services to market.

THE CONCEPT OF CREATIVE ACCOUNTING

Creative accounting is the practice of manipulating financial information to present a more favorable picture of a company's financial performance and condition than is possible under standard accounting principles. Creative accounting involves bending or abusing accounting rules and regulations, often within legal limits, to achieve specific financial goals. Although creative accounting is not inherently illegal, it raises ethical concerns and can mislead investors, creditors, and other

stakeholders. Some common techniques associated with creative accounting include:

Income Smoothing. This involves spreading out income and expenses over multiple periods to create a more stable and positive financial performance (Herrmann & Inoue, 1996). Companies may hold off recognizing certain expenses or accelerate revenue recognition to achieve this effect. This technique can be conducted by a chartered accountant who can manipulate the company's income and expenses to facilitate income smoothing using creative accounting (Abed et al., 2022).

Off-Balance-Sheet Financing. Companies may keep certain liabilities or assets off their balance sheet to make their financial position appear stronger than it actually is. This can be done through various financial instruments, special purpose entities, or partnerships (Cugova, 2020).

Changing Depreciation Methods. Altering depreciation methods can affect the value of assets on the balance sheet and impact the income statement. For instance, a company might switch from straight-line to accelerated depreciation to decrease reported profits in the short term.

Reserving for Contingencies. Companies may establish or release reserves for contingencies to smooth earnings or improve financial metrics. The timing and amount of such provisions can be manipulated.

Changing Accounting Policies. Switching accounting policies, such as from LIFO (Last-In-First-Out) to FIFO (First-In-First-Out), can affect the cost of goods sold and impact reported profits. Ustinova and col. 2022 showed that the results obtained by A. Sweeney (Sweeney, 1994) in her researches reveals that can be used LIFO and FIFO methods in order to influence the company's profit.

Revenue Recognition Timing. Accelerating or delaying the recognition of revenue to achieve specific financial targets, often related to bonuses or investor expectations.

Classification of Expenses. Shifting certain expenses from operational expenses to capital expenses can inflate reported profits in the short term.

Valuation of Assets. Overvaluing assets, such as real estate or inventory, can increase a company's net worth and make it appear financially healthier (Mortură & Dreghiciu, 2018).

While creative accounting can be used to meet short-term financial goals, it often leads to long-term problems. Investors and creditors rely on financial statements to make informed decisions, and when those statements are manipulated, it can erode trust and undermine the stability of the financial markets. Regulatory agencies, such as the U.S. Securities and Exchange Commission (SEC), have put in place strict rules and regulations to prevent and detect creative accounting practices, and Companies that engage in fraudulent accounting practices may face legal consequences. Ethical standards and corporate governance practices are also essential to prevent creative accounting practices and promote transparency and accuracy of financial reporting.

MOTIVATIONS/GENERATOR FACTORS FOR CREATIVE ACCOUNTING

Creative accounting, also known as earnings management or financial engineering, involves manipulating financial statements to present a more favorable picture of a company's financial performance or position than is actually the case. While creative accounting can have negative consequences and can be unethical or even illegal, there are several motivations or generator factors that might lead individuals or organizations to engage in such practices:

Profit Maximization. Creative accounting can be used to increase reported earnings and profits, thereby making the company more attractive to investors and analysts. This is likely to lead to increased stock prices and dividends, benefiting shareholders and possibly related to executive compensation.

Avoiding Debt Covenants. Companies may use creative accounting to avoid breaching debt covenants. By artificially improving financial ratios (e.g., debt-to-equity ratio), they can remain in compliance with loan agreements, thereby avoiding the risk of default and potential financial penalties.

Tax Minimization. Through creative accounting, companies can reduce their taxable income, leading to lower tax liabilities. This can enhance cash flow and the bottom line,

though it should be noted that there are strict legal limitations on what can be done to minimize taxes (Moisescu, 2016).

Managerial Compensation. Executive compensation programs often include bonuses or stock options tied to financial performance metrics. Creative accounting can inflate these figures, leading to larger bonuses or stock options for executives.

Attracting Investment. Companies may engage in creative accounting to attract investments or secure loans. A more favorable financial picture can be more appealing to potential investors, lenders, or creditors.

Meeting Analysts' Expectations. There is often pressure on publicly traded companies to meet or exceed analysts' earnings expectations. Creative accounting can be used to smooth out earnings and make them more predictable, reducing the likelihood of stock price volatility due to earnings surprises.

Hiding Financial Problems. Companies facing financial difficulties may use creative accounting to temporarily hide problems or losses, giving them time to address underlying issues without causing panic among investors, creditors, or employees.

Regulatory Compliance. Companies may engage in creative accounting to comply with complex accounting standards. In some cases, the application of these standards can be subjective, allowing for interpretations that favor the company's interests.

Competitive Advantage. In some industries, creative accounting may be used to maintain a competitive advantage. When competitors are engaging in similar practices, a company may feel compelled to do the same to avoid appearing less profitable or efficient.

Management's Reputation. Creative accounting can be used to maintain or enhance the reputation of a company's management team. This may be particularly important in industries where investor or public perception is closely tied to the integrity and competence of the management.

It's important to note that while there may be motivations for creative accounting, it is often unethical and can lead to legal consequences, damage a company's reputation, and harm stakeholders. Regulatory bodies and auditors play a crucial role in detecting and preventing creative accounting practices. Investors and analysts should be cautious and critically evaluate financial statements to detect possible irregularities.

CREATIVE ACCOUNTING TECHNIQUES

Creative accounting techniques involve manipulating financial and transactional data to present a more favorable or misleading view of a company's performance or financial condition. While these techniques can be unethical and sometimes illegal, they have been employed by some individuals and organizations. It's important to note that engaging in creative accounting can lead to severe consequences, including legal and regulatory action, damage to a company's reputation, and harm to investors and stakeholders. Here are some common creative accounting techniques:

Income Smoothing. This technique involves manipulating income or expenses to achieve a consistent level of reported earnings over several accounting periods. E. Bartov (Bartov, 1993), analyzed the overestimation of income in order to manipulate the moment of recognition of the sale of non-current assets. This technique can be done by postponing or accelerating the recognition of revenue or expenses, thus avoiding fluctuations in reported earnings.

Cookie Jar Reserves. Companies may create "cookie jar" reserves by setting aside excess profits in good years and then releasing them in bad years to boost reported earnings (Okoye&Obioma, 2020). This technique can make a company appear more stable and less volatile in terms of its earnings.

Big Bath Accounting. Under this technique, a company intentionally recognizes larger-than-necessary losses in a particular period, often during a restructuring or difficult year. By doing so, the company sets a low earnings benchmark for future periods, making it easier to show improved performance (Shahid&Ali, 2016).

Off-balance sheet financing. Companies can use off-balance sheet financing by removing assets, liabilities or transactions from the balance sheet to avoid them being recorded in

the financial statements (Kovalova & Michalikova, 2020). This can be used to make a company's financial position appear stronger or less risky than it actually is.

Channel stuffing. This technique involves artificially inflating short-term sales or revenue by shipping more products to distributors or customers than they can sell in a given period of time determined. This may make current sales numbers look better, but it often leads to future returns or unsold inventory.

Revenue Recognition Manipulation. Creative accounting can involve recognizing revenue too early or delaying revenue recognition (Kovalova & Michalikova, 2020). This can be done by changing the terms of sales agreements, issuing fictitious invoices, or recognizing revenue before it is genuinely earned.

Expense Capitalization. Instead of expensing certain costs immediately, some companies may capitalize them by treating them as long-term assets. This practice can make the company's profits appear higher in the short term because expenses are spread out over time.

Changing Depreciation and Amortization Methods. Companies can manipulate depreciation and amortization methods to lower expenses and increase reported earnings. This can involve extending the useful life of assets or using more favorable depreciation methods.

Inventory Valuation Techniques. Altering inventory valuation methods, such as using the LIFO (Last-In-First-Out) method to reduce taxable income, can lead to creative accounting. Companies can also overstate the value of inventory to inflate assets. Creative accounting can be used extensively in inventory, as it is highly subjective. As a result, the difference (positive or negative) in inventory reporting has an impact on the results of the current and future accounting periods (Abed et al, 2022).

Related Party Transactions. Engaging in transactions with related parties (e.g., family members, insiders, or affiliates) at non-market rates can be a way to manipulate financial results. This can involve artificially inflating or deflating expenses, revenues, or asset values.

Aggressive Accruals. Companies may use aggressive accruals by recognizing revenue before it is received or delaying the recognition of expenses (Okoye & Obioma, 2020). This can temporarily inflate earnings.

Income Shifting. Multinational corporations may engage in income shifting by shifting profits from high-tax jurisdictions to low-tax jurisdictions to minimize tax liabilities.

It's important to emphasize that these creative accounting techniques are not only unethical but can also be illegal if they violate accounting and financial reporting regulations. Auditors and regulatory bodies play a crucial role in detecting and preventing such practices, and they can impose penalties on companies and individuals who engage in creative accounting. Investors and analysts should exercise vigilance and conduct thorough due diligence to identify potential red flags in financial statements.

COMPARATIVE ANALYSIS BETWEEN CREATIVE ACCOUNTING AND FRAUD

Creative accounting and fraud are related concepts within the realm of financial reporting and accounting, but they are distinct in their nature, intent, and legal implications (Yadav, B. et all, 2014). Here's a comparative analysis highlighting the key differences between creative accounting and fraud:

Creative Accounting Intent. Creative accounting involves the manipulation of financial data and transactions to present a more favorable, albeit often misleading, view of a company's financial performance or position. The primary intent is to make the financial statements appear better without necessarily breaking the law.

Ethical Consideration. While creative accounting may not necessarily be illegal, it is typically considered unethical as it misrepresents a company's financial health and can deceive investors and stakeholders.

Motivation. The motivations behind creative accounting are often related to profit maximization, tax minimization, debt covenant compliance, and regulatory compliance. It may involve taking advantage of accounting rules' flexibility or subjective interpretation.

Legal Implications. Creative accounting is not inherently illegal, but it may cross ethical and legal boundaries if it involves false or misleading statements (Jan, 2018). Legal

consequences can arise if it violates accounting and reporting standards or securities regulations.

Examples. Income smoothing (Barnea, A. et al., 1976), big bath accounting, and off-balance-sheet financing are common examples of creative accounting techniques.

Fraud intent: Fraud, in the context of financial reporting, involves intentional deception, misrepresentation, or theft with the purpose of gaining financial benefits or causing financial harm. The primary intent is to deceive and commit unlawful activities (Goyal, 2012).

Ethical Consideration (Jan, 2018). Fraud is inherently illegal and unethical. It involves deliberate misrepresentation, embezzlement, or theft.

Motivation. Fraud is typically motivated by personal financial gain, often at the expense of shareholders, investors, or the company itself. It includes activities such as embezzlement, insider trading, and financial statement manipulation with fraudulent intent.

Legal Implications. Fraud is illegal, and it can result in severe legal consequences, including criminal charges, fines, and imprisonment. Legal authorities and regulators actively investigate and prosecute fraudulent activities.

Examples. Fraudulent activities in financial reporting may include cooking the books, embezzlement, insider trading, and financial statement manipulation with the intention to deceive stakeholders.

Regulators, auditors, and the legal system play vital roles in detecting and addressing both creative accounting and fraudulent activities in financial reporting. In contrast to fraud, creative accounting is simply the exploitation of inaccuracies, the incompleteness of accounting records (Gînța & Țirău, 2018).

EFFECTS OF USING CREATIVE ACCOUNTING

The use of creative accounting can have various effects on a company, its stakeholders, and the broader financial market. These effects can be both positive and negative, depending on the circumstances, the intent behind the creative accounting practices, and the ethical and legal boundaries crossed. Here are some of the key effects of using creative accounting:

Positive Effects

Enhanced Financial Performance. Creative accounting can make a company's financial statements appear more robust, which can attract investors and lenders, potentially leading to an increase in stock prices, easier access to capital, and lower borrowing costs (Okoye & Obioma, 2020).

Improved Liquidity. By manipulating financial statements, a company can temporarily improve its cash flow, which may be beneficial in the short term for covering expenses, servicing debt, or funding investments (Guinea, 2016).

Executive Compensation. Company executives who are rewarded based on financial performance metrics may benefit from creative accounting, as it can lead to larger bonuses or stock option payouts.

Debt Covenant Compliance. Creative accounting can help companies avoid breaching debt covenants, preventing the triggering of financial penalties and maintaining access to credit.

Tax Minimization. By reducing taxable income through creative accounting, companies can lower their tax liabilities, freeing up more cash for business operations or other purposes.

Negative Effects

Legal Consequences. Companies are free to take a creative approach to the use of methods that are permitted by the law (Kaminski, 2014). Engaging in creative accounting that involves fraudulent or illegal activities can lead to legal actions, fines, and imprisonment for individuals and significant penalties for companies.

Reputation Damage Discovery of creative accounting practices can severely damage a company's reputation, erode trust with investors, lenders, and the public, and result in a loss of business partners.

Investor Losses. Investors who rely on manipulated financial statements may suffer financial losses when the true financial health of the company is revealed, leading to stock price declines.

Increased Risk. Creative accounting can obscure a company's true financial health, making it difficult for investors to make informed decisions. This can increase the overall risk in the financial markets.

Distorted Capital Allocation. When companies appear more profitable than they are due to creative accounting, capital may be misallocated to companies that do not deserve it, potentially leading to inefficient resource allocation in the economy (Ustinova et al., 2020).

Market Volatility. The uncovering of creative accounting practices can lead to significant market volatility, as investors react to the sudden realization of the true financial state of a company (Groșanu 2013).

Credit Risk. Lenders and creditors may unknowingly extend credit to companies that are riskier than their reported financials suggest, leading to increased credit risk.

Erosion of Confidence in Financial Markets. Repeated instances of creative accounting can erode confidence in financial markets, making investors and stakeholders more skeptical and cautious.

In conclusion, the effects of using creative accounting are complex and multifaceted (Berisha & Shala, 2014). While it may provide short-term benefits for a company or its executives, it often leads to long-term harm, including legal consequences, damage to reputation, and financial losses for investors. For these reasons, regulatory bodies, auditors, and ethical business practices are crucial in ensuring transparency and integrity in financial reporting.

THE INFLUENCE OF SUSTAINABLE FINANCIAL DATA GOVERNANCE, POLITICAL CONNECTIONS ON THE CREATIVE ACCOUNTING PRACTICES

The influence of sustainable financial data governance and political connections on creative accounting practices can have significant implications for a company's financial reporting and ethical behaviour. Let's explore how these factors can affect the likelihood of creative accounting:

1. **Sustainable Financial Data Governance**

a. **Increased Transparency**

Sustainable financial data governance practices promote transparency and accountability in financial reporting (Jan, 2018). When a company has robust governance measures in place, it becomes more challenging to engage in creative accounting without detection.

b. **Ethical Framework**

Companies with a strong commitment to sustainable financial data governance are more likely to follow ethical principles in their financial reporting practices, reducing the incentive for creative accounting.

c. **Reduced Tolerance for Creative Accounting**

Sustainable financial data governance frameworks often have checks and balances that discourage creative accounting. Independent audits, internal controls, and rigorous reporting standards can deter unethical practices.

d. **Stakeholder Trust**

Companies that adhere to sustainable financial data governance are more likely to maintain trust with stakeholders, including investors, customers, and regulators. The risk of damaging this trust through creative accounting practices can be a strong deterrent. Existing practice confirms that most companies today use some form of positive creative accounting. As a result, they are able to ensure that the interests of the company are in line with the expectations of their investors and other stakeholders (Kaminski, 2014).

2. **Political Connections**

a. **Regulatory Influence**

Companies with political connections may have the ability to influence regulatory or legal decisions. This influence can lead to leniency in enforcement or changes in accounting standards that may accommodate creative accounting practices.

b. **Regulatory Capture**

In some cases, political connections can lead to regulatory capture, where regulatory agencies become excessively influenced or controlled by the industry they are supposed to oversee. This can create an environment where creative accounting goes unchecked.

c. **Reduced Scrutiny**

Companies with political connections may face less scrutiny from regulators or law enforcement agencies, making it easier to engage in creative accounting without fear of detection or legal consequences.

d. **Reputation Risk**
While political connections can provide certain advantages, they can also carry a risk of reputational damage. If it becomes public knowledge that a company is using political influence to engage in creative accounting, it can lead to public backlash and investor withdrawals.
e. **Selective Enforcement**
Political connections can lead to selective enforcement of regulations, with authorities choosing to turn a blind eye to certain companies' creative accounting practices. This selective enforcement can create an uneven playing field in the market.
Companies should be vigilant in adhering to ethical financial reporting practices and maintaining transparency, regardless of their political connections, to ensure the integrity of their financial statements and preserve the trust of stakeholders (Ibtihal A. et al., 2022). Regulatory bodies also play a critical role in detecting and preventing creative accounting practices, regardless of political influence. Shah et al. (2011) claimed that in a critical situation, companies can use creative accounting as a powerful weapon, but it is highly dependent on the management and ethical values of the company how this practice is used at this critical point.

ETHICS VERSUS CREATIVE ACCOUNTING

Ethics and creative accounting represent two contrasting approaches to financial reporting and accounting practices. Let's explore the key differences and considerations in the context of ethics versus creative accounting:

1. **Intent and Purpose**
Ethics: Ethical financial reporting emphasizes honesty, integrity, and transparency in financial statements (Amat, O. et al., 1999). The primary intent is to provide accurate and reliable information to stakeholders, such as investors, creditors, and the public. The purpose is to build trust and ensure that all relevant information is fairly and accurately represented (Comândaru, A.M., 2022).
Creative Accounting: Creative accounting involves manipulating financial data to achieve specific outcomes, often to make financial statements appear more favorable than they are (Guinea, 2016). The intent is to present a more positive image of a company's financial position or performance, even if it means bending accounting rules or exploiting loopholes (Richard, J. et al., 2009).

2. **Legal and Regulatory Compliance**
Ethics. Ethical financial reporting is aligned with legal and regulatory requirements. Companies following ethical practices adhere to accounting standards, laws, and regulations to ensure compliance and avoid legal consequences.
Creative Accounting. Creative accounting may involve actions that are legal but ethically questionable. However, it can also cross the line into illegal practices, such as fraud or securities violations. Companies engaged in creative accounting may take advantage of the flexibility in accounting rules or push the boundaries of legal compliance.

3. **Long-Term Consequences**
Ethics. Ethical financial reporting considers the long-term health and sustainability of a company. It fosters trust and positive relationships with stakeholders, which can lead to stable growth and a strong reputation.
Creative Accounting. Creative accounting often prioritizes short-term gains or appearances over long-term sustainability. While it may temporarily boost stock prices or profitability, it can erode trust, damage a company's reputation, and lead to negative consequences in the long run.

4. **Stakeholder Trust**:
Ethics. Ethical financial reporting is crucial for building and maintaining trust with shareholders, investors, creditors, employees, and other stakeholders. Trust is a cornerstone of successful and sustainable business relationships.
Creative Accounting. Engaging in creative accounting practices can erode stakeholder trust when discovered, as it demonstrates a lack of transparency and integrity in financial reporting.

5. **Legal and Ethical Accountability**

Ethics: Ethical financial reporting practices align with ethical standards and principles (Okoye & Obioma, 2020). Violations of ethics may result in reputational damage but may not necessarily lead to legal consequences.

Creative Accounting. Creative accounting practices, especially if they cross legal boundaries, can lead to serious legal and regulatory consequences, including fines, penalties, and even imprisonment for individuals and legal actions against companies (Umobong & Ironkwe, 2017).

AUDITOR ROLE IN VERIFYING UNETHICAL ACCOUNTING PRACTICES

Auditors play an important role in verifying and detecting unethical accounting practices. Their primary responsibility is to provide an independent and objective assessment of a company's financial statements, ensuring that they fairly represent the company's financial position and performance (Al Rawashdeh, N.H.H., 2013). Here's how auditors fulfil this role in the context of unethical accounting practices:

Independence and Objectivity. Auditors must maintain independence and objectivity in their work (Groșanu, 2013). This means they should not have any financial or personal interests that could compromise their impartiality. Independence is vital in detecting and reporting unethical practices.

Risk Assessment. Auditors assess the risks associated with unethical accounting practices by analysing the company's internal control systems, financial records, and industry-specific factors. They identify areas where there is a higher risk of material misstatement due to unethical behaviour.

Audit Procedures. Auditors design audit procedures to address the identified risks. These procedures may include substantive testing, analytical procedures, and tests of controls to ensure that the financial statements are free from material misstatement, whether due to error or fraud.

Fraud Detection. Auditors are specifically responsible for detecting fraud, including unethical accounting practices. They look for signs of fraudulent financial reporting or misappropriation of assets. This includes assessing the risk of management override of controls (Groșanu, 2013).

Professional Skepticism. Auditors employ professional skepticism when evaluating the financial statements (Rabin, C.E., 2005). They maintain a questioning mindset and exercise judgment when examining the evidence. This skepticism helps auditors uncover and investigate irregularities or inconsistencies.

Whistleblower Reporting:

Auditors are often required to report any instances of fraud or unethical behaviour they discover during the audit (Groșanu, 2013). If auditors suspect unethical accounting practices, they may escalate their concerns to the company's audit committee or the board of directors.

Materiality Assessment. Auditors assess materiality, which is the threshold at which financial misstatements would be considered significant enough to impact the decision-making of users of the financial statements. If unethical practices result in material misstatements, auditors are more likely to detect them.

Communication with Management. Auditors communicate with company management throughout the audit process. They may request additional information or explanations if they identify areas of concern or if they suspect unethical practices.

Documentation. Auditors maintain thorough documentation of their audit work, including the procedures performed, findings, and conclusions. This documentation provides a record of their assessment of the financial statements.

Reporting to Regulators. In some cases, auditors may be required to report unethical practices to regulators or law enforcement, depending on the severity of the problem discovered (Dechow&Skinner, 2000).

Audit Report. The final audit report issued by the auditor gives an opinion on the integrity of the financial statements (Okoye & Obioma, 2020). If auditors discover unethical accounting practices, they are required to include a statement regarding the issues found in the report.

If auditors uncover unethical accounting practices, they have a professional and ethical duty to report their findings to the appropriate parties, which may include the company's board of directors, audit committee, regulators, or law enforcement agencies, depending on the circumstances and severity of the issues. The auditor's role in uncovering unethical practices is critical in maintaining the integrity of financial reporting and protecting the interests of stakeholders.

CONCLUSIONS

The influence of sustainable financial data governance tends to discourage creative accounting by promoting transparency, adherence to ethical principles, and stakeholder trust. On the other hand, political connections can have a more ambiguous impact, as they can either facilitate or discourage creative accounting, depending on the extent of influence and regulatory capture.

Creative accounting and fraud both involve manipulation of financial data and transactions but differ significantly in terms of intent, ethics, motivation, and legal consequences. Creative accounting, while often unethical, may not necessarily be illegal, whereas fraud is inherently illegal and entails deliberate deception and financial harm.

Ethics and creative accounting represent opposite approaches to financial reporting. Ethical financial reporting prioritizes accuracy, transparency, and long-term sustainability while creative accounting often seeks short-term gains through manipulation of financial data. Companies that prioritize ethics are more likely to build trust and maintain positive relationships with their stakeholders, while those that engage in creative accounting may face with long-term legal, reputational and financial risks.

REFERENCES

Abed, Ibtihal A., Nazimah Hussin, Mostafa A. Ali, Hossam Haddad, Maha Shehadeh, and Elina F. Hasan, 2022. Creative Accounting Determinants and Financial Reporting Quality: Systematic Literature Review. Risks 10: 76.https://doi.org/10.3390/risks10040076

Al Rawashdeh, N.H.H., 2013. The Role of the Auditor in Verified of the Unethical Practices in Accounting, International Journal of Scientific and Research Publications, Volume 3, Issue 7, July 2013 1 ISSN 2250-3153

Amat, O.; Blake, J.; Dowds, J., 1999. The ethics of creative accounting. Econ. Work. Paper, 715–736.

Barnea, A., Ronen, J. and Sadan, S., 1976. Classificatory smoothing of income with extraordinary items, The Accounting Review, January, 110-122

Bartov, Eli, The Timing of Asset Sales and Earnings Manipulations (October 23, 1993). The Accounting Review, October, Vol. 68 Issue 4, pp. 840-855, 1993, Available at SSRN: https://ssrn.com/abstract=2972514

Berisha, V., Shala., A., 2014. Effect of creative accounting on the company, 8th International Scientific Conference on Economic and Social Development and 4th Eastern European ESD Conference: Building Resilient Economy, Zagreb, Croatia

Comândaru, A.M., 2022. Implicațiile contabilității creative asupra calității informațiilor furnizate de situațiile financiare anuale ale entităților economice din industria farmaceutică, Teză de doctorat, Universitatea Valahia, Târgoviște

Cugova, A., Cug,J., 2020. Motivation for the use of creative accounting techniques in the conditions of the globalized business environment, https://doi.org/10.1051/shsconf/20207401004

Dechow, P.M. and Skinner, D.J.: 2000. Creative accounting: reconciling the views of accounting academics, practitioners and regulators, Accounting Horizons, Vol. 14, Issue 2, 235-51

Faragalla, W.A., 2015. Practici de contabilitate creativă în viziunea profesioniștilor contabili, Profesioniștii despre contabilitatea creative Audit financiar, XIII, Nr. 7(127) ISSN: 1583-5812; ISSN on-line: 1844-8801, 59-72

Gînța, A.I., Țirău, A.I., 2018. Analysis of Creative Accounting - Faithful Image Relationship and Creative Accounting - Fraud Relationship, Ovidius· University Annals, Economic Sciences Series Volume XVIII, Issue 2

Goyal, M.M., 2012. Creative Accounting: A devious tool of accounting, IRJMSH Volume 3 Issue 1 online ISSN 2277 – 9809

Groșanu, A., 2013. Contabilitate creativă, Colecția Cercetare avansată postdoctorală în științe economice, Ed. ASE București, ISBN 978-606-505-705-0

Guinea, F.A. , 2016. Study Regarding the Creative Accounting Techniques in Management Accounting, Audit Financiar, vol. XIV, no. 10(142)/2016, pp. 1136-1148, DOI: 10.20869/AUDITF/2016/142/1136

Herrmann, D., Inoue, T., 1996. Income smoothing and incentives by operating condition: An empirical test using depreciation changes in Japan. Journal of International Accounting, Auditting and Taxation 5, 161-177

Ibtihal A. Abed, Nazimah Hussin, Mostafa A. Ali, Hossam Haddad, Maha Shehadeh and Elina F. Hasan, 2022. Creative Accounting Determinants and Financial Reporting Quality: Systematic Literature

Review. Risks 10: 76. https://doi.org/10.3390/risks10040076
Jan, C.I., 2018. An Effective Financial Statements Fraud Detection Model for the Sustainable Development of Financial
Markets: Evidence from Taiwan, Journal of Sustainability, 10, 513; doi:10.3390/su10020513
Kovalov, E., Michalíková, K.F., 2020. The creative accounting in determining the bankruptcy of Business Corporation, SHS Web of Conferences 74, 01017, https://doi.org/10.1051/shsconf/20207401017
Moisescu, F., 2016. The art of "creative accounting" in the current economic context, The Journal Contemporary Economy ISSN 2537 – 4222, ISSN-L 2537 – 4222
Mortură, L.A., Dreghiciu, A.E., 2018. Possibilities of Influencing the Results of an Entity through Creative Accounting at Stock Level, "Ovidius· University Annals, Economic Sciences Series, Volume XVIII, Issue 2/2018
Okoye, E., Obioma, J., 2020. Impact of Creative Accounting Techniques on Firm Financial Performance: A Study of Selected Firms in Nigeria, Journal of Accounting and Financial Management E-ISSN 2504-8856 P-ISSN 2695-2211 Vol 6. No. 2 2020
Rabin, C.E., 2005. Determinants of auditors' attitudes towards creative accounting. Medit. Account. Res.,13, 67–88
Richard, J., Devinney, M., Yip, S. & Johnson, G. (2009). Measuring organizational performance: Towards methodological best practice. Journal of Management, 2 (1), 6-11.
Shah, S., Butt S., Tariq, Y.B., 2011. "Use or abuse of creative accounting techniques." International Journal of Trade, Economics and Finance, 2(6), 531-536
Shahid, M., Ali, H., 2016. Influence of Creative Accounting on Reliability and Objectivity of Financial Reporting (Factors Responsible for Adoption of Creative Accounting Practices in Pakistan), Journal of Accounting and Finance in Emerging Economies ISSN: 2519-0318ISSN (E) 2518-8488 Volume 2: Issue 2 December 2016
Sweeney, A. P., 1994. Debt-covenant violations and managers' accounting responses. Journal of Accounting and Economics, 17(3), 281-308. https://doi.org/10.1016/0165-4101(94)90030-2
Umobong, A. A., Ironkwe, U. (2017). Creative accounting practices and financial performance of firms. International Journal of Innovative Finance and Economics Research, 5(1), 1-10.
Ustinova, Y., Gupta, C.M., Shaposhnikov, A., 2022. Creative Accounting Detection Methods: Foreign Experience, Advances in Social Science, Education and Humanities Research, volume 632
Yadav, B., Kumar, A., Bhatia, B.S. (2014). "Concept of creative accounting and its different tools". International Journal of Management and Social Sciences Research (IJMSSR), 3(2), 66-74

Scientific Bulletin. Series F. Biotechnologies, Vol. XXVII, No. 2, 2023
ISSN 2285-1364, CD-ROM ISSN 2285-5521, ISSN Online 2285-1372, ISSN-L 2285-1364

ADVANCES IN GRAPE MARC COMPOSTING: A REVIEW

Alexandru Cristian GROSU[1], Constantin Alexandru ALDEA[1], Florentina MATEI[1, 2], Narcisa Elena BĂBEANU[1]

[1]University of Agronomic Sciences and Veterinary Medicine of Bucharest, 59 Mărăști Blvd, District 1, Bucharest, Romania
[2]Faculty of Food Industry and Tourism, Transilvania University of Brașov, 140 Castelului Street, Brașov, Romania

Corresponding author email: florentina.matei@unitbv.ro

Abstract

During the winemaking process, large amounts of waste are produced, including pomace, skin, stalk, wastewater, less and shoots, which contribute significantly to environmental degradation. Grape marc, which accounts for 25% of grape weight and is left over after juice extraction, can be used in a circular approach for green energy, component extraction, energy recovery and a variety of applications such as fuel, alcohol, biosurfactants and composting for natural fertiliser. There are numerous techniques for obtaining a final compost, including static systems (aerated static piles, continuous vertical reactors and in-vessel tunnels) and turned or agitated systems (turned windrow, agitated bed and rotating drum). Enhancing compost value involves co-composting the grape marc with organic byproducts like manures, olive oil industry waste, coffee grounds and more. Vermicomposting, that can be applied indoors and outdoors, yields nutrient-rich organic material for crops efficiently. This method offers advantages such as year-round composting and quicker production of superior-quality nutrients.

Key words: *grape marc, co-composting, vermicomposting*

INTRODUCTION

Waste and wastewater are produced in large quantities during the winemaking process. Before they can go back into the environment, these residues need to be recycled or treated. The winery waste chemical composition is affected by the conditions and moment of the harvest. Harvest and post-harvest are the two distinct times that are usually taken into consideration in the winemaking process. Compared to the post-harvest period, the amount of solid waste and wastewater generated during harvest is significantly higher (Oliveira & Duarte, 2016). Among the main waste products from the winery are grape marc (or pomace), skin and stalk. In addition to these waste products, a significant amount of waste, including water, lees, shoots and some filtration residue produced by wineries, is another major factor contributing to environmental degradation (Ahmad et al., 2020; Musee et al., 2007).

The wastewater from wineries is created during processing and cleaning procedures, such as washing grapes during crushing and pressing, rinsing fermentation tanks, filtration, cleaning barrels, bottling and aging (Flores et al., 2023; Andreottola et al., 2009; Musee et al., 2006). Significant wastewater flows are produced during the grape-processing phase (vintage and racking) and considerable amounts of water are used in the months that follow, as bottling and container cleaning are nearly constant activities (Bolognesi et al., 2020; Masi et al., 2015). The wine industry is one of the industries which uses high amounts of water; taking into account all of the operations that were previously mentioned, a wastewater/wine ratio of 14 L/0.5 L was reported (Bolognesi et al., 2020; Oliveira & Duarte, 2010), with an average value of 4 L wastewater/1 L of wine (Flores et al., 2023). The wastewater from wineries is acidic (with a pH less than 5.5), phytotoxic and contains salts, organic matter, trace elements like magnesium, calcium and sodium. It also has a high level of biological oxygen demand, a significant amount of sugars, organic acids, glycerol and alcohols, as well as a microbial population of yeasts and bacteria (Bharathiraja et al., 2020; Lucas et al., 2009; Vlyssides et al., 2005).

The vine shoots are agricultural byproducts of vine pruning, with a global production of 15

million tonnes each year. Typically, they are burned to stop phytopathogen growth or ground and left in the field as an organic fertiliser (David et al., 2020). They are made up of cellulose, hemicellulose and lignin and may be utilised to create a variety of biobased products including proteins, bioactive compounds, biofuels like as ethanol and biogas (Baptista et al., 2023; Pachón et al., 2020; Jesus et al., 2017; Pérez-Rodríguez et al., 2016), oligosaccharides (Dávila et al., 2016), proteins, polyphenols (Rajha et al., 2014), lactic acid (Garita-Cambronero et al., 2021), xylitol (Rivas et al., 2007) and biosurfactants (Cortés-Camargo et al., 2016).

The grape marc or pomace is composed of grape skins, seeds and stalks, as well as the distrupted cells from the grape pulp (Crespo-López et al., 2022; Perra et al., 2022; Gómez-Brandón et al., 2019), which represents approximately 25% of the total grape weight used in the process (Salgado et al., 2019). It is the waste that remains after the juice from grape pressing is collected for wine production and it is made up of polyphenols, pectic polysaccharides, heteroxylans, cellulose, alcohol, unfermented sugars, tannins, pigments and other valuable products (Pinter et al., 2019; Corbin et al., 2015). It has been estimated that the production of 6 L of wine generates approximately 1 kg of grape marc, accounting for an annual global production of 10.5-13.1 Mtons of grape marc (Gómez-Brandón et al., 2019). The quantity and quality of grape marc will vary depending on the size of the winery and the winemaking methods used (Muhlack et al., 2018). According to Frîncu et al. (2019), when researching the feed properties of winemaking byproducts, standardised analysis methods such as dry matter, crude protein, aminoacids, crude fat, fatty acids, crude fibre, ash, calcium, copper, iron, manganese and zinc, phosphorus, gross energy, polyphenols concentration and antioxidant capacity are typically recommended. Grape marc, as an organic product high in lignocellulosic compounds, can be used in a circular approach as an attractive feedstock for green energy production, extraction for useful components, thermochemical and biological treatments for energy recovery, fuel or beverage alcohol production, biosurfactants production, as well as in composting to obtain natural fertiliser (Muhlack et al., 2018).

Composting is a biological process that turns organic wastes into a homogenous, plant-available material in an environmentally friendly way under aerobic conditions with suitable temperature and moisture. In the presence of oxygen, nitrogen and carbon, a variety of microorganisms carry out complex metabolic processes to create their own microbial biomass throughout the composting process. Additionally, the microorganisms produce heat and compost (a solid substrate that has less carbon and nitrogen but is more stable) during this process (Meena et al., 2021). There are mainly four stages to the composting process: a mesophilic initial phase, during which simple compounds like sugars or amino acids are broken down by mesophilic bacteria and fungi by quickly raising the temperature; a thermophilic second phase, during which thermophilic microorganisms break down organic matter (fats, cellulose, hemicellulose and lignin). The reduction in the feedstock's organic carbon content during this phase is attributed to the metabolic activities of heat-tolerant microorganisms. Finally, a lowered temperature and diminished microbial activity are the hallmarks of the cooling phase. Within this, mesophilic microorganisms recolonize the compost pile and break down the remaining sugars, cellulose and hemicellulose, to produce compounds that resemble humic substances. After this, the maturation phase occurs, during which the rate of organic matter decomposition decreases and the rates of organic compound polymerization and humification increase. (Rastogi et al., 2020; Albrecht et al., 2010).

The compost quality is determined by the raw materials used to create a stabilised end result of the biological aerobic transformation of organic matter till the humification process, which is evaluated at the maturity phase. The compost is defined by two characteristics: stability and maturity. The stability correlates to the change of an initially unstable organic matter into a stable organic matter at the completion of composting. The maturity refers to safe use as a result of the absence of phytotoxic compounds (Salgado et al., 2019; Bazrafshan et al., 2016; Martínez et al., 2016; Wichuk and McCartney, 2010).

APPROACHES OF GRAPE MARC COMPOSTING

When talking about grape marc composting, two main approaches are taken into account: the static systems and the turned/agitated systems. Their characteristics and specificities will be described below.

Static systems

Aerated static pile

Aerated static pile composting removes the necessity to turn the compost pile by introducing airflow into the piles (Abdoli et al., 2019). The aeration system in aerated static pile composting technology is made up of a system of perforated pipes connected to timer-controlled blowers. The blowers provide direct process control, maintaining an oxygen level of 5-15% without turning the pile. The piles are frequently topped with a layer of matured compost to avoid heat loss from the upper layers and to provide smell management. The active composting period can be completed in three to five weeks if the pile is properly formed and enough air is supplied (Makan & Fadili, 2020).

Continuous vertical reactors

In this method, the materials are typically loaded through the reactor's top and discharged through its bottom. The composting mass is oxygenated by forcing air up from the bottom. These reactors can handle massive quantities of material (up to 2000 cubic metres) and can reach heights of nine metres. However, height is extremely important, and masses greater than three metres cause serious ventilation problems (Dominguez et al., 1997). Temperatures and other variables can be tracked via ports installed along the vertical wall. Because air warms up as it passes through the composting mixture, the control over moisture is limited. When the heated air reaches the cool mass of new material, it condenses at the top. The composting process takes about 14 days (Arvanitoyannis et al., 2006).

In-vessel tunnels

According to Diaz et al. (2007), in-vessel tunnels are rectangular containers that have typical dimensions of 4-5 m long, 3-4 m high and up to 30 m long. They have dedicated doors for the loading and unloading of materials and are constructed of brick, concrete or metal. Every day, the material for composting is loaded through the loading door. A hydraulic piston is then used to push the feedstock in the direction of the door on the other side. The majority of tunnel designs come with sensors for measuring oxygen and moisture content. Compressors are frequently used to supply air to the feedstock. A computer is used to oversee the entire procedure (Makan & Fadili, 2020; Noble & Gaze, 1997).

Turned or agitated systems

The composting materials in turned (agitated) systems are regularly combined, agitated or "turned" at frequencies that can vary from daily to every two months. As a result, compounds are homogenised by dispersing moisture, transporting materials from the oxygen-poor inside to the oxygen-rich outside and mixing high and low temperature elements. Some compost turning methods may assist to decrease the particle size and rebuild a windrow or pile. Turning is the process of lifting composting feedstocks into the air, mixing them and allowing them to fall back to the ground. Front-end loaders, augers, dedicated turning machines and other equipment may be used (Michel et al., 2022). Turning compost piles raises temperatures due to aeration, which is beneficial during composting (Mulidzi, 2021).

Turned windrow

Turned windrow composting is the oldest and most basic composting technology and it is widely used for stabilising and converting organic substrates to usable and value-added products. Turning windrow composting requires specialised equipment on a regular basis. Diffusion and convection naturally ventilate the elongated piles known as windrows. Despite its simplicity, this technology has significant limitations that should be highlighted, including high labour costs, long lead times and the use of valuable land space (Makan & Fadili, 2020). The turned windrow composting method has benefits such as the possibility to handle a huge volume of material, it is simple to implement and use,

involves minimal capital expenses, needs the least amount of infrastructure and equipment, is easily flexible to accommodate demand, enabling small to large-scale operations, is simple to begin and finish and produces high-quality compost (Vigneswaran et al., 2016).

Agitated bed

Agitated bed is a horizontal composting system. It consists of an aerated bed contained within a horizontal bin. The sludge introduced into the bin can be mechanically turned up on a regular basis and removed after 21 days. Curing then occurs outside the bins in an open or covered area (Arvanitoyannis et al., 2006).

Rotating drum

The rotating drum incorporates a tilted rotating cylinder that allows for downward material displacement. A drum's typical dimensions are 45 m long and 2-4 m in diameter. The rotational speed is approximately 0.2-2 rpm. Some drums have internal vanes that, when combined with the rotating action of the drum, force the material towards the exit and contribute to size reduction and feedstock mixing. Moisture and oxygen concentrations in the reactor are monitored and kept at or near optimal levels. This type of reactor is typically used for the active phase of composting, and the composting process can be accelerated by carefully controlling the oxygen and moisture contents (Diaz et al., 2007). The final product that is produced is therefore uniform and consistent and free of any problems related to leachate or odour (Makan & Fadili, 2020).

ADDED VALUE BROUGHT BY DIFFERENT WASTES DURING GRAPE MARC COMPOSTING

Co-composting winery wastes with other organic materials may help neutralise the acidity associated with grape marc, thereby improving the dynamics of the process of composting along with the overall quality of the final product (Gómez-Brandón et al., 2019). Table 1 shows the various organic matter that can be added to the grape marc (pomace) composting process in order to improve the final compost quality.

Table 1. Co-composting winery wastes with other organic matter to improve compost quality

Mixture	Reference
Grape marc + Grape stalks	Pinto et al., 2023
Grape marc + Animal manure	Eon et al., 2023
Grape marc + Coffee grounds	Karapantzou et al., 2023
Grape mill waste + Olive mill waste	Chrysargyris et al., 2023
Grape marc + Sugarbeet Vinasse	Díaz et al., 2002
Grape marc + Organic fraction of municipal solid waste	Hungría et al., 2017
Grape marc + Mature vermicompost	Gómez-Brandón et al., 2023
Grape marc + Goat manure + Leaves from garden raking + Alfalfa	Pinter et al., 2019
Grape pomace + Pig manure + Biochar + Fe_2O_3	Zhang et al., 2023
Grape pomace + Goat and Horse manure	Salgado et al., 2019
Grape pomace + Corn straw + Pig manure	Xu et al., 2022
Grape pomace + Wheat straw + Swine manure	Ivanović et al., 2022
Grape marc + Hose leaves + Sheep manure	Barros et al., 2021
Exhausted grape pomace + Cattle manure or Poultry manure	Bustamante et al., 2008
Grape marc + Goat manure + Leaves from garden raking + Alfalfa	Pinter et al., 2019
Grape marc + Sewage sludge + Pelletized wheat straw	Dume et al., 2023
Grape marc + Green herbaceous crop residues + Pruning residues	Alfonzo et al., 2022
Grape marc + Grass clippings + Orange fruit waste	Coelho et al., 2021
Grape marc + Olive mill wastewater + Green waste	Majbar et al., 2017
Grape marc compost + Perlite + Soil + Pumice	Tassoula et al., 2021
Grape marc + Green waste + Straw + Soil + Biochar	Kessler et al., 2021
Grape marc + Waste from orange juice / Waste from tomato soup production / Cattle manure / Sheep manure	Pérez-Murcia et al., 2021
Substrate mixed with Grape marc + Pumice + Pure sphagnum peat + Soil	Paraskevopoulou et al., 2021
Winery wastewater sludge + Grape stalks + Biochar	Pinto et al., 2021
Grape skin + Stalks + Vineyard pruning waste + Zeolite	Cataldo et al., 2023
Green waste + Sugarcane bagasse + Exhausted grape marc	Zhang & Sun, 2016

Co-composting grape marc with residues from olive oil production can provide numerous benefits to the final compost. Blending grape marc with olive oil wastewater to create compost may be a useful fertiliser for lawns. Research by Paplomatas et al. (2004) shows that this mixture can inhibit the growth of *Rhizoctonia solani*, the agent that causes brown patch disease in two types of lawns: *Festuca arundinacea* 'Tomahawk' and *Lolum perenne* 'Applaud'. Furthermore, composts made with wastes from olive mills have several advantageous agronomic traits: low phytotoxicity and the potential to suppress soil-borne illnesses; and these can be used as soil amendments and can effectively replace a portion of the peat moss used in growing media without adversely affecting crop yields (Aviani et al., 2010). Majbar et al. (2018) investigated the compost produced by co-composting grape marc and olive mill wastes. According to the authors, the co-composting process progressed well, with biodegradation of organic matter and bioconversion of unstable matter into a mature, stable product rich in nutrients, with no phytotoxic effect. Also, physicochemical analysis revealed that these composts are of high quality, high in nutrients, particularly N, P, K, Ca, Mg and Fe (Majbar et al., 2018).

The spent coffee grounds and grape marc contain high levels of polyphenols, tannins, cellulose and hemicellulose, consequently laccases and peroxidases are more active in the composts made from them (Sanchez-Hernandez & Domínguez, 2017). As a result, the compost produced by co-composting grape marc and used coffee grounds could be used in the bioremediation of polluted soils (Gómez-Brandón et al., 2019).

Zhang et al. (2023) claim that co-composting wine grape marc, which has a high phenol content, with pig manure can enhance the pig manure's composting process and increase its conversion to humic acid. Another study (Xu et al., 2022) showed that by promoting advantageous interactions between microorganisms, the grape marc effectively extended the thermophilic period and enhanced humification production and compost maturity in the co-composting process with pig manure. The authors believe that using 40% grape marc in the composting mix was the best way to conserve nitrogen, with a 95% germination index as a result. Additionally, Ivanovi et al. (2022) suggests that 40% grape marc is the optimal amount when swine manure and wheat straw are used as co-substrates. Salgado et al. (2019) investigated the grape marc co-composting process with goat and horse manure. They noticed that the compost made from grape pomace and goat or horse manure could be used as organic fertilisers or amendments because it had been proved safe in terms of human pathogens, faecal indicators and phytotoxicity (with more than 90% germination rate in all the treatments). Pérez-Murcia et al. (2021) examined the effects of applying different composts (one made with exhausted grape marc and cattle manure, another with exhausted grape marc and sheep manure) over two different varieties of almonds over the course of a two-year experiment. The results of this study demonstrated that these composts enhanced the biotic activity and nutrient content of the soil, while having minimal effects on the nitrate and salinity levels.

Hungría et al. (2017) studied the co-composting of grape marc and the organic fraction of municipal solid waste (OFMSW) in a 50:50 w/w ratio. They used a pilot scale dynamic respirometer that was operated under aerobic conditions and monitored physico-chemical, respirometric and olfactometric parameters. It has been discovered that combining grape marc with OFMSW neutralises the acidity of grape marc while creating a final composted product that is high in phosphorus and nitrogen, increasing its potential for reuse as an organic fertiliser. When grape marc and OFMSW are composted together, odour emissions are also found to be lower than when OFMSW is composted alone in similar circumstances (Muhlack et al., 2018).

Fernández et al. (2008) investigated the carbon biodegradation of exhausted grape marc in combination with other organic wastes using the turned pile composting system. Four different piles were built (exhausted grape marc, exhausted grape marc mixed with cow manure and straw, exhausted grape marc mixed with municipal solid waste and exhausted grape marc mixed with grape stalks). According to the results, co-composting significantly reduced

the remanent amount of carbon after composting all of the piles while increasing the readily biodegradable carbon fractions from 35% when the exhausted grape marc was composted alone to 50 and 60% when the municipal solid waste or the grape stalks were added. Furthermore, the authors believe that grape stalks are the best option for co-composting with exhausted grape marc because of their availability in the winery, faster carbon biodegradation rates and higher quality of the final compost obtained.

VERMICOMPOSTING THE GRAPE MARC

Vermicomposting, unlike composting, is based on the cooperative action of detritivorous earthworms and microorganisms and does not include a thermophilic phase (Gómez-Brandón et al., 2019). The microorganisms play a role in the biochemical decomposition of organic matter by producing enzymes, whereas earthworms contribute to a greater population of microorganisms by fragmentation and ingestion of fresh organic matter (Vuković et al., 2021). The vermicomposting process involves two distinct phases in terms of earthworm activity: an active phase in which earthworms ingest, process and digest organic matter, thereby changing its physical-chemical and microbial composition and a maturation phase in which earthworms move towards fresher layers of the substrate while microorganisms decompose the earthworm-processed substrate (Gómez-Brandón et al., 2019; Lores et al., 2006). Compared to other waste management techniques, vermicomposting offers several advantages. For instance, it can be done both indoors and outdoors, enabling composting to occur all year round. In addition, this process allows for the production of organic nutrients for crops in less time, which are more nutritionally, physically and biochemically efficient than other composts (Alshehrei & Ameen, 2021; Rodríguez-Canché el al., 2010; Yadav et al., 2010). The final vermicompost quality is determined by a number of variables, including the earthworm species involved, the management techniques used, the length of the conditioning period and the chemical and physical properties of the substrate fed to the earthworms (Santana et al., 2020; Domínguez & Gómez-Brandón, 2013; Bisen et al., 2011).

Gómez-Brandón et al. (2020) investigated the feasibility of using vermicomposting for processing grape marc obtained from the red winemaking of Menca grapes for the purpose to produce a high-quality, polyphenol-free organic vermicompost that might be utilised as an environmentally friendly fertiliser. Their results indicate that the grape marc seems to be a good substrate for earthworm feeding, offering ideal growth and reproduction conditions as well as enough energy to support substantial populations. Additionally, the authors report that during the vermicomposting process, which lasted 112 days, earthworm activity helped to stabilise the grape marc, resulting in a final vermicompost that had a lower polyphenol content and a higher concentration of macro- and micronutrients. At the end of the composting process, lower microbial activity values were noted, which are indicating the stability of the compost.

Using 16S rRNA high-throughput sequencing, Kolbe et al. (2019) reported the first characterization of bacterial succession during white grape marc vermicomposting. To obtain the compost, the authors used a metal pilot-scale vermireactor, located in a greenhouse without any temperature control over the course of 91 days. Prior to adding the grape marc, the earthworms (*Eisenia andrei*) were placed in a 12 cm layer of vermicompost. After, a 12-centimeter layer of fresh grape marc was added to the bed. In order to facilitate earthworm migration and enable grape marc sampling, a plastic mesh was utilised to separate the vermicompost bedding from the fresh grape. This prevented processed grape marc from being mixed with the vermicompost bedding. The results of this study indicate that vermicomposting significantly alters the composition of bacterial communities and increases bacterial diversity, as well as the bacterial community's metabolic capacity or specific metabolic processes such as cellulose metabolism, plant hormone synthesis and antibiotic synthesis.

CONCLUSIONS

Composting is a biological process that converts organic waste into a material that can be used by plants. Grape marc, which is high in lignocellulosic compounds, is a valuable feedstock in green energy and compost production methods. Composting techniques such as aerated static piles, continuous vertical reactors and turned windrows can produce stabilised end products with defined stability and maturity characteristics. Co-composting grape marc with organic byproducts improves the quality of the final compost. Acidity is neutralised and compost dynamics are improved by adding materials such as grape stalks, animal manure or coffee grounds. Olive oil production residues combined with grape marc provide advantages such as low phytotoxicity and disease suppression. Exhausted grape marc combined with cattle or sheep manure can improve the soil biotic activity and nutrient content. Grape marc vermicomposting shows promise as it produces an organic vermicompost with increased nutrient concentration that is free of polyphenols. Overall, wineries can manage waste more efficiently, reduce their environmental impact and encourage the sustainable use of byproducts in a variety of applications by implementing composting and vermicomposting techniques as well as thoughtful co-composting practises.

REFERENCES

Abdoli, M. A., Omrani, G., Safa, M., & Samavat, S. (2019). Comparison between aerated static piles and vermicomposting in producing co-compost from rural organic wastes and cow manure. *Int. J. Environ. Sci. Technol., 16*, 1551-1562.

Ahmad, B., Yadav, V., Yadav, A., Rahman, M. U., Yuan, W. Z., Li, Z., & Wang, X. (2020). Integrated biorefinery approach to valorize winery waste: A review from waste to energy perspectives. *Science of The Total Environment, 719*.

Albrecht, R., Périssol, C., Ruaudel, F., Petit, J. L., & Terrom, G. (2010). Functional changes in culturable microbial communities during a co-composting process: Carbon source utilization and co-metabolism. *Waste Management, 30*(5), 764–770.

Alfonzo, A., Laudicina, V. A., Muscarella, S. M., Badalucco, L., Moschetti, G., Spanò, G. M., & Francesca, N. (2022). Cellulolytic bacteria joined with deproteinized whey decrease carbon to nitrogen ratio and improve stability of compost from wine production chain by-products. *Journal of Environmental Management, 304*, 114194.

Alshehrei, F., & Ameen, F. (2021). Vermicomposting: A management tool to mitigate solid waste. *Saudi Journal of Biological Sciences, 28*(6), 3284-3293.

Andreottola, G., Foladori, P., & Ziglio, G. (2009). Biological treatment of winery wastewater: an overview. *Water Science & Technology, 60*(5), 1117–1125.

Arvanitoyannis, I. S., Ladas, D., & Mavromatis, A. (2006). Wine waste treatment methodology. *International Journal of Food Science and Technology, 41*, 1117-1151.

Aviani, I., Laor, Y., Medina, S., Krassnovsky, A., & Raviv, M. (2010). Co-composting of solid and liquid olive mill wastes: management aspects and the horticultural value of the resulting composts. *Bioresource Technology, 101*(17), 6699-6706.

Baptista, S. L., Romaní, A., Cunha, J. T., & Domingues, L. (2023). Multi-feedstock biorefinery concept: Valorization of winery wastes by engineered yeast. *Journal of Environmental Management, 326*, 116623.

Barros, E. S., de Amorim, M. C., Olszevski, N., & Silva, P. T. (2021). Composting of winery waste and characteristics of the final compost according to Brazilian legislation. *Journal of Environmental Science and Health, Part B, 56*(5), 447-457.

Bazrafshan, E., Zarei, A., Mostafapour, F. K., Poormollae, N., Mahmoodi, S., & Zazouli, M. A. (2016). Maturity and Stability Evaluation of Composted Municipal Solid Wastes. *Health Scope, 5*(1).

Bharathiraja, B., Iyyappan, J., Jayamuthunagai, J., Kumar, R. P., Sirohi, R., Gnansounou, E., & Pandey, A. (2020). Critical review on bioconversion of winery wastes into value-added products. *Industrial Crops and Products, 158*, 112954.

Bisen, J. S., Singh, A. K., Kumar, R., Bora, D. K., & Bera, B. (2011). Vermicompost quality as influenced by different species of earthworm and bedding material. *Two and a Bud, 58*, 137-140.

Bolognesi, S., Cecconet, D., & Capodaglio, A. G. (2020). 5 - Agro-industrial wastewater treatment in microbial fuel cells. *Integrated Microbial Fuel Cells for Wastewater Treatment*, 93-133.

Bustamante, M. A., Paredes, C., Marhuenda-Egea, F. C., Pérez-Espinosa, A., Bernal, M. P., & Moral, R. (2008). Co-composting of distillery wastes with animal manures: Carbon and nitrogen transformations in the evaluation of compost stability. *Chemosphere, 72*(4), 551-557.

Cataldo, E., Fucile, M., Manzi, D., Masini, C. M., Doni, S., & Mattii, G. B. (2023). Sustainable soil management: effects of clinoptilolite and organic compost soil application on eco-physiology, quercitin, and hydroxylated, methoxylated anthocyanins on Vitis vinifera. *Plants, 12*(4), 708.

Chrysargyris, A., Hajisolomou, E., Xylia, P., & Tzortzakis, N. (2023). Olive-mill and grape-mill waste as a substitute growing media component for unexploded vegetables production. *Sustainable Chemistry and Pharmacy, 31*, 100940.

Coelho, L., Reis, M., Guerrero, C., & Dionísio, L. (2021). Biological control of turfgrass diseases with organic composts enriched with Trichoderma atroviride. *Biological Control, 159*, 104620.

Corbin, K. R., Hsieh, Y. S., Betts, N. S., Byrt, C. S., Henderson, M., Stork, J., . . . Burton, R. A. (2015). Grape marc as a source of carbohydrates for bioethanol: Chemical composition, pre-treatment and saccharification. *Bioresource Technology, 193*, 76-83.

Cortés-Camargo, S., Pérez-Rodríguez, N., Oliveira, R. P., Huerta, B. E., & Domínguez, J. M. (2016). Production of biosurfactants from vine-trimming shoots using the halotolerant strain Bacillus tequilensis ZSB10. *Industrial Crops and Products, 79*, 258-266.

Crespo-López, L., Martínez-Ramirez, A., Sebastián, E., & Cultrone, G. (2023). Pomace from the wine industry as an additive in the production of traditional sustainable lightweight eco-bricks. *Applied Clay Science, 243*, 107084.

David, G., Vannini, M., Sisti, L., Marchese, P., Celli, A., Gontard, N., & Angellier-Coussy, H. (2020). Eco-Conversion of Two Winery Lignocellulosic Wastes into Fillers for Biocomposites: Vine Shoots and Wine Pomaces. *Polymers, 12*(7), 1530.

Dávila, I., Gordobil, O., Labidi, J., & Gullón, P. (2016). Assessment of suitability of vine shoots for hemicellulosic oligosaccharides production through aqueous processing. *Bioresource Technology, 211*, 636-644.

Diaz, L., Savage, G., Eggerth, L., & Chiumenti, A. (2007). Systems used in composting. *Waste Management Series, 8*, 67-87.

Diaz, M. J., Madejon, E., Ariza, J., Lopez, R., & Cabrera, F. (2002). Cocomposting of beet vinasse and grape marc in windrows and static pile systems. *Compost science & utilization, 10*(3), 258-269.

Domínguez, J., & Gómez-Brandón, M. (2013). The influence of earthworms on nutrient dynamics during the process of vermicomposting. *Waste Management & Research, 31*(8), 859-868.

Dominguez, J., Edwards, C. A., & Subler, S. (1997). A comparison of vermicomposting and composting. *Biocycle, 38*, 57-59.

Dume, B., Hanc, A., Svehla, P., Michal, P., Chane, A. D., & Nigussie, A. (2023). Composting and vermicomposting of sewage sludge at various C/N ratios: technological feasibility and end-product quality. *Ecotoxicology and Environmental Safety, 263*, 115255.

Eon, P., Deogratias, J.-M., Robert, T., Coriou, C., Bussiere, S., Sappin-Didier, V., . . . Cornu, J.-Y. (2023). Ability of aerated compost tea to increase the mobility and phytoextraction of copper in vineyard soil. *Journal of Environmental Management, 325*, 116560.

Fernández, F. J., Sánchez-Arias, V., Villaseñor, J., & Rodríguez, L. (2008). Evaluation of carbon degradation during co-composting of exhausted grape marc with different biowastes. *Chemosphere, 73*(5), 670-677.

Flores, L., Josa, I., García, J., Pena, R., & Garfí, M. (2023). Constructed wetlands for winery wastewater treatment: A review on the technical, environmental and socio-economic benefits. *Science of The Total Environment, 882*, 163547.

Frîncu, M., Teodorescu, R. I., Bărbulescu, D. I., Tudor, V., Dumitrache, C., Hîrjoabă, L. N., & Matei, F. (2019). The Grape Marc as Potential Source of Biologically Active Compounds. *Scientific Papers. Series B. Horticulture, 63*(2).

Garita-Cambronero, J., Hijosa-Valsero, M., Paniagua-García, A. I., & Díez-Antolínez, R. (2021). Revisiting the production of L(+)-lactic acid from vine shoots: bioconversion improvements by employing thermotolerant bacteria. *Applied Microbiology and Biotechnology, 105*, 9385-9402.

Gómez-Brandón, M., Lores, M., & Domínguez, J. (2023). Recycling and valorization of distilled grape marc through vermicomposting: a pilot-scale study. *J Mater Cycles Waste Manag, 25*, 1509-1518.

Gómez-Brandón, M., Lores, M., Insam, H., & Domínguez, J. (2019). Strategies for recycling and valorization of grape marc. *Critical Reviews in Biotechnology, 39*(4), 437-450.

Gómez-Brandón, M., Lores, M., Martínez-Cordeiro, H., & Domínguez, J. (2020). Effectiveness of vermicomposting for bioconversion of grape marc derived from red winemaking into a value-added product. *Environmental Science and Pollution Research, 27*, 33438-33445.

Hungría, J., Gutiérrez, M., Siles, J., & Martín, M. (2017). Advantages and drawbacks of OFMSW and winery waste co-composting at pilot scale. *Journal of Cleaner Production, 164*, 1050-1057.

Ivanović, M., Stefanović, G., & Savić, D. (2022). Potentials of Grape Pomace for Compost Production on the Territory of the Three Morava Tegion: A case study. *Facta Universitatis, Series: Working and Living Environmental Protection*, 085-099.

Jesus, M. S., Romaní, A., Genisheva, Z., Teixeira, J. A., & Domingues, L. (2017). Integral valorization of vine pruning residue by sequential autohydrolysis stages. *Journal of Cleaner Production, 168*, 74-86.

Karapantzou, I., Mitropoulou, G., Prapa, I., Papanikolaou, D., Charovas, V., & Kourkoutas, Y. (2023). Physicochemical Changes and Microbiome Associations during Vermicomposting of Winery Waste. *Sustainability, 15*(9), 7484.

Kessler, S., Kanthak, S., & Zimmer, S. (2021). Co2Mpositiv-Optimization Of Organic Materials Cycles Of Viticulture In Luxembourg.

Kolbe, A. R., Aira, M., Gómez-Brandón, M., Pérez-Losada, M., & Domínguez, J. (2019). Bacterial succession and functional diversity during vermicomposting of the white grape marc Vitis vinifera v. Albariño. *Scientific Reports, 9*, 7472.

Lores, M., Gómez-Brandón, M., Pérez-Díaz, D., & Domínguez, J. (2006). Using FAME profiles for the characterization of animal wastes and vermicomposts. *Soil Biology and Biochemistry, 38*(9), 2993-2996.

Lucas, M. S., Peres, J. A., Lan, B. Y., & Puma, G. L. (2009). Ozonation kinetics of winery wastewater in a

pilot-scale bubble column reactor. *Water Research, 43*(6), 1523-1532.

Majbar, Z., Lahlou, K., Ben Abbou, M., Ammar, E., Triki, A., Abid, W., . . . Rais, Z. (2018). Co-composting of olive mill waste and wine-processing waste: an application of compost as soil amendment. *Journal of Chemistry, 2018*.

Majbar, Z., Rais, Z., Haji, M. E., Abbou, M. B., Bouka, H., & Nawdali, M. (2017). Olive mill wastewater and wine by-products valorization by co-composting. *Journal of Materials and Environmental Sciences, 8*(9), 3162-3167.

Makan, A., & Fadili, A. (2020). Sustainability assessment of large-scale composting technologies using PROMETHEE method. *Journal of Cleaner Production, 261*, 121244.

Martínez, M., Ortega, R., Janssens, M., Angulo, J., & Fincheira, P. (2016). Selection of maturity indices for compost derived from grape pomace. *J. Soil Sci. Plant Nutr., 16*(2), 262-267.

Masi, F., Rochereau, J., Troesch, S., Ruiz, I., & Soto, M. (2015). Wineries wastewater treatment by constructed wetlands: a review. *Water Sci. Technol., 71*(8), 1113-1127.

Meena, A. L., Karwa, M., Dutta, D., & Mishra, R. (2021). Composting: Phases and Factors Responsible for Efficient and Improved Composting. *Agriculture and Food: e-Newsletter, 3*(1), 85-90.

Michel, F., O'Neill, T., Rynk, R., Gilbert, J., Wisbaum, S., & Halbach, T. (2022). Chapter 5 - Passively aerated composting methods, including turned windrows. *The Composting Handbook*, 159-196.

Muhlack, R. A., Potumarthi, R., & Jeffery, D. W. (2018). Sustainable wineries through waste valorisation: A review of grape marc utilisation for value-added products. *Waste Management, 72*, 99-118.

Mulidzi, A. R. (2021). Evaluating Sustainable Use and Management of Winery Solid Wastes through Composting. *South African Journal of Enology and Viticulture, 42*(2), 193-200.

Musee, N., Lorenzen, L., & Aldrich, C. (2005). Decision support for waste minimization in wine-making processes. *Environmental Progress, 25*(1), 56-63.

Musee, N., Lorenzen, L., & Aldrich, C. (2007). Cellar waste minimization in the wine industry: a systems approach. *Journal of Cleaner Production, 15*(5), 417-431.

Noble, R., & Gaze, R. H. (1997). Composting in aerated tunnels for mushroom cultivation: Influences of process temperature and substrate formulation on compost bulk density and productivity. *International Symposium on Composting & Use of Composted Material in Horticulture, 469*, 417-426.

Oliveira, M., & Duarte, E. (2010). Guidelines for the management of winery wastewaters. *Treatment and Use of Non-conventional Organic Residues in Agriculture*, 148-151.

Oliveira, M., & Duarte, E. (2016). Integrated approach to winery waste: waste generation and data consolidation. *Front. Environ. Sci. Eng., 10*(1), 168-176.

Pachón, E. R., Mandade, P., & Gnansounou, E. (2020). Conversion of vine shoots into bioethanol and chemicals: Prospective LCA of biorefinery concept. *Bioresource Technology, 303*, 122946.

Paplomatas, E., Malandrakis, A., & Nektarios, P. (2004). Compost Management of Brown Patch Disease in Turfgrass. *Acta Horticulturae, 661*, 487-489.

Paraskevopoulou, A. T., Zafeiriou, S., & Londra, P. A. (2021). Plant growth of Atriplex portulacoides affected by irrigation amount and substrate type in an extensive green roof system. *Ecological Engineering, 165*, 106223.

Pérez-Murcia, M. D., Bustamante, M. Á., Orden, L., Rubio, R., Agulló, E., Carbonell-Barrachina, Á. A., & Moral, R. (2021). Use of agri-food composts in almond organic production: Effects on soil and fruit quality. *Agronomy, 11*(3), 536.

Pérez-Rodríguez, N., García-Bernet, D., & Domínguez, J. (2016). Effects of enzymatic hydrolysis and ultrasounds pretreatments on corn cob and vine trimming shoots for biogas production. *Bioresource Technology, 221*, 130-138.

Perra, M., Bacchetta, G., Muntoni, A., Gioannis, G. D., Castangia, I., Rajha, H. N., . . . Manconi, M. (2022). An outlook on modern and sustainable approaches to the management of grape pomace by integrating green processes, biotechnologies and advanced biomedical approaches. *Journal of Functional Foods, 98*, 150276.

Pinter, I. F., Fernández, A. S., Martínez, L. E., Riera, N., Fernández, M., Aguado, G. D., & Uliarte, E. M. (2019). Exhausted grape marc and organic residues composting with polyethylene cover: Process and quality evaluation as plant substrate. *Journal of Environmental Management, 246*, 695-705.

Pinto, R., Brito, L. M., Mourão, I., Tavares, T., Sá, F., Gonçalves, F., . . . Coutinho, J. (2021). Effect of winery waste composted with biochar on seedling and plant growth of organic Brassica spp. crops. *III International Organic Fruit Symposium and I International Organic Vegetable Symposium, 1354*, 285-292.

Pinto, R., Correia, C., Mourão, I., Moura, L., & Brito, L. M. (2023). Composting Waste from the White Wine Industry. *Sustainability, 15*(4), 3454.

Rajha, H. N., Boussetta, N., Louka, N., Maroun, R. G., & Vorobiev, E. (2014). A comparative study of physical pretreatments for the extraction of polyphenols and proteins from vine shoots. *Food Research International, 65*, 462-468.

Rastogi, M., Nandal, M., & Khosla, B. (2020). Microbes as vital additives for solid waste composting. *Heliyon, 6*(2).

Rivas, B., Torrado, A., Rivas, S., Moldes, A. B., & Domínguez, J. M. (2007). Simultaneous lactic acid and xylitol production from vine trimming wastes. *Journal of the Science of Food and Agriculture, 87*(8), 1603-1612.

Rodríguez-Canché, L., Vigueros, L. C., Maldonado-Montiel, T., & Martínez-Sanmiguel, M. (2010). Pathogen reduction in septic tank sludge through vermicomposting using Eisenia fetida. *Bioresource Technology, 101*(10), 3548-3553.

Salgado, M. M., Blu, R. O., Janssens, M., & Fincheira, P. (2019). Grape pomace compost as a source of organic

matter: Evolution of quality parameters to evaluate maturity and stability. *Journal of Cleaner Production, 216*, 56-63.

Sanchez-Hernandez, J. C., & Domínguez, J. (2017). Vermicompost derived from spent coffee grounds: assessing the potential for enzymatic bioremediation. *Handbook of Coffee Processing By-Products*, 369-398.

Santana, N. A., Jacques, R. J., Antoniolli, Z. I., Martínez-Cordeiro, H., & Domínguez, J. (2020). Changes in the chemical and biological characteristics of grape marc vermicompost during a two-year production period. *Applied Soil Ecology, 154*, 103587.

Tassoula, L., Papafotiou, M., Liakopoulos, G., & Kargas, G. (2021). Water use efficiency, growth and anatomic-physiological parameters of Mediterranean xerophytes as affected by substrate and irrigation on a green roof. *Notulae Botanicae Horti Agrobotanici Cluj-Napoca, 49*(2), 12283-12283.

Vigneswaran, S., Kandasamy, J., & Johir, M. (2016). Sustainable Operation of Composting in Solid Waste Management. *Procedia Environmental Sciences, 35*, 408-415.

Vlyssides, A., Barampouti, E., & Mai, S. (2005). Wastewater characteristics from Greek wineries and distilleries. *Water Sci Technol, 51*(1), 53-60.

Vuković, A., Velki, M., Ečimović, S., R., V., Štolfa Čamagajevac, I., & Lončarić, Z. (2021). Vermicomposting—Facts, Benefits and Knowledge Gaps. *Agronomy, 11*(10), 1952.

Wichuk, K., & McCartney, D. (2010). Compost stability and maturity evaluation- a literature review. *Can. J. Civ. Eng., 37*(11), 1505-1523.

Xu, Y., Bi, Z., Zhang, Y., Wu, H., Zhou, L., & Zhang, H. (2022). Impact of wine grape pomace on humification performance and microbial dynamics during pig manure composting. *Bioresource Technology, 358*, 127380.

Yadav, K. D., Tare, V., & Ahammed, M. M. (2010). Vermicomposting of source-separated human faeces for nutrient recycling. *Waste Management, 30*(1), 50-56.

Zhang, L., & Sun, X. (2016). Improving green waste composting by addition of sugarcane bagasse and exhausted grape marc. *Bioresource Technology, 218*, 335-343.

Zhang, Y., Liu, L., Huang, G., Yang, C., Tian, W., Ge, Z., . . . Zhang, H. (2023). Enhancing humification and microbial interactions during co-composting of pig manure and wine grape pomace: The role of biochar and Fe2O3. *Bioresource Technology*, 130120.

Scientific Bulletin. Series F. Biotechnologies, Vol. XXVII, No. 2, 2023
ISSN 2285-1364, CD-ROM ISSN 2285-5521, ISSN Online 2285-1372, ISSN-L 2285-1364

IN VITRO ANTIMICROBIAL ACTIVITY OF DIFFERENT ESSENTIAL OILS FROM *Lavandula* sp.

Ionuț Georgică MARCHIDAN, Elisabeta Elena POPA, Mihaela GEICU-CRISTEA, Oana-Alina BOIU-SICUIA, Nicoleta RADU, Narcisa Elena BĂBEANU

University of Agronomic Sciences and Veterinary Medicine of Bucharest, 59 Marasti Blvd, District 1, Bucharest, Romania

Corresponding author emails: elena.eli.tanase@gmail.com, mihaela_geicu@yahoo.com

Abstract

The objective of this work was to highlight the antimicrobial properties of lavender essential oil (EO) obtained from the George 90 cultivar, which is the newest lavender cultivar approved in Romania for oil production. In order to highlight its antimicrobial properties, the essential oil obtained from the George 90 cultivar was tested in comparison with two other essential oils (EOs) obtained from the species Lavandula latifolia and Lavandula angustifolia, these two lavender species being the parents of the new George 90 cultivar. Three food pathogenic fungi (Aspergillus brasieliensis ATCC 16404*, Fusarium oxysporum and Penicillium expansum), two food spoilage bacteria (Bacillus cereus and Bacillus subtilis) and six species of phytopathogenic molds (Alternaria sp., Botrytis cinerea, Fusarium culmorum FC46, Fusarium oxysporum f. sp. radicis lycopersici ZUM2407 (FORL), Macrophomina phaseolina and Sclerotinia sclerotiorum) were used in this study. In all the interactions with phytopathogenic agents and bacteria studied, a close relationship was observed between the antifungal potential and the dose of essential oils tested. Thus, as expected, increasing the dose of lavender essential oil is directly proportional to the inhibition effectiveness of the tested microorganisms.*

Key words*: antimicrobial properties, essential oil, new lavender cultivar.*

INTRODUCTION

Lavender is part of the Laminaceae family, Lamiales order, it originates in SW-SE Europe, being a thermophilic subshrub that grows in the form of a 40-1.60 cm tall bush, with a diameter of 100-150 cm. The leaves are persistent, linear, 2-6 cm long and have a silver-gray color in the cold weather and silver-green in the hot weather. In the months of May-June-July, depending on the climate, it emits flower stalks of 40-60 cm, finished with spikes of bluish-violet flowers of ± 8-10 cm, very pleasantly scented.

The essential oils obtained from *Lavandula* × *intermedia*, which result from natural crosses between *Lavandula latifolia* and *Lavandula angustifolia* species, contain a mixture of monoterpenes present in both parental lines, and are mainly utilized in personal care and hygiene products including soaps, shampoos, mouth washes, and industrial and household cleaners, among others (Sarker et al., 2012).

Essential oil obtained from *L.* × *intermedia* has been widely investigated and is known to vary in composition (Balajan and Pirbalouti, 2015), but according to our knowledge, no documented reports on variation of chemical composition of the essential oils from different populations of *L.* × *intermedia* leaves and flower cultivated in Romania are available.

The George 90 cultivar comes from a foreign lavender variety, which was acclimatized in Romania, being registered in 2017 in the official Catalog of the State Institute for the Testing and Registration of Varieties, within the Ministry of Agriculture and Rural Development in Romania. Regarding the phenological observations and biometric determinations specific to the lavender culture, the characteristics of the cultivar George 90 are highlighted in comparison with *L. latifolia* and *L. angustifolia,* these two lavender species being considered its parents. The George 90 lavender cultivar presents the following characteristics, which result from the technical examination report:

- plant growth type is stretched;
- the plant size is very large;
- the intensity of the green color of the foliage is medium;

- the gray intensity of the foliage is low;
- the bearing of the external flower stems, at full bloom, is spread out;
- density at full bloom is medium;
- the length of the flowering stalk, including the spike is very long;
- intensity of the green color of the stalk is medium;
- side branches, above the foliage, at the stem level are present;
- the number of side branches, at the stem level is small;
- the length of the largest branch, including the spike is very long;
- the spike has a cylindrical shape, with a large number of flowers and vertices, many flowers on the terminal verticil and a very long distance between vertices;
- regarding the flower, it can be observed that the calyx color is greenish, the calyx pubescence is strong represented and the corolla color is purple.

There are various studies that shows that lavender essential oils present antibacterial (Danh et al., 2012; Gismondi et al., 2021; Jianu et al., 2013; Man et al., 2019; Mesic et al., 2021; Valkova et al., 2021) and antifungal activity (Behmanesh et al., 2015; Císarová et al., 2016; Dhaouadi et al., 2018; Erdogan et al., 2016; Schroder et al., 2018; Zuzarte et al., 2012). For example, a study conducted by Ciocarlan et al. (2021) showed that *L. angustifolia* L. essential oil presented good antibacterial activity at 300 µg/mL concentration against *Erwinia carotovora*, *Bacillus subtilis*, *Xanthomonas campestris*, *Pseudomonas fluorescens*, and at 150 µg/mL concentration against *Candida utilis* and *Erwinia amylovora*. Another study conducted by Badr et al. (2021) determined the antimicrobial activity of *Lavandula spica* essential oil on different microorganisms (Gram-positive and Gram-negative bacteria, fungi and yeast), and the results showed a minimum inhibitory concentration (MIC, mg/L) of 3150 against *Salmonella typhimurium* and 3000 against *Staphylococcus aureus*. Regarding *Candida albicans*, lavender essential oil presented EC_{50} (mg/L) of 561.26. The *in vitro* antifungal activity was conducted using *Aspergillus flavus* and *Aspergillus niger* fungi and the results showed values of EC_{50} of 1265.38 and 1492.93 respectively (Badr et al., 2021). Jeddi et al. (2023) also studied the antimicrobial activity of *L. angustifolia* essential oil. Therefore, several microorganisms were tested (*Micrococcus luteus*, *Bacillus cereus*, *Salmonella enterica*, *S. aureus*, *Klebsiella aerogenes*, *Escherichia coli*, *Candida tropicalis* and *C. albicans*) and the results showed good activity of the tested essential oil, with inhibition zones varying between 19.1 to 13.4 for bacteria and between 24.10 and 19.05 for fungi. Essential oil extracted from *L. latifolia* L. was tested for its antimicrobial activity against five phytopathogenic fungi by Al-Ansari et al. (2021). The results showed that the essential oil presented inhibitory activity, with MIC (µg/mL) values of 2.5 for *A. flavus*, >10 for *Aspergillus nidulans*, 0.125 for *Trichophyton mentagrophytes*, 5 for *Leptosphaeria maculans*, 2.5 for *Rhizoctonia solani* and >10 for *Fusarium oxysporum*. Antimicrobial activity of essential oil obtained from flowers and leaves of *Lavandula officinalis* was determined by Martucci et al. (2015). The results of their study showed a MIC (µg/mL) of 2000 on *E. coli* and of 1000-1200 on *S. aureus*. Sumalan et al. (2020) determined the antifungal activity of lavender essential oil on *Penicillium digitatum*, with results showing a minimum fungicidal dose of 350 µl.

MATERIALS AND METHODS

Materials

The tested essential oils were obtained by hydrodistillation from *L. latifolia* (LL), *L. angustifolia* (LA) and *L.* × *intermedia* George 90 cultivar (GE).

Lavender EOs exhibits high volatility at ambient temperature as well as a good solubility in organic solvents and alcohol. EOs can also be entrained by water vapor despite the fact that they have low solubility in water. The lavender EOs used in this scientific work were transparent and had a strong smell and flavor. Two approaches have been used during the experiments. The studied essential oils were tested in terms of antimicrobial activity on both food pathogenic strains (*Aspergillus brasieliensis* ATCC 16404, *Fusarium oxysporum*, *Penicillium expansum*, *Bacillus subtilis*, *Bacillus cereus*) and phytopathogenic

strains (*Alternaria* sp., *Botrytis cinerea*, *Fusarium culmorum* FC46, *Fusarium oxysporum* f. sp. *radicis lycopersici* (FORL) ZUM2407, *Macrophomina phaseolina*, *Sclerotinia sclerotiorum*).

Methods

A GC–MS/MS TRIPLE QUAD Agilent 7890 A equipment (Santa Clara CA, USA) was used in the analysis of the essential oil's chemical composition according to the method presented by Schroder et al. (2022).

In order to determine the antifungal activity of the food pathogenic strains, the fungi were cultivated on Potato Dextrose Agar (purchased from Scharlau) for 7 days at 25°C, and then they were used in the experiments as a form of spore suspension, which was obtained in aseptic conditions, having a concentration of 10^6 spores/ml. The working method used was the disc diffusion method. Briefly, on solidified culture media, filter paper discs (Φ = 6 mm) were placed and essential oil was distributed in quantities varying between 5 and 10 µl/disc in order to achieve the specific testing quantity per plate (20, 25, 30, 35, 40, 45 µl). The plate was then inoculated in the center with 2 µl of spore suspension. The plates thus obtained were incubated at 25°C for 7 days, and the colony growth determination was realized by measuring the colony diameters at the end of the incubation period. The degree of inhibition was expressed as a percentage and calculated using the formula:

$$Inhibition\ degree, \% = \frac{A_c - A_i}{A_c} \times 100$$

where A_c represents the average for Control colonies and A_i represents the average for sample colonies.

For antibacterial activity determination, the used bacterial strains were cultivated on Nutrient Agar (purchased from Scharlau) culture media for 24 h at 35°C and then used. Briefly, on the solidified culture media 100 µl of bacterial suspension was distributed using a Drigalski spatula, and the plates were left at rest for 30 minutes to facilitate the microorganism incorporation. Filter paper discs (Φ = 6 mm) were then applied, and the essential oil quantity was distributed equally on the discs, varying between 2.5 and 10 µl/disc, the function of the necessary quantity for testing. The plates were then incubated for 24h at 35ºC, and the antibacterial activity was evaluated by assessment of the zone of inhibition in the Petri dish.

The lavender EOs were also analyzed for their antifungal activity against phytopathogenic fungi. Potato-Glucose-Agar medium (purchased from Roth) was used, for refreshing the phytopathogenic cultures and evaluation of lavender oils' antifungal activity. For the antifungal evaluation, test plates were prepared by inoculating the fungi one cm away from the edge of the Petri, using mycelial plugs of 6 mm in diameter. Diametrically opposite, 1 cm away from the edge of the Petri plates, one well was made in each plate, using a sterile corkborer, in which different doses (20, 30, 40 µl) of lavender EO were introduced. Control plates were similarly prepared, for each phytopathogenic fungi. However, no EOs were added in the wells of the control plates. Both test and control plates were incubated at 25°C. At different incubation times, after 5, 7, and 12 days respectively, biometric measurements were made to determine the mycelial growth in the test plates, and control cultures. The antifungal activity of the EOs was quantified as efficacy (E%) to inhibit or limit the growth of the studied phytopathogens. The following formula was used for the calculation:

$$E\% = \frac{R_C - R_I}{R_C} \times 100$$

Where R_C represents the radius of the mycelial colony in the Control plate, and R_I represents the radius of the mycelial colony, grown in the test plate, towards the essential oil.

RESULTS AND DISCUSSIONS

Morphological characteristics

Lavender is a thermophilic species, sensitive to excess water, therefore demanding of heat, loving direct sunlight, warm, stony, and calcareous soils but rich in macroelements. In our country, thanks to the soils rich in macro-microelements and minerals, lavender has adapted perfectly even to the frosty weather conditions in winter.

Following the phenological observations and biometric determinations, the characteristics of the cultivar George 90 were highlighted in comparison with its parents, namely *L. latifolia* and *L. angustifolia,* and the results are shown in Table 1, expressed as the average of the 5-year measurements (respectively 2017, 2018, 2019, 2020 and 2021).

Table 1. Characteristics of tested lavender varieties (average of 5 years)

Phenological characteristics determined	*Lavandula latifolia*	*Lavandula angustifolia*	George 90 lavender cultivar
Plant height (cm)	131	75	129
Inflorescence length (cm)	12.5	10	12
Flower stem length (cm)	69	43	70
The number of vertices on the floral spike (cm)	84	70	88
Plant diameter in the 5th year of cultivation (cm)	131	75	129
Variety/Production of inflorescences/ha (kg)	15730	6435	18590
Variety/oil content %/ ha	169.8	49	324.8

Lavender has an important ornamental interest, being very loved for the color and fragrance of the flowers, but at the same time appreciated for the persistence of the foliage. The ornamental lavender has many cultivars, which have special ornamental characteristics: richer flowering, flowers more colorful or with a different color than the type species - red, white, and dark blue. Figure 1 shows the appearance of a flowering lavender field from the George 90 lavender cultivar.

Figure 1. The appearance of flowering lavender field belonging to the George 90 cultivar

Chemical composition of the essential oils

Following a study on *L.* × *intermedia* var. George 90 essential oil that was obtained from a lavender plantation in Suceava county, Romania, the chemical composition and the proportion of their compounds were analyzed by Gas Chromatography-Mass Spectrometry (GC–MS), and the major identified compounds are shown in Table 2.

Table 2. Chemical composition of the essential oils

Major Components	*Lavandula angustifolia*	*Lavandula latifolia*	George 90 cultivar
Eucalyptol (%)	14.49	18.04	15.62
Camphor (%)	13.33	11.43	11.49
B-Linalool (%)	31.59	28.39	31.95
P-Menth-1-en-4-ol (%)	6.92	8.63	9.32

It can be observed that eucalyptol, camphor, and B-linalool are the three major components of the studied essential oils. The highest percent of eucalyptol was obtained from *L. latifolia* at 18.04%, while the highest content of camphor was obtained for *L. angustifolia* at 13.88 %. Shunying et al. (2005) studied the chemical composition and antimicrobial activity of the essential oils obtained from *Chrysanthemum indicum*, which showed greater bacteriostatic activity due to the higher percentage of camphor identified in the oil obtained from the processed flowers.

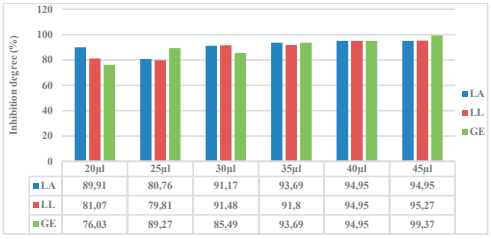
Linalool is an unsaturated monoterpene alcohol with the specific odor description; “light and refreshing, floral-woody, with a faint citrusy note” (Kamatou and Viljoen, 2008). The highest content in B-linalool was found George 90 cultivar with 31.95%. Linalool is also the principal component of many essential oils known to exhibit several biological activities such as antibacterial and antiplasmodial effects (van Zyl et al., 2006).

The p-Menth-1-en-4-ol, also known as terpinen-4-ol or 1-para-menthen-4-ol, belongs to the class of organic compounds known as menthane monoterpenoids is one of the major compounds identified in the studied lavender EOs, which is present in highest quantity in George 90 cultivar, respectively 9.32%.

Antifungal activity against food pathogenic fungi

The amounts of lavender oil tested showed an inhibitory effect on the growth of *Fusarium oxysporum* (Figure 2).

At a concentration of 20 µl of lavender oil/Petri plate, it is observed that all three oils inhibit the growth of the fungus, the strongest degree of inhibition being manifested by LA (89.91%), followed by LL (81.07%) and GE (76.03%).



	20µl	25µl	30µl	35µl	40µl	45µl
LA	89,91	80,76	91,17	93,69	94,95	94,95
LL	81,07	79,81	91,48	91,8	94,95	95,27
GE	76,03	89,27	85,49	93,69	94,95	99,37

Figure 2. Inhibition degree of tested essential oils against *Fusarium oxysporum*

There is a tendency in increasing the degree of inhibition with the increase in the amount of tested lavender oil. When using 40 µl of lavender oil per plate, the degree of inhibition is of 94.95% for all tested lavender oil (Figure 1), while at a quantity of 45 µl per plate, the highest inhibition degree was obtained for the GE essential oil. The aspect of the plates inoculated with the fungus *Fusarium oxysporum* in the presence of the tested essential oils, at the end of the incubation period is presented in Figure 3.

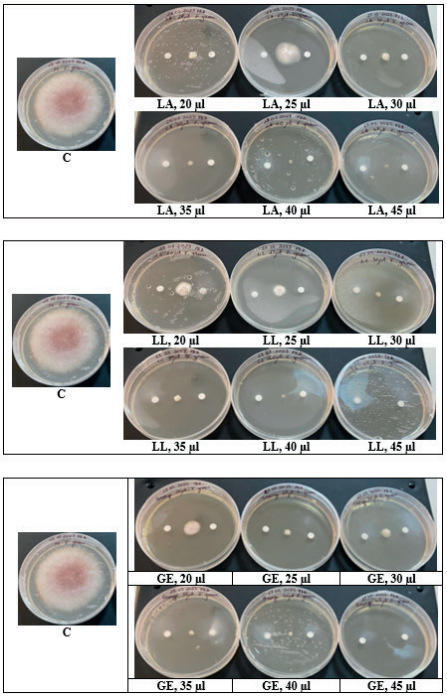
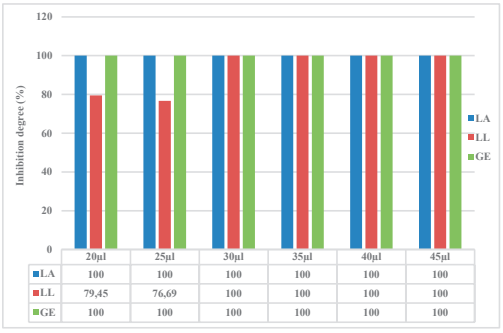


Figure 3. Appearance of plates inoculated with the fungus *Fusarium oxysporum* in the presence of the tested essential oils

Figure 4 shows the fact that LA, as well as GE samples, had a strong antifungal effect on *Aspergillus brasiliensis* ATCC 16404 fungus, showing a degree of inhibition of 100% for tested quantities. Regarding the antifungal potential of LL essential oil, it can be observed that for 20 µl and 25 µl per plate, the degree of inhibition varies in the range of 79.45-76.69%, while for higher quantities, the degree of inhibition was of 100%.



	20µl	25µl	30µl	35µl	40µl	45µl
LA	100	100	100	100	100	100
LL	79,45	76,69	100	100	100	100
GE	100	100	100	100	100	100

Figure 4. Inhibition degree of tested essential oils against *Aspergillus brasiliensis* ATCC 16404

In Figure 5, the appearance of plates inoculated with the *Aspergillus brasiliensis* ATCC 16404 fungus in the presence of the tested essential oils, at the end of the incubation period, can be seen.

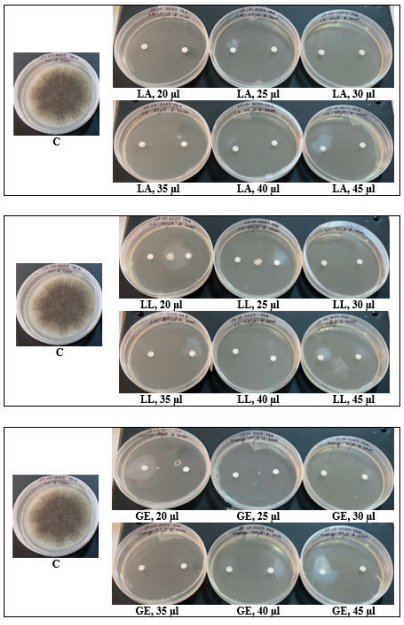
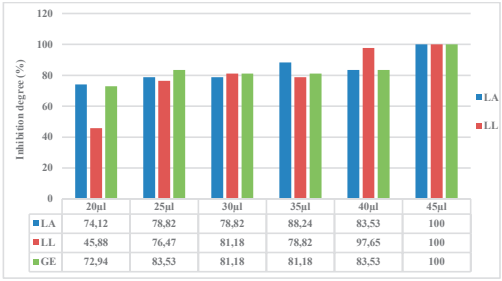


Figure 5. Appearance of plates inoculated with the fungus *Aspergillus brasiliensis* ATCC 16404 in the presence of the tested essential oils

In the case of the *Penicillium expansum* fungi, it was observed that the degree of inhibition is maximum (100%) when 45 µl of essential oil was applied, for all tested essential oils, which denotes a higher natural resistance of this fungus (Figure 6).



	20µl	25µl	30µl	35µl	40µl	45µl
LA	74,12	78,82	78,82	88,24	83,53	100
LL	45,88	76,47	81,18	78,82	97,65	100
GE	72,94	83,53	81,18	81,18	83,53	100

Figure 6. Inhibition degree of tested essential oils against *Penicillium expansum*

In Figure 7, the appearance of the plates inoculated with the *Penicillium expansum* fungus in the presence of the tested essential oils, at the end of the incubation period, can be seen.

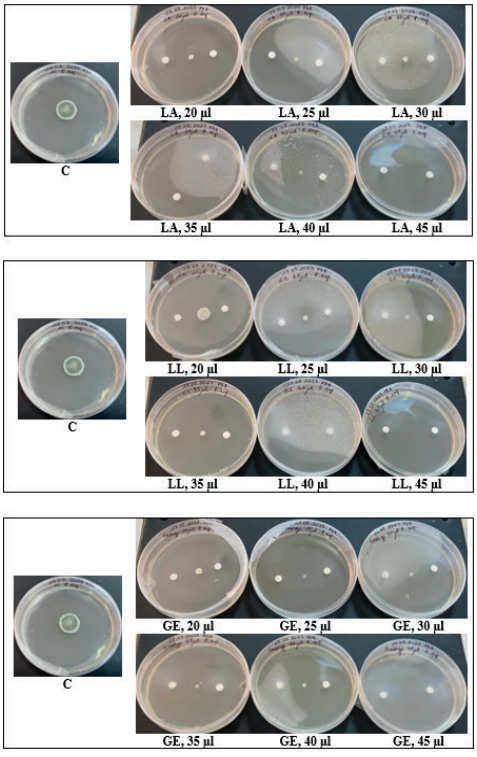


Figure 7. Appearance of plates inoculated with the fungus *Penicillium expansum* in the presence of the tested essential oils

Antibacterial activity against food pathogenic bacteria

Regarding the antibacterial activity of the studied essential oils, several observations were made during the experiments. It was shown that all tested samples showed an antibacterial effect when 2.5 µl of essential oil was applied in the presence of *Bacillus cereus* strain but not on *Bacillus subtilis*. The antibacterial effect on the two *Bacillus* strains tested increased as the amount of lavender essential oil was increased, and the strongest antibacterial effect was shown by LL samples, the diameters of inhibition zones measured being clearly larger than those recorded for LA and GE essential oils. The results are presented in Table 3 and the aspect of the plates inoculated with the tested bacterial strains in the presence of the studied essential oils is presented in Figure 8.

Tabel 3. Antibacterial activity of the tested essential oils

Bacterial strain	Essential oil quantity	Diameter of inhibition zone (cm)		
		LA	LL	GE
Bacillus cereus	2.5 µl	0.65	1.10	0.73
	5 µl	0.83	1.35	1.03
	7.5 µl	1.43	1.63	1.35
	10 µl	1.63	1.80	1.50
Bacillus subtilis	2.5 µl	0	0	0
	5 µl	1.25	0	1.03
	7.5 µl	2.43	2.40	1.60
	10 µl	3.60	4.08	3.68

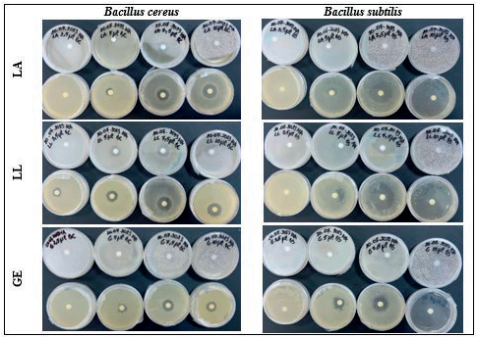


Figure 8. The appearance of plates inoculated with the two bacteria under study in the presence of the tested essential oils

Antifungal activity against phytopathogenic fungi

Regarding the antifungal activity against phytopathogenic fungi, a first visual analysis was made after 3 days of incubation. No microbial growth was observed in the test plates and the fungal radius in the Control plates was also

reduced. Therefore, incubation was continued at 25°C, and the first biometric measurements were collected after 5 days of incubation. Studies have shown the potential of lavender essential oil to inhibit phytopathogenic fungi, regardless of the lavender species from which the oil was extracted (Table 4).

Table 4. Biometry of phytopathogenic fungi grown in the presence of lavender extracts after 5 days of incubation at 25°C

Variant	Dose of EOs	Alt.	B.c.	*F.c.*	*FORL*	*M.p.*	*S.s.*
		Colony radius (mm)					
Control	-	17	58	31	30	60	55
LA	20 µl	3	0	0.5	5	7	5
	30 µl	0.5	0	0	2	2	1
	40 µl	0	0	0	1.5	0.5	0
LL	20 µl	0	1	0	8	4	2
	30 µl	0	0.5	0	3	3	0
	40 µl	0	0	0	2	2	0
GE	20 µl	0	0	0	3	5	0
	30 µl	0	0	0	2	1	0
	40 µl	0	0	0	1.5	0	0

Legend: LA = *Lavandula angustifolia* essential oil, LL = *Lavandula latifolia* essential oil, GE = essential oil of lavender George 90 cultivar, EOs = essential oils, Alt. = *Alternaria* sp., B.c. = *Botrytis cinerea*, F.c. = *Fusarium culmorum* FC46, FORL = *Fusarium oxysporum* f.sp. *radicis lycopersici* ZUM2407, M.p. = *Macrophomina phaseolina*, S.s. = *Sclerotinia sclerotiorum*.

After 7 days of incubation, the antifungal efficacy of the EOs was calculated. The inhibition of the *Alternaria* sp. phytopathogen was maintained in the first 7 days of incubation in the presence of lavender EOs, however after prolonged incubation, up to 12 days, the inhibitory efficacy was reduced (Figure 9).

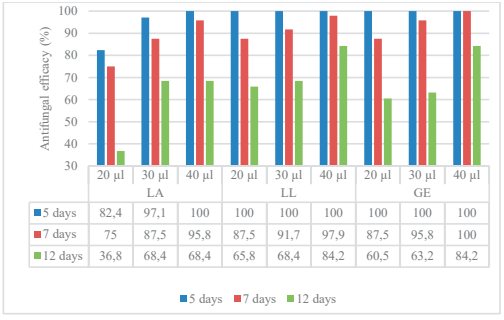


Figure 9. *In vitro* antifungal efficacy of the lavender EOs against *Alternaria*, after 5 to 12 days of incubation

The best results were obtained when using the EO of lavender cultivar George 90. All tested doses of GE completely inhibited the growth of the phytopathogen in the first 5 days of incubation. After 7 days, only the highest tested dose was able to maintain a complete fungal growth inhibition, while after 12 days of incubation, the efficacy of *Alternaria* sp. growth inhibition was reduced to 84.2%, when 40 µl dose of GE was used (Figure 10).

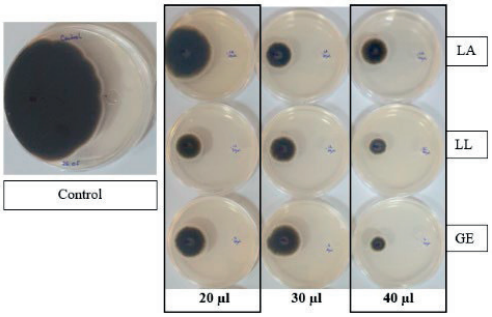


Figure 10. Inhibitory activity of lavender EOs against *Alternaria* sp. phytopathogen, after 12 days of incubation at 25°C

Against the *Botrytis cinerea* phytopathogen, the best results were also obtained when using GE essential oil (Figure 11). The EO of lavender cultivar George 90 completely inhibited the growth of grey mold during the 12 days of incubation at 25°C, for all tested doses (Figure 11). The *L. angustifolia* EO (LA) completely inhibited *B. cinerea* growth during the first 7 days of incubation, at all doses (20-40 µl). After 12 days, the complete inhibition was maintained only when using the 30 and 40 µl EO/plate, while at the lower dose (20 µl LA/plate), the antifungal efficacy was reduced to 99.2% compared to the Control culture (Figure 12). The *L. latifolia* EO (LL) at the highest tested dose (40 µl/plate) showed complete inhibition of *B. cinerea* fungal growth during the first 12 days of incubation. However, lower dose of LL against this mold is fungistatic. The lower doses of LL, 20 and 30 µl EO/plate, revealed an antifungal efficacy of 98.3% and 99.2% respectively after 7 days of incubation. The inhibitory activity decreased with time, and after 12 days of incubation the efficacy was reduced to 94.2% at the dose of 20 µl EO/plate, and at 98.3% when 30 µl EO/plate were used. However, the highest dose of 40 µl EO/plate completely inhibited the growth of *B. cinerea* (Figure 13).

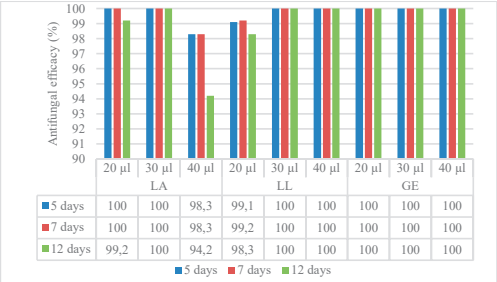


Figure 11. *In vitro* antifungal efficacy of lavender EOs against *Botrytis cinerea* phytopathogen

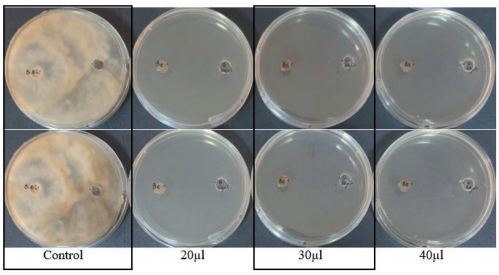


Figure 12. The inhibitory activity of the GE essential oil against *Botrytis cinerea*, after 7 days (upper line) and 12 days (bottom line) of incubation at 25°C

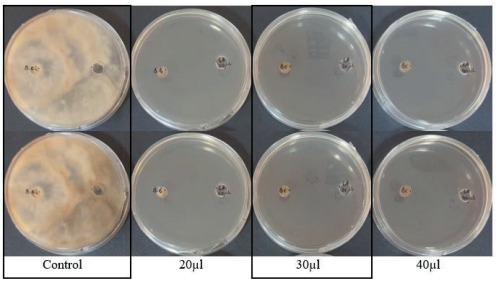


Figure 13. The inhibitory activity of the LA essential oil against *Botrytis cinerea*, after 7 days (upper line) and 12 days (bottom line) of incubation at 25°C

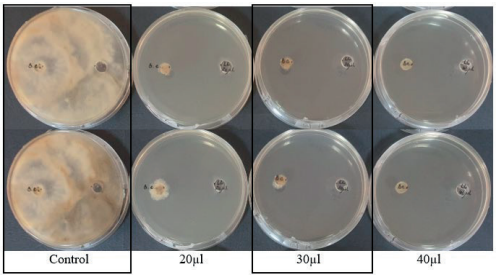


Figure 14. The inhibitory activity of the LL essential oil against *Botrytis cinerea*, after 7 days (upper line) and 12 days (bottom line) of incubation at 25°C

All tested doses of EOs completely inhibited *Fusarium culmorum* FC46 growth in the first 5 days of incubation (Figure 15).

Figure 15. *In vitro* antifungal efficacy of lavender EOs against *Fusarium culmorum* FC46 phytopathogen

The *L. latifolia* EO (LL) maintained a complete inhibition of *F. culmorum* FC46 after 12 days of incubation, however the antifungal efficacy decreased when lower doses were used. At 20 and 30 µl LL/plate, the efficacy against *F. culmorum* FC46 decreased to 71.7% and 73.3% respectively (Figure 16).

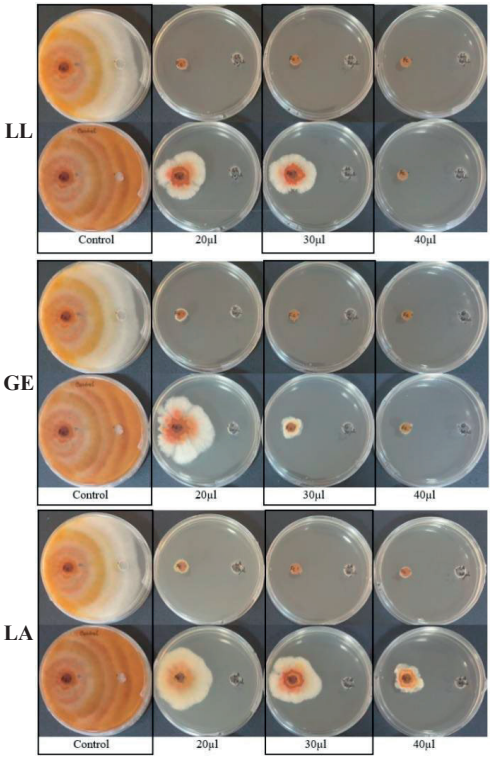
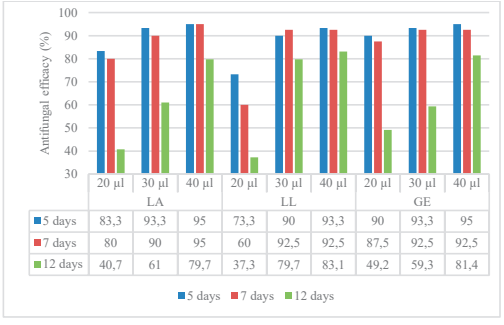


Figure 16. Inhibitory activity of EOs against *F. culmorum*, after 7 days (upper line) and 12 days (bottom line) of incubation at 25°C

The GE essential oil, maintained a complete inhibition of *F. culmorum* FC46 growth in the first 5 days of incubation at all tested doses, as well as after 7 days of incubation, at the dose of 30 and 40 µl EO/plate. After 12 days of incubation, the fungistatic efficacy of this essential oil began to decrease, up to 99.2% at 40 µl GE/plate and to 90% at 30 µl GE/plate, respectively. At 20 µl GE/plate the efficacy considerably dropped down to 53.3% fungal inhibition (Figure 15). The LA essential oil completely inhibited the growth of *F. culmorum* FC46 only during the first 7 days of incubation, when applied in 30 and 40 µl dose. After 12 days of incubation the antifungal efficacy decreased to 81.7% at 40 µl LA/plate, 66.7% when using 30 µl LA/plate, respectively 61.7% at 20 µl LA/plate (Figure 15).

The *F. oxysporum* f.sp. *radicis lycopersici* ZUM2407 (FORL) phytopathogen was less sensitive to the applied lavender EOs treatment. When using doses of 30 µl and 40 µl EOs/plate, no significant differences were observed between treatments after 5 and 7 days of incubation. However, after 12 days of incubation, the antifungal efficacy decreased considerably (Figure 16). Thus, at the highest dose tested (40 µl EO/ plate), LL revealed 83.1% antifungal efficacy (Figure 17), GE revealed 81.4% efficacy, while LA revealed 79.7% antifungal efficacy against FORL (Figure 18).

The growth of *Macrophomina phaseolina* phytopathogen was inhibited by the tested lavender essential oils only during the first 5 days of incubation (Figure 20).



	20 µl	30 µl	40 µl	20 µl	30 µl	40 µl	20 µl	30 µl	40 µl
	LA			LL			GE		
5 days	83,3	93,3	95	73,3	90	93,3	90	93,3	95
7 days	80	90	95	60	92,5	92,5	87,5	92,5	92,5
12 days	40,7	61	79,7	37,3	79,7	83,1	49,2	59,3	81,4

5 days 7 days 12 days

Figure 17. *In vitro* antifungal efficacy of lavender EOs against FORL phytopathogen

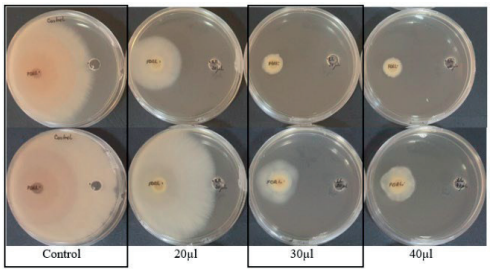


Figure 18. Inhibitory activity of LL essential oils against FORL, after 7 days (upper line) and 12 days (bottom line) of incubation at 25°C

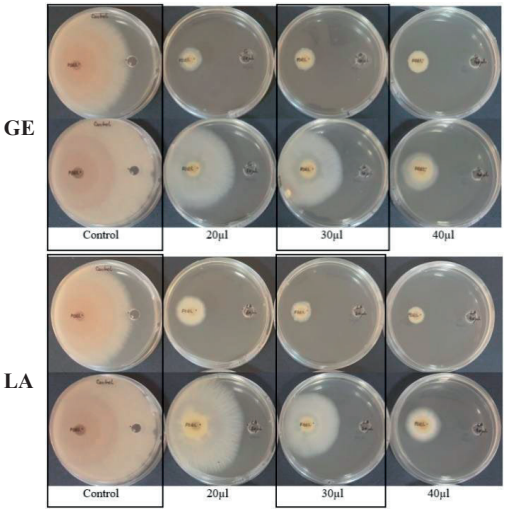
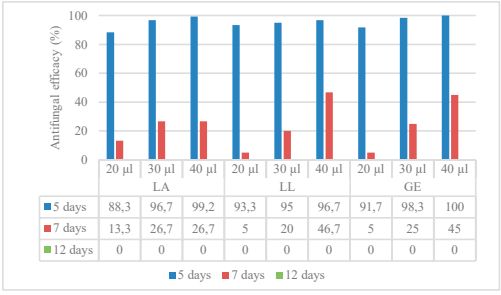


Figure 19. Inhibitory activity of GE and LA essential oils against FORL, after 7 days (upper line) and 12 days (bottom line) of incubation at 25°C



	20 µl	30 µl	40 µl	20 µl	30 µl	40 µl	20 µl	30 µl	40 µl
	LA			LL			GE		
5 days	88,3	96,7	99,2	93,3	95	96,7	91,7	98,3	100
7 days	13,3	26,7	26,7	5	20	46,7	5	25	45
12 days	0	0	0	0	0	0	0	0	0

5 days 7 days 12 days

Figure 20. *In vitro* antifungal efficacy of lavender EOs against *Macrophomina phaseolina* phytopathogen

When using GE essential oil in a dose of 40 µl / plate, the fungal growth was completely inhibited in the first 5 days of incubation.

The antifungal efficacy considerably decreased to 45%, after 7 days of incubation, while after 12 days, GE completely lost its inhibitory effect. However, the GE essential oil was the only one inhibiting the pigmentation of the pathogen (Figure 21).
Similar antifungal results were observed when testing the LA and LL essential oils, with the difference that the fungal pigmentation was not inhibited by this EOs.

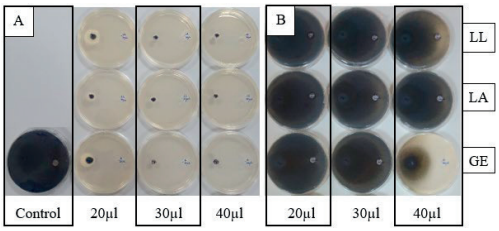


Figure 21. The antifungal activity of the lavender EOs against *Macrophomina phaseolina* after 5 days (A), respectively 12 days (B) of incubation at 25°C

GE lavender essential oil showed very good inhibitory activity against *Sclerotinia sclerotiorum* (Figure 22).

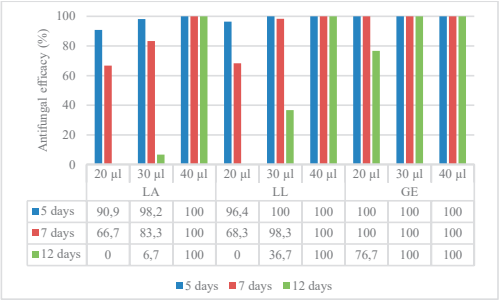


Figure 22. *In vitro* antifungal efficacy of lavender EOs against *Sclerotinia sclerotiorum* phytopathogen

Compared to the other tested lavender essential oils, GE completely inhibited the growth of the pathogen during the first 12 days of incubation, at both 30 and 40 µl/plate. Moreover, when using the dose of 20 µl GE/plate, the pathogen was completely inhibited during the first 7 days of incubation, while after 12 days the efficacy decreased to 76.7%, which is well above the inhibitory potential of the other two tested lavender oils (Figure 23).

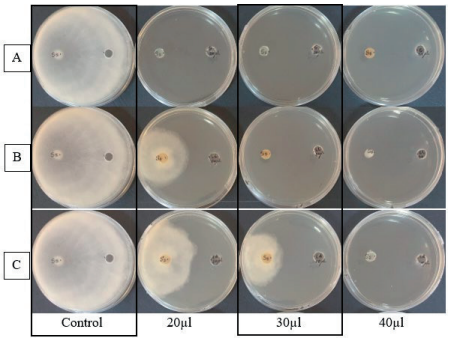


Figure 23. The antifungal activity of GE (A), LL (B) and LA (C) lavender essential oils against *Sclerotinia sclerotiorum*, after 7 days of incubation at 25°C

The antifungal effects of LA and LL essential oils against *S. sclerotiorum*, were similar compared to each other. However, LL was slightly more effective.

CONCLUSIONS

The highest sensitivity in terms of the antifungal activity of lavender essential oils was recorded by the fungus *Aspergillus brasiliensis* ATCC 16404, its growth being completely inhibited (100%) when adding to the plate an amount of 20 µl LA or GE essential oil. The fungi *Penicillium expansum* and *Fusarium oxysporum* showed greater resistance, the highest degree of inhibition being obtained when 45 µl of essential oil was added to the plate, thus obtaining an inhibition rate of 94.95% for LA, 95.27% for LL and 99.37% for GE, and in the case of *Penicillium expansum* the inhibition rate was 100% for all tested oils. Regarding the antibacterial activity, a complete inhibition of the development of the two bacteria under study was not observed in the case of the tested essential oils, the bacteria *Bacillus cereus* being less sensitive to their presence compared to *Bacillus subtilis*. The halo formed around the essential oil-soaked disc increased as the amount of applied essential oil increased. Its largest diameters were measured for the amount of 10 µl of essential oil LL, namely 1.80 cm in the case of *Bacillus cereus* bacteria and 4.08 cm in the case of *Bacillus subtilis* bacteria.

In all interactions with phytopathogenic agents, a close relationship was observed between the antifungal potential and the dose of essential oil tested. Thus, as expected, increasing the dose of lavender essential oil is directly proportional to the effectiveness of inhibiting phytopathogenic fungi. The three tested lavender essential oils inhibited the growth of the analyzed phytopathogenic fungi. At 40 µl EO/plate, all tree tested lavender EOs completely inhibited the growth of the *Botrytis cinerea*, *Fusarium culmorum* FC46 and *Sclerotinia sclerotiorum* phytopathogens in the first 7 days of incubation. Against *Alternaria* sp., only GE lavender essential oil maintained a complete fungal inhibition at 40 µl EO/plate in the first 7 days of incubation. *Macrophomina phaseolina* and *Fusarium oxysporum* f.sp. *radicis lycopersici* ZUM2407 phytopathogens were less sensitive to lavender essential oil treatments. Their inhibition was possible only in the first 5 days of incubation.

REFERENCES

Al-Ansari, M.M., Andeejani, A.M.I., Alnahmi, E., AlMalki, R.H., Masood, A., Vijayaraghavan, P., Rahman, A.A. & Choi, K.C. (2021). Insecticidal, antimicrobial and antioxidant activities of essential oil from *Lavandula latifolia* L. and its deterrent effects on *Euphoria leucographa*. *Industrial Crops and Products*, *170*, 113740.

Badr, M.M., Badawy, M.E.I. & Taktak, N.E.M. (2021). Characterization, antimicrobial activity, and antioxidant activity of the nanoemulsions of *Lavandula spica* essential oil and its main monoterpenes. *Journal of Drug Delivery Science and Technology*, *65*, 102732.

Balajan, I. & Pirbalouti, A.G. (2015). Variation in chemical composition of essential oil of populations of *Lavandula × intermedia* collected from Western Iran. *Industrial Crops and Products*, *69*, 344–347.

Behmanesh, F., Pasha, H., Sefidgar, A.A., Taghizadeh, M., Moghadamnia, A.A., Rad, H.A. & Shirkhani, L. (2015). Antifungal Effect of Lavender Essential Oil (*Lavandula angustifolia*) and Clotrimazole on *Candida albicans*: An *In Vitro* Study. *Scientifica*, *2015*, 261397.

Ciocarlan, A., Luparscu, L., Aricu, A., Dragalin, I., Popescu, V., Geana, E.I., Ionete, R.E., Vornicu, N., Duliu, O.G., Hristozova, G. & Zinicovscaia, I. (2021). Chemical Composition and Assessment of Antimicrobial Activity of Lavender Essential Oil and Some By-Products. *Plants*, *10*(9), 1829.

Císarová, M., Tančinová, D. & Medo, J. (2016). Antifungal activity of lemon, eucalyptus, thyme, oregano, sage and lavender essential oils against *Aspergillus niger* and *Aspergillus tubingensis* isolated from grapes. *Potravinarstvo*, *10*(1), 83-88.

Danh, L.T., Han, L.N., Triet, N.D.A., Zhao, J., Mammucari, R. & Foster N. (2012). Comparison of Chemical Composition, Antioxidant and Antimicrobial Activity of Lavender (*Lavandula angustifolia* L.) Essential Oils Extracted by Supercritical CO_2, Hexane and Hydrodistillation. *Food and Bioprocess Technology*, *6*, 3481-3489.

Dhaouadi, S., Rouissi, W., Mougou-Hamdane, A., Hannachi, I. & Nasraoui, B. (2018). Antifungal activity of essential oils of *Origanum majorana* and *Lavender angustifolia* against *Fusarium* wilt and root rot disease of melon plants. *Tunisian Journal of Plant Protection*, *13*(1), 39-55.

Erdogan, O., Celik, A. & Zeybek A. (2016). *In vitro* antifungal activity of mint, thyme, lavender extracts and essential oils on *Verticillium dahliae* kleb.. *Fresenius Environmental Bulletin*, *25*(11), 4856-4862.

Gismondi, A., Di Marco, G., Redi, E.L., Ferruci, L., Cantonetti, M. & Canini, A. (2021). The antimicrobial activity of *Lavandula angustifolia* Mill. essential oil against *Staphylococcus* species in a hospital environment. *Journal of Herbal Medicine*, *26*, 100426.

Jeddi, M., El Havhlafi, N., El Fadili, M., Benkhaira, N., Al-Mijalli, S.H., Kandsi, F., Abdallah, E.M., Ouaritini, Z.B., Bouyahya, A., Lee, L.H., Zengin, G., Mrabti, H.N. & Fikri-Benbrahim, K. (2023). Antimicrobial, antioxidant, α-amylase and α-glucosidase inhibitory activities of a chemically characterized essential oil from *Lavandula angustifolia* Mill.: *in vitro* and *in silico* investigations. *Biochemical Systematics and Ecology*, *111*, 104731.

Jianu, C., Pop, G., Gruia, A.T. & Horhat, F.G. (2013). Chemical Composition and Antimicrobial Activity of Essential Oils of Lavender (*Lavandula angustifolia*) and Lavandin (*Lavandula x intermedia*) Grown in Western Romania. *International Journal of Agriculture & Biology*, *15*, 772-776.

Kamatou, G.P.P. & Viljoen, A.M. (2008). Linalool – A Review of a Biologically Active Compound of Commercial Importance. *Natural Product Communications, 3*(7), 118-1192.

Man, A., Satacroce, L., Iacob, R., Mare, A. & Man, L. (2019). Antimicrobial Activity of Six Essential Oils Against a Group of Human Pathogens: A Comparative Study. *Pathogens*, *8*(1), 15.

Martucci, J.F., Gende, L.B., Neira, L.M. & Ruseckaite, R.A. (2015). Oregano and lavender essential oils as antioxidant and antimicrobial additives of biogenic gelatin films. *Industrial Crops and Products*, 71, 205-213.

Mesic, A., Mahmutović-Dizdarević, I., Tahirović, E., Durmišević, I., Eminovic, I., Jerković-Mujkić, A. & Bešta-Gajević, R. (2019). Evaluation of toxicological and antimicrobial activity of lavender and immortelle essential oils. *Drug and chemical toxicology*, *44*(2), 2021.

Sarker, L.S., Galata, M., Demissie, Z.A. & Mahmoud, S.S., (2012). Molecular cloning and functional characterization of Borneol dehydrogenase from the glandular trichomes *of Lavandula × intermedia. Archives of Biochemistry and Biophysics*, *528*, 163–170.

Schroder, T., Gaskin, S., Ross, K. & Whiley H. (2018). Antifungal activity of essential oils against fungi isolated from air. *International Journal of Occupational and Environmental Health*, *23*(3), 181-186.

Schroder, V., Radu, N., Cornea, P.C., Coman, O.A., Pirvu, L.C., Mohammed, M.S.O., Stefaniu, A., Pintilie, L., Bostan, M., Caramihai, M.D. & Roman, V. (2022). Studies Regarding the Antimicrobial Behavior of Clotrimazole and Limonene. *Antibiotics (Basel)*, *11*(12), 1816.

Shunying, Z., Yang, Y. Huaidong, Y., Yue, Y. & Guolin, Z., (2005). Chemical composition and antimicrobial activity of the essential oils of *Chrysanthemum indicum*. *Journal of Ethnopharmacology*, *96*, 151–158.

Sumalan, R.M., Kuganov, R., Obistioiu, D., Popescu, I., Radulov, I., Alexa, E., Negrea, M., Salimzoda, A.F., Sumalan, R.L. & Cocan, I. (2020). Assessment of Mint, Basil, and Lavender Essential Oil Vapor-Phase in Antifungal Protection and Lemon Fruit Quality. *Molecules*, *25*(8), 1831.

van Zyl, R.L., Seatlholo, S.T., van Vuuren, S.F. & Viljoen, A.M. (2006). The biological activities of 20 nature identical essential oil. *Journal of Essential Oil Research*, *18*, 129-133.

Valkova, V., Ďúranová, H., Galovičová, L., Vukovic, N.L., Vukic, M. & Kačániová, M. (2021). *In Vitro* Antimicrobial Activity of Lavender, Mint, and Rosemary Essential Oils and the Effect of Their Vapours on Growth of *Penicillium* spp. in a Bread Model System. *Molecules*, *26*(13), 3859.

Zuzarte, M., Vale-Silva, L., Gonçalves, M.J., Cavaleiro, C., Vaz, S., Canhoto, J., Pinto, E. & Salgueiro, L. (2012). Antifungal activity of phenolic-rich *Lavandula multifida* L. essential oil. *European Journal of Clinical Microbiology & Infectious Diseases*, *31*, 1359-1366.

Scientific Bulletin. Series F. Biotechnologies, Vol. XXVII, No. 2, 2023
ISSN 2285-1364, CD-ROM ISSN 2285-5521, ISSN Online 2285-1372, ISSN-L 2285-1364

PREVALENCE OF BRUCELLOSIS IN BUFFALOES FROM IRAQ

Ionuţ Nicolae RANGA, Diana-Gabriela GROPOȘILĂ-CONSTANTINESCU, Alishaqi MOHAMMED

University of Agronomic Sciences and Veterinary Medicine of Bucharest, 59 Marasti Blvd, District 1, Bucharest, Romania

Corresponding author email: bth.diana@yahoo.com

Abstract

Brucellosis, also known as Malta fever or undulant fever, is a bacterial infection caused by the genus Brucella. It primarily affects animals but can be transmitted to humans through direct contact with infected animals or their products, consumption of contaminated food or unpasteurized dairy products, or inhalation of infected aerosols. Brucellosis in buffalo milk in Iraq represents a significant public health concern. Buffalo brucellosis caused by Brucella abortus can be transmitted to humans through the consumption of unpasteurized or contaminated buffalo milk and dairy products. To diminish the effects of brucellosis in buffalo milk in Iraq, it is crucial to implement strict measures to ensure the safety of dairy products. This includes promoting pasteurization of milk to eliminate the bacteria, enforcing hygiene practices in milk production and processing, and conducting regular testing and monitoring of buffalo herds for brucellosis. Additionally, educating the public about the risks associated with consuming unpasteurized dairy products and promoting safe milk practices can help reduce the incidence of brucellosis transmission.

Key words*: brucellosis, buffalo, Brucella sp.*

INTRODUCTION

Throughout history, brucellosis has significantly influenced human communities, impacting cattle production, trade and public health.

The serious zoonotic disease known as brucellosis, sometimes called Malta fever, Mediterranean fever or undulant fever, is caused by bacteria of the genus *Brucella* (Sayer, 2016). Many species of animals are affected by this infectious disease, but domestic animals including cattle, goats and pigs are the main victims. Other mammals such as dogs, lambs and wild animals such as deer and bison can also contract the disease (Yon et al., 2019).

There are many species of *Brucella* that can infect both humans and animals (Corbel, 2020). The four primary *Brucella* species that pose the greatest threat to human health are as follows (Hull & Schumaker, 2018):

- *Brucella melitensis*. Although it can infect other animals, it mainly affects sheep and goats. It is considered the most virulent species and is responsible for most brucellosis infections in humans worldwide (Pisarenko et al., 2018).
- *Brucella abortus*. Cattle are the major source, but zebras and other animals are also susceptible. The most common ways people get sick are direct contact with sick animals or by eating contaminated dairy products (Jamil et al., 2017).
- *Brucella suis*. This species primarily affects pigs, although it can infect other animals such as dogs, rodents and wild boar. In humans, it is usually associated with occupational exposure to pig farmers, veterinarians and slaughterhouse workers (James et al., 2017).
- *Brucella canis*. This species primarily infects dogs. Although relatively rare, human infections can occur through direct contact with infected dogs, especially during the birthing process or through close contact with reproductive fluids (Hensel et al., 2018).

All these *Brucella* species have similar characteristics, including the ability to survive and replicate in host cells, especially macrophages. They have a tropism for reproductive organs, leading to abortion (Figure 1) or stillbirths in animals, which is a significant factor in transmission (Sarma & Singh, 2022).

Each species of *Brucella* has specific animal reservoirs, but they can also infect humans through different routes, including direct contact with infected animals, consumption of

contaminated animal products (especially raw or unpasteurised dairy products), inhalation of infectious aerosols or laboratory accidents (Figure 2). Understanding the causative agents of brucellosis is crucial for implementing effective control and prevention measures (Berhanu & Pal, 2020).

Figure 1. Aborted fetus due to *Brucella abortus* biovar infection (Megid et al., 2010)

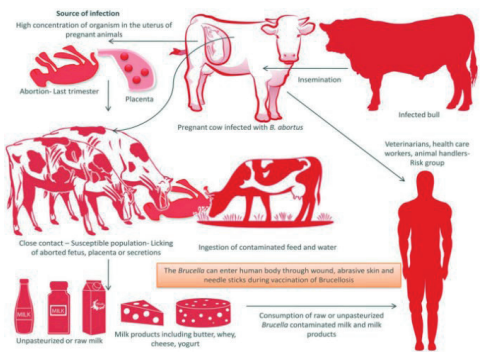


Figure 2. Transmission of brucellosis (Khurana et al., 2021)

Brucellosis can manifest as an acute or chronic disease in humans and its clinical presentation can vary greatly. Symptoms and severity of the disease may differ depending on different factors, such as the species, the dose of bacteria and the individual's immune response (Baldwin & Goenka, 2006).

The clinical presentation of brucellosis can be diverse and may mimic other infectious or inflammatory diseases, making diagnosis difficult. If brucellosis is suspected on the basis of symptoms and risk factors, laboratory tests, including blood cultures, serological tests (detection of antibodies against *Brucella*) and molecular techniques (Polymerase chain reaction - PCR) are used for confirmation (Al-Shemmari, 2018).

Brucellosis-causing bacteria can persist in the environment under specific circumstances, although they are vulnerable to a variety of environmental variables. Other diagnostic methods, such as bone marrow culture, synovial fluid culture or biopsy of affected tissues, may be used in specific cases, particularly when there is suspicion of localised infection (Pal et al., 2020).

It is important to interpret the laboratory results in the context of the clinical findings and patient history. Positive blood cultures or serological tests, together with compatible clinical symptoms, lead to the diagnosis of brucellosis (Barbuddhe et al., 2020).

Prompt diagnosis and treatment are crucial to prevent complications and ensure a favourable outcome. If brucellosis is suspected, healthcare professionals should consult with infectious disease specialists or microbiologists to guide appropriate testing and management (Pal et al., 2020).

MATERIALS AND METHODS

Several areas in Iraq were targeted in this study. Milk and blood samples were collected from buffaloes of different ages and sexes as shown in Table 1. The study was conducted during the year 2022.

Table 1. Areas in Iraq from which samples were collected

No. crt.	Area	No and type of samples
1.	Erbil area	80 samples of raw milk
2.	Governorate Basra	250 blood samples
3.	Governorate Mosul	400 blood samples
4.	Governorate Salahaldeen	205 blood samples

Raw milk samples were tested by the Milk Ring Test (MRT) method for the identification of *Brucella* antibodies (Mohamand et al., 2014). Thus 100 ml of milk were collected under sterile and hygienic conditions and placed in sterile plastic containers with screw caps. Samples were tested by culturing in Petri dishes at 37^0C, 24-48 hours, in the presence of a specific *Brucella* antigen. The formation of a ring or clusters around the antigen drop indicates the presence of *Brucella* antibodies in the milk sample, suggesting infection. Another method of testing is to add a drop of antigen to

1 ml of raw milk in a test tube. Specific antibodies attach to the antigen and rise to the surface, forming a blue ring. In the absence of antibodies, the mixture remains uniformly blue-white throughout the test tube.

The tests also aimed to isolate and identify *Brucella* strains present in raw milk by culturing on specific solid media at 37^0C for 7 days, followed by biochemical testing of the isolates.

The following tests were used to detect *Brucella* presence in milk and blood samples: a) Milk Ring Test; b) Microbiological tests, by cultivating the samples on specific culture media; c) Elisa test; d) Rose Bengal Test.

Blood samples were taken from male and female buffaloes aged 1-5 years. Using sterile syringes, 5 ml of blood was drawn from the jugular vein and placed into glass tubes. The plasma was then separated by centrifuging the blood samples at 3000 rpm/min for 5 minutes, after which it was stored in sterile plastic tubes at -20^0C until serological tests were performed.

The Rose Bengal test was performed on *Brucella abortus* antigens. Positive plasma samples were tested by a method using a phenol solution, which was prepared by dissolving 5 g phenol and 8.5 g sodium chloride in 1000 ml distilled water. *Brucella* antigen was used to test tubular agglutination, while it was diluted 1:10 using a mercaptan solution.

The tests in the Salahaldeen Governorate region involved 205 mature male and female buffaloes from which blood samples were taken. All the characteristics of the samples collected from buffaloes in this region (sex, physiological and pathological characteristics) are listed in Table 2.

Table 2. Characteristics of the samples collected from buffaloes from Salahaldeen area

Status of buffalo	Number of samples
Adult males	63
Adult females	84
Female abortion	21
Females pregnancy	37

RESULTS AND DISCUSSIONS

Bovine brucellosis is of great economic importance because the losses it causes can be very high, through miscarriages, prenatal morbidity, reduced milk and meat production, uneconomical use of production and slaughter and surveillance costs.

But the health importance of this disease is also particularly great, as it is one of the most feared zoonoses and at the same time one of the most feared professional diseases, and the toll paid by the veterinary profession over the years has been enormous.

Tests carried out on raw buffalo milk collected from the Erbil area showed a brucellosis incidence of 7.5% out of 80 samples tested (Table 3).

Table 3. Occurrence of *Brucella* antibodies among buffaloes raw milk from Erbil area according to Milk Ring Test method

Type of Milk	Number of samples	Positive samples (%)	Negative evidence (%)
Buffalo	80	7.5	92.5

Brucella species were identified in 6.3% (respectively 5 positive samples out of 80) of raw buffalo milk samples. Of the 5 *Brucella* strains found in raw milk samples, 3 strains of *Brucella abortus* (60%) and 2 strains of *Brucella melitensis* (40%) were identified. The data are presented in Table 4.

Table 4. Results obtained after the isolation of *Brucella* species from buffaloes raw milk

Type of milk	Buffaloes
Number	80
Positive (%)	6.3
Negative (%)	93.7
B. abortus (%)	60
B. melitensis (%)	40

Results obtained by the MRT method were compared with the microbiological results using milk samples. The MRT method identified 7.5% more cases of brucellosis in buffaloes.

Table 5 gives the sensitivity, specificity, positive predictive value (PPV) and negative predictive value (NPV) of the MRT method. Compared to the classical microbiological method, the accuracy of the MRT method in identifying bovine brucellosis is 97%, making it a viable alternative screening/diagnostic method (Table 5, Figure 3).

Table 5. Comparison between result of MRT and isolation of *Brucella* species from buffalo milk

Type of Milk	Buffalo
Number	80
MRT positive (n/%)	6/7.5
Culture positive (n/%)	5/6.3
Sensitivity (%)	83.3
Specificity (%)	98.7
Predictive value positive (%)	83
Predictive value negative (%)	98.5
Efficiency (%)	97

Figure 3. Milk ring test (MRT) result

The Rose Bengal Test results showed that of the 250 samples collected from the Basra area, 27 of them, representing 10.8%, were positive (Table 6).

Table 6. Brucellosis in buffaloes of Basra by Rose Bengal Test

Number of samples	Rose Bengal Test		
	Seropositive (%)	Seronegative (%)	Suspicious (%)
250	10.8	64.8	24.4

The Elisa test was performed on 88 plasma samples collected from buffaloes in Basra area, which included the 27 positive and 61 suspect plasma previously detected using the Rose Bengal Test.

In Figure 4, sample 3 represent positive control, sample 5 represent negative control, while samples 1, 2, 4, represent the positive cases detected.

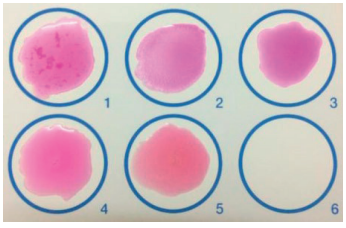


Figure 4. Positive results of infected animals using Rose Bengal Test

The Elisa test showed that of the 88 samples tested, 21, representing 23.8% were positive (Table 7). Table 7 also contains information on the regions from which samples were collected.

Table 7. Result of Elisa for *Brucella abortus* in buffaloes from Basra Guvernorate

Region	Number of probes	Number positive Elisa Test (%)
Al Hartha	30	6.8
Al Qurna	15	5.7
Al Dear	18	4.5
Al Zubier	10	3.4
Al Medaiana	7	2.3
Al Tanooma	8	1.1
Total	88	23.9
Significance		P < 0.05%

From Table 8 it can be observed that a significant impact on the severity of infection was the age of the animals tested. Of the 88 samples from animals aged between 1.5-8.5 years, the category > 5-8.5 years was strongly affected, with 12 positive results out of the 40 samples tested, representing 13.6% of the total number of samples tested (88). Although the proportion of young animals, of 1.5-5 years, was higher than that of older animals, a lower infection with *Brucella abortus* was recorded for them, only ~10% of the total number of samples tested (9 positive tests out of 48 animals tested). Out of the total of 88 samples, 67 tested negative, 66% of the total (Table 8).

Thus, *Brucella abortus* infection was detected in 24% of the buffalo tested, which indicates a serious health problem that requires prompt intervention.

Table 8. Infection rate of *Brucella abortus* according to age groups

Age (years)	Number of animals	Elisa test results	
		Positive (%)	Negative (%)
1.5 - 5	48	10.2	44.3
> 5 - 8.5	40	13.6	31.9
Total	88	23.8	76.2

The same Rose Bengal Test was also carried out on 400 buffalo in the Mosul area. For 52 tests the results were positive, representing 13% of the total number of tests (Table 9).

Table 9. Test result of Rose Bengal Test

Number of probes	Positive results (%)	Negative results (%)
400	13	87

According to the Rose Bengal Test, the highest incidence of infection was reported in females, with 8.29% positive tests, compared to 2.44% in males.

Positive Elisa results were obtained in 5.37% of females, while for males, 2.44% were positive tests (Table 10).

Table 10. Results of RBT and Elisa test of buffalo in Salahaldeen area

Buffalo status	Number of sample	Positive results		Negative results (%)
		Rose Bengal Test (%)	Elisa Test (%)	
Adults male	63	2.44	2.44	25.85
Adults female	84	3.41	2.93	34.63
Aborted female	21	2.93	0.98	6.30
Pregnant female	37	1.95	1.46	14.63
Total	205	10.73	7.81	81.46

CONCLUSIONS

- Brucellosis in buffalo milk in Iraq represents a significant public health concern.
- To diminish the effects of brucellosis in buffalo milk in Iraq, it is crucial to implement strict measures to ensure the safety of dairy products. This includes promoting pasteurization of milk to eliminate the bacteria, enforcing hygiene practices in milk production and processing, and conducting regular testing and monitoring of buffalo herds for brucellosis.
- Educating the public about the risks associated with consuming unpasteurized dairy products and promoting safe milk practices can help reduce the incidence of brucellosis transmission.

REFERENCES

Al-Shemmari, I. G. M. (2018). Comparative study between conventional and molecular tests to detect the incidence of brucellosis in cattle and buffaloes in Babylon and Karbala provinces. *Scientific Journal of Medical Research*, 2(5), 7-13.

Baldwin, C. L., & Goenka, R. (2006). Host immune responses to the intracellular bacteria *Brucella*: does the bacteria instruct the host to facilitate chronic infection? *Critical Reviews in Immunology*, *26*(5), 407-442.

Barbuddhe, S. B., Vergis, J., Rawool, D. B. (2020). Immunodetection of bacteria causing brucellosis. In *Methods in Microbiology,* 47, 75-115.

Berhanu, G., & Pal, M. (2020). Brucellosis: A highly infectious zoonosis of public health and economic importance. *Journal of Emerging Environmental Technologies and Health Protection*, 3, 5-9.

Corbel, M. J. (2020). Microbiology of the genus *Brucella*. In *Brucellosis,* 53-72, CRC Press.

Hensel, M. E., Negron, M., & Arenas-Gamboa, A. M. (2018). Brucellosis in dogs and public health risk. *Emerging infectious diseases*, *24*(8), 1401.

Hull, N. C., & Schumaker, B. A. (2018). Comparisons of brucellosis between human and veterinary medicine. *Infection ecology & epidemiology*, *8*(1), 1500846, 1-12.

James, D. R., Golovsky, G., Thornton, J. M., Goodchild, L., Havlicek, M., Martin, P., ... & Mor, S. M. (2017). Clinical management of *Brucella suis* infection in dogs and implications for public health. *Australian veterinary journal*, *95*(1-2), 19-25.

Jamil, T., Melzer, F., Njeru, J., El-Adawy, H., Neubauer, H., & Wareth, G. (2017). *Brucella abortus*: Current research and future trends. *Current Clinical Microbiology Reports*, *4*, 1-10.

Khurana, S. K., Sehrawat, A., Tiwari, R., Prasad, M., Gulati, B., Shabbir, M. Z., Chhabra R., Karthik K. ... & Chaicumpa, W. (2021). Bovine brucellosis–a comprehensive review. *Veterinary Quarterly*, *41*(1), 61-88.

Megid J, Mathias L. A., Robles C.A. Clinical manifestations of brucellosis in domestic animals and humans. Bentham Open, 2010.

Mohamand, N., Gunaseelan, L., Sukumar, B., & Porteen, K. (2014). Milk Ring Test for spot identification of Brucella abortus infection in single cow herds. *Journal of Advanced Veterinary and Animal Research*, *1*(2), 70-72.

Pal, M., Kerorsa, G. B., Desalegn, C., & Kandi, V. (2020). Human and Animal Brucellosis: A Comprehensive Review of Biology, Pathogenesis, Epidemiology, Risk Factors, Clinical Signs, Laboratory Diagnosis. *American Journal of Infectious Diseases*, *8*(4), 118-126.

Pisarenko, S. V., Kovalev, D. A., Volynkina, A. S., Ponomarenko, D. G., Rusanova, D. V., Zharinova, N. V., ... & Kulichenko, A. N. (2018). Global evolution and phylogeography of *Brucella melitensis* strains. *BMC genomics*, *19*(1), 1-10.

Sarma, O., & Singh, P. (2022). Brucellosis: A major abortion causing disease of livestock with zoonotic potential. *Pharm. Innov. J*, *11*(8), 270-277.

Sayer, K. (2016). Brucellosis in fact and fiction: The story of a zoonosis. *Veterinary History*, *18*(2), 165-183.

Yon, L., Duff, J. P., Ågren, E. O., Erdélyi, K., Ferroglio, E., Godfroid, J., ... & Gavier-Widén, D. (2019). Recent changes in infectious diseases in European wildlife. *Journal of wildlife diseases*, *55*(1), 3-43.

Scientific Bulletin. Series F. Biotechnologies, Vol. XXVII, No. 2, 2023
ISSN 2285-1364, CD-ROM ISSN 2285-5521, ISSN Online 2285-1372, ISSN-L 2285-1364

ROMANIAN CONSUMER ATTITUDES REGARDING ALTERNATIVE PROTEIN SOURCES

Mihaela GEICU-CRISTEA, Elisabeta Elena POPA, Paula Gabriela MANEA, Mihaela Cristina DRĂGHICI, Amalia Carmen MITELUȚ, Paul Alexandru POPESCU, Mona Elena POPA

University of Agronomic Sciences and Veterinary Medicine of Bucharest, 59 Marasti Blvd, District 1, Bucharest, Romania

Corresponding author email: elena.eli.tanase@gmail.com

Abstract

By 2050, the global population is set to reach 9.8 billion, which means high food demands. Therefore, the necessity of using alternative protein sources for food development, instead of meat, is of great interest. Meat alternatives which are generally based on proteins that are not of animal source present chemical characteristics very similar to animal protein and they intend to present same flavour, texture and appearance. These types of proteins are gaining more attention due to their health benefits, sustainability from an environmental point of view and ethics. Their sources are of plant origin (edible seeds, cereals, pseudo-cereals, tubers, legumes), microorganisms (bacteria and fungi), unconventional alternative sources (by-products of agro-industrial processes), algae, microalgae and insects. This study aimed at presenting consumer attitudes and perceptions related to alternative protein sources, and in this respect, a questionnaire was developed and distributed to be completed on-line. The results of our study showed that consumers are not totally informed about alternative protein sources and the majority are reluctant to some of these sources, but a small percent would be willing to pay more and consume alternative protein-based products.

Key words*: alternative protein sources, consumer attitude, questionnaire.*

INTRODUCTION

In the last years, the number of studies related to the use of alternative proteins in human nutrition has increased. Among the reasons cited in terms of supporting the development of alternative sources of protein are demographic growth and limited resources along with degradation of environmental protection, deterioration of animal welfare, increase in flexitarianism and human health reasons. Lately, the increase of human population numbers and depletion of natural resources have become among the most critical issues to be faced worldwide (Quintieri et al., 2023). Regarding the demographic growth, United Nations reports that the current world population comprises 7.5 billion people and is foreseen to increase up to 9-10 billion by 2050 (United Nations, 2019), which will require the meat industry to increase production by 50–73% to meet the growing food demands (Bonny et al., 2017). With respect to degradation of environmental protection, many studies argue that animal agriculture emits greenhouse gases such as carbon dioxide, methane and nitrous oxide respectively (Takefuji, 2021). Their emissions account for 14.5% of total emissions, including those of other sectors, indicating that animal agriculture is a contributor to global warming (FAO, 2017). Also, in recent times entire areas on Earth have faced very long periods of drought, water being a very important resource in raising animals and also being known that for every kilogram of livestock meat grown, water of 20.7 tons is required for beef, 5.9 tons for pork and 4.5 tons for poultry for every kilogram of feed crops (Oki et al., 2003). Alternatives and cultured meat, are generally considered to be healthier and more environmentally friendly than traditional animal-derived proteins (Aiking, 2011). That being said, the benefits of alternative protein production have not yet been fully scientifically documented, particularly with respect to the environment (Onwezen et al., 2021).

Flexitarianism, or "occasional vegetarianism", is an increasingly popular plant-based diet that claims to reduce carbon footprint and improve

health through a diet that is mostly vegetarian but still allows for occasional consumption of meat (Delaney, 2018). For those who want to adopt this diet, meat alternatives are welcome. In terms of aspects related to human health, it is well known that excessive consumption of meat and meat products is often associated with overconsumption of energy and fat, resulting in excess weight, obesity and an increased risk of chronic diseases, such as cardiovascular disease and type 2 diabetes. In addition, certain components of fresh and processed red meat may further increase the risk of these diseases and predispose the consumer to cancer, particularly colorectal cancer (Salter, 2018).

Gravely and Fraser (2018) stated in a study that in comparison to meat, the market shares of alternative proteins remain low, even despite the fact that super-markets and restaurants increasingly offer alternatives to traditional meat products or dishes, such as plant-based burgers or wraps with beans (Curtain and Grafenauer, 2019). The growth of plant-based meat market is projected to increase from $4.6 billion in 2018 to $85 billion in 2030 (UBS, 2019) and, as a milestone by year 2026, reach $30.9 billion (Watson, 2019). This new market appears to be well positioned for further expansion and innovation.

In this context, the aim of the present study was to assess the familiarity level of Romanian consumers with alternative sources of protein, such as vegetable protein, edible insects, algae and laboratory-grown meat. Also, the authors of this study wanted to obtain a detailed picture of consumer preferences and attitudes regarding the use of alternative protein sources in their own diet in order to identify key barriers and opportunities in their adoption, as well as their concerns regarding the impact of meat consumption on the environment.

MATERIALS AND METHODS

In order to investigate consumer attitudes towards alternative protein sources a survey in form of an online questionnaire was designed and uploaded on “Google Forms” platform, this being distributed in the online environment on different social networks, in order to collect responses covering a diverse sample of consumers of different ages, genders and education levels.

The questionnaire has a short completion time of several minutes and consists of a small number of logical questions that aim to provide information about buying habits, preferences, behaviour and even the profile of consumers (Colibaba, 2001). The questionnaire contains 26 questions, strategically designed to explore different aspects of consumer acceptability. A number of 20 questions were included in the questionnaire regarding respondents' knowledge and information about alternative protein sources, their personal preferences regarding these sources, their eating habits, their motivations and concerns related to their consumption, as well as factors that could influence the decision to adopt or reject alternative protein sources, but also 6 other questions aimed at collecting socio-demographic information such as: age, gender, income, environment from which they come, etc. The questions are closed, simple, clear, some with a single answer and others with multiple answers.

RESULTS AND DISCUSSIONS

This study was conducted in 2023 and following the completion of the questionnaire a total sample of 178 answers was gathered, from which 71.9% were women and 27% men, while 1.1% preferred not to declare their gender. Regarding the age of the participants in this study, 51.7% are between 18-25 years old and 20.8% are between 26-35 years old. Respondents aged 36-45 years, as well as those aged 46-60 years presented equal percentage, namely 12.9%, while respondents over 60 years old represent 1.7%. The majority of the respondents were employed (73%), while another important part of them were students (18.5%). Other respondents were entrepreneurs (3.4%), pensioners (2.8%) and unemployed people (2.2%). Regarding the monthly income, 49.4% have between 2500 - 4500 RON, 27.3% have low incomes below 1500 RON and 23.32% of consumers earn more than 4500 RON per month. The education level was of 52.2% for higher education graduation, 27% graduated high school, 16.9% graduated a professional school, while 2.2% graduated

primary school, respectively 1.7% graduated secondary education school. Further, 87.6% of the respondents mentioned they live in urban areas, and 12.4% represent the number of those from rural areas. The socio-demographic profile of the respondents is presented in Table 1.

Table 1. The socio-demographic profile of the respondents

Age group	51.7% - 18-25 years 20.8% - 26-35 years 12.9% - 36-45 years 12.9% - 46-60 years 1.7% - more than 60 years
Gender	71.9% - female 27% - male 1.1% - preferred not to declare their gender
Highest level of education completed	52.2% - University 27% - High school 16.9% - Professional School 2.2% - Primary education School 1.7% - Secondary education School
Professional status	73% - employed 18.5% - student 3.4% - entrepreneur 2.8% - pensioner 2.2% - unemployed
Where do you live?	87.6% - urban area 12.4% - rural area
Household's monthly net to income	49.4% - between 2500 - 4500 RON 27.3% - below 2500 RON 23.3 % - over 4500 RON

Table 2 shows the answers that reflect the probability of the respondents to pay more for alternative proteins than for animal protein, or adopt the long-term consumption of these alternative proteins in their diet. Therefore, 16.4% are very likely to pay more for alternative proteins than for traditional animal-based proteins, while 36.7% are somewhat likely, 33.9% are not very likely and 13% are not likely at all to pay more for these products.

Table 2. The probability to pay more for alternative proteins or to switch the diet to these for a long-term period

Question	**Answers***			
	Very likely	**Somewhat likely**	**not very likely**	**not likely at all**
How likely are you to pay more for alternative proteins than you would for traditional animal-based proteins?	16.4%	36.7%	33.9%	13%
How likely are you to switch to alternative protein sources as your main long-term protein source?	6.8%	23.7%	50.8%	18.6%

* - % of respondents

The outlook on transitioning to alternative protein sources in the long term varies among respondents. When asked how likely they are to make this transition, the results indicate that a small sample, namely 6.8% of respondents, say they are very likely to make this transition. At the same time, 23.7% indicated that they are somewhat likely to adopt alternative protein sources in the long term. However, the majority of respondents, representing approximately 50.8%, indicated that they were not very likely to make this transition, and 18.6% indicated that they were not at all likely to do so.

A series of decisive factors in choosing the consumption of alternative protein were analysed considering their degree of importance for respondents and the results obtained are presented in Table 3. It seems that taste is very important for consumers, influencing their purchase decision, thus 33.9% of the respondents consider the taste similarity between the alternative protein and traditional

animal – based products to be very important, while 34.5% consider this issue somewhat important, 20.3% not very important and only 11.3% not important at all. Further, 27% of respondents consider that, in the case of alternative protein, texture is very important to be similar to traditional animal – based products, while 37.1% consider to be somewhat important, 20.8% not very important and 15.2% not important at all. People are willing to buy alternative protein products if they are available at the store/in the restaurants, etc., thus 49.7% of respondents consider very important the availability of this products, while 37.5% consider it somewhat important, 10.2% not very important and 5.6% not important at all. For the majority of consumers, the high protein content of alternative protein products matters, thus 27.1% consider it very important, 46.3% somewhat important, 16.9% not very important and 9.6% not important at all (Table 3). The costs of alternative proteins should reflect their quality and should be correct, thus 33.2% consider it very important, while 37.1% is somewhat important, 18.5% not very important and 11.2% not important at all (Table 3).

Table 3. The degree of importance of some decisive factors in choosing the consumption of alternative protein

Question	**Answers***			
	Very important	**Somewhat important**	**Not very important**	**Not important at all**
How important is it to you that alternative protein taste similar to traditional animal products?	33.9%	34.5%	20.3%	11.3%
How important is it to you that alternative protein have a similar texture to traditional animal protein?	27%	37.1%	20.8%	15.2%
How important is it to you that alternative sources of protein are easily accessible (eg, available at grocery stores, restaurants, etc.)?	49.7%	37.5%	10.2%	5.6%
How important is it to you that alternative sources of protein have a high protein content?	27.1%	46.3%	16.9%	9.6%
How important is the cost of alternative proteins to you?	33.2%	37.1%	18.5%	11.2%

* - % of respondents

The majority of the surveyed consumers stated that they consumed alternative sources of proteins (88.2%), they heard about insect-based protein as an alternative source of protein (66.9%), but they don't want to try them (52.2%), also they have information and heard of cultured meat (64%), but they are reluctant in terms of its consumption (53.9%). Therefore, 27%, respectively 24.2% of respondents are not sure that they want to consume insect-based protein respectively cultured meat as an alternative protein source. The results reflected the fact that 51.1% of the respondents never thought about the environmental impact of consuming traditional meat products (Table 4).

Table 4. Denying/Affirming the information about alternative proteins and the availability to consume them

Question	**Answers***		
	Yes	**No**	**I am not sure**
Have you ever consumed alternative sources of protein such as legumes (peas, chickpeas, beans, etc.), plant-based proteins (ex: soy, tofu, etc.), algae, insect protein, or lab-grown meat?	88.2%	11.8%	-
Have you ever thought about the environmental impact of consuming traditional meat products?	48.9%	51.1%	-
Have you ever heard of insect based protein as an alternative source of protein?	66.9%	33.1%	-
Would you be willing to try insect-based protein?	20.8%	52.2%	27%
Have you ever heard of cultured meat (also known as lab-grown meat)?	64%	36%	-
Would you be willing to try cultured meat as an alternative protein source?	21.9%	53.9%	24.2%

* - % of respondents

In this study, the authors wanted to determine the frequency with which people consume animal protein products or alternative protein sources. The obtained results revealed that only 14.1% of respondents use to consume daily alternative sources of protein, while 33.9% use to consume it a few times a week, 27.7% a few times a month, respectively 19.2% rarely and 5.1% never. Regarding how often the consumers use to eat traditional meat products, the results recorded a daily consume in the case of 29.8% of respondents, while 58.4% use to consume a few times a week this category of products, respectively 8.4% a few times a month, 2.2% rarely and 1.1% never (Table 5).

Table 5. The frequency of alternative protein consumption versus animal protein

Question	Answers*				
	Daily	A few times a week	A few times a month	Rarely	Never
How often do you consume alternative sources of protein?	14.1%	33.9%	27.7%	19.2%	5.1%
How often do you eat traditional meat products (beef, chicken, pork, etc.)?	29.8%	58.4%	8.4%	2.2%	1.1%

* - % of respondents

Regarding the alternative proteins that Romanians are used to consume, the questionnaire provided a question with multiple answers, and the collected data mostly count legumes (93.3%), respectively plant based-proteins (52.1%), at the opposite pole, being seaweed consumption (11.7%), respectively insect proteins (1.2%) and cultivated meat (0.6%), as shown in Figure 1.

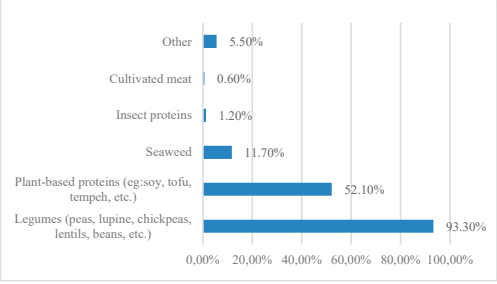


Figure 1. The chosen alternative proteins of the respondents

Consumers who answered that they did not consume alternative proteins until now were asked what was the main reason for this choice (Figure 2).

Thus, it seems that the main reasons were the lack of availability (11.4%), different concerns about taste (27.8%) and the lack of interest (21.5%). Among other reasons why some consumers were not tempted to try alternative proteins are health issues (10.1%) and other personal opinions and reasons (29.1%).

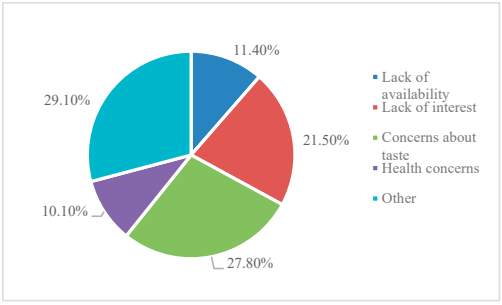


Figure 2. The main reason the consumers did not tried alternative proteins

Onwezen et al. (2019) show that affective drivers are more relevant for innovative alternative proteins of insects and seaweed (compared to less innovative alternative proteins of pulses and fish), indicating that acceptance of innovative alternative proteins is based more on feelings than the acceptance of less innovative alternative proteins is.

Among the reasons listed by reluctant consumers regarding what could motivate them to try alternative protein sources is counted curiosity (33.5%), health issues (31.2%), animal welfare concerns (20.2%), environmental concerns (12.7%) and other personal reasons (31.2%) (Figure 3).

The acceptance of all alternative proteins is affected by food neophobia - which is defined as being the aversion to trying novel foods, and is a key barrier for the consumption of insects (Onwezen et al., 2021).

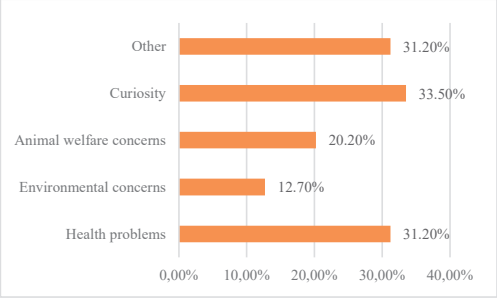


Figure 3. What motivates you to try alternative protein sources?

All respondents were asked in what form would alternative sources of protein be more appealing to incorporate into their diet, and the data collected highlighted that consumers prefer protein bars (52.3%), burgers (34.3%), chips (30.2%), flour (20.9%) and other categories of products (25.6%) (Figure 4).

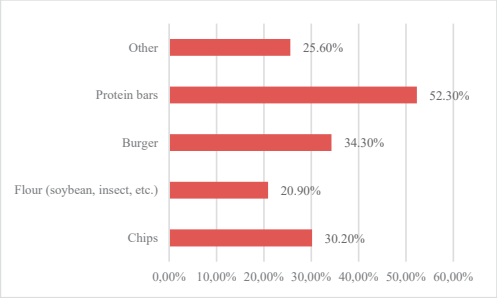


Figure 4. In what form would alternative sources of protein be more appealing to incorporate into your diet?

The world is currently facing the challenge of satisfying the food needs of a growing population, without consuming excessive natural resources, or extensively the ecological environment beyond repair (Zhang et al., 2021). Regarding the level of concern about the impact of animal husbandry on the environment, the largest share, 41.8% of the respondents, stated that they show a moderate concern regarding this aspect, 29.9% indicated a level low level of concern, while 7.9% chose the option "very concerned" and 1.1% indicated the highest level of concern, "extremely concerned". On the other hand, 19.2% of respondents chose the "not at all concerned" option, signifying a lack of awareness of the impact of animal husbandry on the environment (Figure 5).

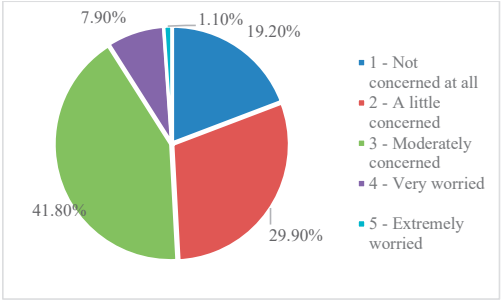


Figure 5. The concern of the respondents regarding the impact that raising animals have on the environment

Environmental impact and concerns about maintaining a healthy lifestyle are important factors in consumers' decision to adopt alternative protein sources. Awareness of the consequences of meat and dairy production has led to an openness to alternatives that reduce the ecological footprint.

CONCLUSIONS

The responses received from all the participants to this study were analysed and interpreted in this paper, with the aim of drawing relevant conclusions and gaining a clearer understanding of the determinants of protein consumption from alternative sources. Information and education are key in promoting alternative proteins, as consumers need clear and accessible knowledge of the benefits and options available so they can make informed decisions and adopt these alternatives consciously. Identified advantages of alternative protein sources include their high protein content, comparable to traditional products, but with reduced environmental impact and sometimes additional health benefits. Disadvantages identified include possible differences in taste and texture compared to traditional products, as well as the higher costs associated with alternative proteins. These aspects may represent obstacles to the long-term adoption of a diet with alternative protein sources and require continued development to provide more affordable products with a similar sensory experience. In conclusion, this paper highlighted an increase in consumer interest and positive attitude towards alternative protein sources, particularly in terms of environmental

impact. However, continued efforts are needed to improve the acceptance and adoption of alternative protein sources through innovations in product development, affordable pricing policies, and ongoing consumer information and education.

REFERENCES

Aiking, H. (2011). Future protein supply. *Trends in Food Science & Technology*, *22*(2–3), 112–120.

Bonny, S.P., Gardner, G.E., Pethick, D.W. & Hocquette, J.-F. (2017). Artificial meat and the future of the meat industry. *Animal Production Science*, *57*, 2216–2223.

Colibaba, D. (2001). Chestionarul - instrument valoros în cercetarea pieței, *Revista Informatică Economică*.

Curtain, F. & Grafenauer, S. (2019). Plant-based meat substitutes in the flexitarian age: An audit of products on supermarket shelves. *Nutrients*, *11*(11), 2603.

Delaney, E. (2018). BBC Good Food: What is flexitarian diet? Available online: https://www.bbcgoodfood.com/howto/guide/what-flexitarian-diet, accessed on 20.11.2022.

FAO (2017). Livestock solutions for climate change. In 2013. idem, Tackling climate change through livestock (p. 7). Rome: FAO, 2017 http://www.fao.org/3/I8098EN/i8098en.pdf http://www.fao.org/3/i3437e/i3437e.pdf.

Gravely, E. & Fraser, E. (2018). Transitions on the shopping floor: Investigating the role of Canadian supermarkets in alternative protein consumption. *Appetite*, *130*, 146–156.

Oki, T., Sato, M., Kawamura, A., Miyake, M., Kanae, S. & Musiake, K. (2003). Virtual water trade to Japan and in the world. In: Virtual water trade: Proceedings of the international expert meeting on virtual water trade (p. 1-13).

Onwezen, M.C., Bouwman E.P., Reinders M.J. & Dagevos H. (2021). A systematic review on consumer acceptance of alternative proteins: Pulses, algae, insects, plant-based meat alternatives, and cultured meat. *Appetite*, *159*, 105058.

Onwezen, M.C., van den Puttelaar, J., Verain, M.C.D. & Veldkamp, T. (2019). Consumer acceptance of insects as food and feed: The relevance of affective factors. *Food Quality and Preference*, *77*, 51–63.

Quintieri, L., Nitride, C., De Angelis, E., Lamonaca, A., Pilolli, R., Russo, F. & Monaci, L. (2023). Alternative Protein Sources and Novel Foods: Benefits, Food Applications and Safety Issues. *Nutrients*, *15*, 1509.

Salter, A.M. (2018). The effects of meat consumption on global health. *Revue scientifique et technique/ Office international des épizooties*, *37*(1), 47-55.

UBS (2019). Market news. Accessed https://www.ubs.com/global/en/wealth-management/marketnews/home/article.1441202.html/

United Nations. Report of the Secretary-General on the Work of the Organization (A/74/1, 74th Session); United Nations: New York, NY, USA, 2019. Available online: https://www.un.org/annualreport/2019/files/2019/09/Annual-report-SG-2019-EN-Complete-Web.pdf.

Takefuji, Y. (2021). Sustainable protein alternatives. *Trends in Food Science & Technology*, *107*, 429–431.

Watson, J. (2019). Plant-based meat market to reach USD 30.92 Billion by 2026. Reports and Data. Accessed https://www.globenewswire.com/news-release/2019/10/14/1929284/0/en/Plant-based-Meat-Market-To-Reach-USD-30-92-Billion-By-2026-Reports-And-Data.html/.

Zhang, T., Dou, W., Zhang, X., Zhao, Y., Zhang, Y., Jiang L. & Sui X. (2021). The development history and recent updates on soy protein-based meat alternatives. *Trends in Food Science & Technology*, *109*, 702–710.

Scientific Bulletin. Series F. Biotechnologies, Vol. XXVII, No. 2, 2023
ISSN 2285-1364, CD-ROM ISSN 2285-5521, ISSN Online 2285-1372, ISSN-L 2285-1364

PLA/PHBV ACTIVE PACKAGING APPLICATION ON FRESH MINCED CHICKEN MEAT

Elisabeta Elena POPA[1], Mihaela GEICU-CRISTEA[1], Amalia MITELUȚ[1], Maria RAPA[2], Mihaela DRĂGHICI[1], Paul Alexandru POPESCU[1], Mona Elena POPA[1]

[1]University of Agronomic Sciences and Veterinary Medicine of Bucharest, 59 Marasti Blvd, District 1, Bucharest, Romania
[2]Faculty of Material Science and Engineering, University Politehnica of Bucharest, 313 Spl. Independentei, Bucharest, Romania

Corresponding author email: mihaela.geicu@usamv.ro

Abstract

Meat spoilage is of great concern along the food chain, in respect to both consumers health and food waste, and also from an economic point of view. Spoilage mainly occurs due to the growth of microorganisms, from which the necessity of developing new packaging materials with antimicrobial properties have gained more and more interest. Active packaging materials can incorporate different ingredients that could interact with the food products and with the packaging environment in an active way. It presents great properties in respect to shelf-life prolongation, due to the variety of active substances that can be added (essential oils, natural antimicrobials, bacteriocins, etc.). The aim of the present study was to determine the effect that an active packaging material have on the properties and quality of fresh minced chicken meat. In this respect, a film based on PLA/PHBV and a nanoemulsion containing nisin and dill essential oil was applied on the studied meat, and its quality indicators (physical-chemical and microbiological parameters) were monitored during 7 days of storage at 4±1°C. The results showed that the packaging film maintained the chicken meat properties during the monitored storage period.

Key words*: fresh minced chicken meat, active packaging, PLA, PHBV, nisin, dill essential oil.*

INTRODUCTION

The production and consumption of broilers at a global level is increasing every year, with a prediction of production for 100 million tons and consumption of 98 million tons in 2022. Chicken meat is preferred by consumers due to its high nutritional value and unique taste and flavour (Shao et al., 2023). It is the most consumed meat in many countries due to its low-fat content, availability and relatively low cost. Fresh chicken meat is highly perishable due to high content in protein, free amino acids, vitamins and water (Mohamed-Noor et al., 2012; Wang et al., 2023), the main reason for spoilage being related to psychrotrophic microbial growth and physicochemical changes (Katiyo et al., 2020). The microbial contamination is primary generated from the bird's gastrointestinal track microbiota that can spread on the meat and equipment surface and also air within the processing area and during further processing steps through portioning and packaging (Hrustemović et al., 2022; Dourou et al., 2023). Furthermore, microbial spoilage can occur anytime during processing, retail and at the end consumer generating great economic losses besides health-related outbreaks. Due to this facts, low shelf life and increasing consumer demands for safer food products, research has been conducted to develop various technologies and/or packaging materials for chicken meat preservation.

Tsafrakidou et al. (2021) determined the evolution of spoilage microbiota of minced chicken meat during 10 days of storage at 4°C. The evaluation was made in association with pH changes and acetic and lactic acids in retail oxygen free modified atmosphere packaging (30 CO_2: 70 N_2). It was shown that the modified atmosphere packaging led to a growth inhibition of lactic acid bacteria (under 6.5 log CFU/g) and a total inhibition of *Enterobacteria*, *Pseudomonas*, *Enterococci*, *Staphylococci* and yeasts.

Karaca et al. (2023) developed real time CO_2 freshness indicators to be used on chicken breast, based on phenol red and bromothymol

blue. The developed indicators were applied to monitor chicken meat spoilage packed in polyamide/polyethylene pouches under air and 100% N_2 at 4°C for 10 days. The results of the study showed that the bromothymol blue based indicator was highly efficient in showing changes in meat quality, having three stage colour transition, namely dark blue-turquoise-green. The results shown by the freshness indicator were supported by the physicochemical, microbiological and sensorial properties of the tested samples.

Another area of research is related to the application of essential oils, which is the case of a study conducted by Chaichi et al. (2021), who tested the synergistic antibacterial effect of cinnamon, shirazi-thyme and clove essential oils both *in vitro* and *in vivo* in chicken breast meat against *Escherichia coli*, *Pseudomonas fluorescens* and *Staphylococcus aureus*. The most sensitive bacteria was proved to be *S. aureus*, while the most resistant to the essential oils action was *P. fluorescens*. The combination of the three essential oils presented a synergistic effect with a 6-8 folds reduction in the minimum inhibitory concentration compared to double combination or single use of the tested essential oils. The results were sustained by the *in vivo* evaluation of essential oils action, preventing the growth of *P. fluorescens* in chicken breast for 12 days. Takma and Korel (2019) developed an active film based on PET, chitosan and alginate coating, incorporated with black cumin oil. The developed material showed antimicrobial activity against *E. coli* and *S. aureus*, and modified quality indicators in chicken meat packed in the presence of the films were also observed. Another study, performed by Mulla et al. (2017), used clove essential oil as coating for LLDPE, which was previously treated at its surface with chromic acid. The results of the study showed that the material presented great antimicrobial activity when used as packaging for chicken meat, against *Listeria monocytogenes* and *Salmonella typhimurium*.

Another approach in this domain is the application of bacteriocins in food packaging. Guo et al. (2014) coated PLA films with mixtures of chitosan (in acid solution) and acetic, levulinic and lactic acids. Further, nisin and lauric arginate ester (LAE) were added to the solution alone or in combination. The developed films were applied on turkey meat. The results showed that the coatings containing chitosan and LAE reduced *Listeria innocua* with about 4.5 log CFU/cm^2, while nisin showed less efficiency than LAE. Zhou et al. (2015) studied two application methods of plantaricin BM-1 and its effect on *L. monocytogenes* and spoilage bacteria in sliced vacuum packed cooked ham. The application on the surface of the ham of plantaricin BM-1 was more effective than the incorporation of the bacteriocin in the product. However, regardless of the application method, plantaricin BM-1 inhibited the development of *L. monocytogenes* during the first 21 days of refrigerated storage. In another study, Barbiroli et al. (2017) developed an active packaging containing sakacin-A, which was further applied on thin-cut veal meat slices with the purpose of *Listeria* inhibition. The results of the study showed a 1.5 log reduction of *L. innocua* after 48 hours incubation at 4°C in the veal slices packed using the developed active packaging, compared to control samples.

Therefore, the aim of the present study was the evaluation of the application of an active packaging film based on PLA/PHBV and nanoemulsion containing nisin and dill essential oil on fresh minced chicken meat quality.

MATERIALS AND METHODS

To carry out this experiment, boneless skinless chicken breast purchased from a butcher shop in Bucharest was minced and then packed, forming the following samples:

(1) The film obtained by ES (PLA coated by electrospinning with nanoemulsion with dill essential oil and nisin) placed at the base of the PET casserole in direct contact with the tested sample - **Chicken PLA/Nisin/Dill EO**.

(2) The film obtained by ES (PHBV coated by electrospinning with nanoemulsion with dill essential oil and nisin) placed at the base of the PET casserole in direct contact with the tested sample - **Chicken PHBV/Nisin/Dill EO**.

(3) The PET casserole considered as a Control - **Chicken Control**.

In each casserole, 100 g of minced chicken meat was packed and stored at 4±0.5°C, to

determine the quality and shelf life during storage (Figure 1).

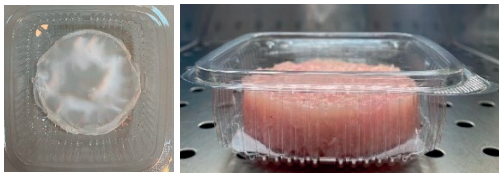


Figure 1. Experimental design

Further, free acidity determination was performed using the titration method with NaOH 0.1N in the presence of phenolphthalein as color indicator. pH was determined using a WTW INOLAB 720 series type pH meter, equipped with an automatic temperature compensator. Dry matter content (DM) was determined using a thermobalance type RADWAG MAC 50. A NOVASINA equipment was used for water activity (aw) determination. Freshness analysis consisting of the determination of free ammonia was evaluated using the Nessler reagent. Color determination was assessed with a HunterLab colorimeter, Miniscan XE Plus at room temperature. Furthermore, microbiological analysis were performed, consisting of TVC (total viable count), Enterobacteriaceae and *E. coli*/Coliforms determination, using dry medium plates (Compact Dry).

RESULTS AND DISCUSSIONS

The pH values (Table 1) had an increasing trend during the refrigerated storage period, for all the studied samples. However, values close to the initial pH value were obtained, the variations being insignificant. Further, the values obtained after determining the acidity of minced chicken meat samples showed a decreasing trend during the refrigerated storage period, the results being correlated with those obtained for the pH of the studied samples.

Table 1. Evolution of pH and free acidity of minced chicken meat during the refrigeration storage

Sample / Moment of analysis	pH				Acidity (oleic acid/100 g)			
	Day 0	Day 3	Day 5	Day 7	Day 0	Day 3	Day 5	Day 7
Chicken Control	6.07 ± 0.028	5.93 ± 0.000	6.42 ± 0.014	6.41 ± 0.183	6.805 ± 0.615	7.065 ± 0.190	5.93 ± 0.254	6.590 ± 0.735
Chicken PLA/Nisin/Dill EO	6.07 ± 0.028	5.99 ± 0.000	6.65 ± 0.063	6.66 ± 0.205	6.805 ± 0.615	7.445 ± 0.586	6.395 ± 0.360	6.230 ± 0.268
Chicken PHBV/Nisin/Dill EO	6.07 ± 0.028	5.94 ± 0.000	6.53 ± 0.000	6.62 ± 0.049	6.805 ± 0.615	6.915 ± 0.190	6.705 ± 0.289	6.705 ± 0.445

After analysing the data in Table 2, no significant changes were observed in the values obtained for dry matter for the analysed samples. As for aw, a trend of decreasing values can be observed for the chicken samples analysed and stored in a refrigerated state at a temperature of 4±0.5°C. This leads to the conclusion that the water available for the development of microorganisms has decreased, thus preventing the alteration of the product from a microbiological point of view.

Table 2. The values for dry matter (DM) and water activity (aw) of the tested samples during storage

Sample / Moment of analysis	DM%				a_w			
	Day 0	Day 3	Day 5	Day 7	Day 0	Day 3	Day 5	Day 7
Chicken Control	25.402 ± 2.013	25.615 ±0.327	25.991 ± 0.060	23.827 ± 0.779	0.988 ± 0.001	0.978 ± 0.002	0.972 ± 0.002	0.975 ± 0.000
Chicken PLA/Nisin/Dill EO	25.402 ± 2.013	25.365 ± 0.332	29.199 ± 4.570	25.140 ± 1.140	0.988 ± 0.001	0.978 ± 0.004	0.974 ± 0.000	0.972 ± 0.004
Chicken PHBV/Nisin/Dill EO	25.402 ± 2.013	27.611 ± 0.545	24.024 ± 0.837	25.349 ± 0.823	0.988 ± 0.001	0.973 ± 0.000	0.974 ± 0.000	0.971 ± 0.002

Regarding freshness analysis (Table 3), chicken samples packaged in the presence of films based on PLA and nisin and PHBV and nisin showed no signs of product degradation on day 7 (the last day of analysis), compared to the Control sample which showed signs of alteration on day 5 of analysis.

Table 3. NH3 presence in minced fresh chicken meat during refrigeration storage

Sample / Moment of analysis	Day 0	Day 3	Day 5	Day 7
Chicken Control	Negative	Negative	Weakly positive	Weakly positive
Chicken PLA/Nisin/Dill EO	Negative	Negative	Negative	Negative
Chicken PHBV/Nisin/Dill EO	Negative	Negative	Negative	Negative

Following the analysis of the colour of the minced chicken meat samples packaged in the presence of the studied films and stored in a refrigerated state at 4±0.5°C, it can be observed that the values of the parameters L*, a* and b* did not change significantly during the period of storage compared to the values obtained for the sample analysed on the day of packaging (Table 4), the samples maintaining their initial appearance during the storage period (Figure 2).

Table 4. The values of the L*, a* and b* parameters for the tested samples during refrigeration storage

Moment of analysis / Sample	Chicken Control			Chicken PLA/Nisin/Dill EO			Chicken PHBV/Nisin/Dill EO		
	L*	**a***	**b***	**L***	**a***	**b***	**L***	**a***	**b***
Day 0	56.71 ± 0.94	3.03 ± 0.40	14.57 ± 0.62	56.71 ± 0.94	3.03 ± 0.40	14.57 ± 0.62	56.71 ± 0.94	3.03 ± 0.40	14.57 ± 0.62
Day 3	56.33 ± 0.23	3.81 ± 0.24	15.70 ± 0.28	55.40 ± 0.40	4.87 ± 0.32	14.20 ± 0.14	55.41 ± 0.45	5.22 ± 0.25	15.91 ± 0.21
Day 5	57.64 ± 0.16	4.56 ± 0.18	15.26 ± 0.29	57.63 ± 0.21	4.43 ± 0.32	15.48 ± 0.27	55.40 ± 0.36	5.47 ± 0.16	16.13 ± 0.10
Day 7	56.94 ± 0.20	4.16 ± 0.36	14.57 ± 0.14	56.56 ± 0.25	4.56 ± 0.16	16.15 ± 0.20	55.58 ± 0.41	4.38 ± 0.09	16.75 ± 0.34

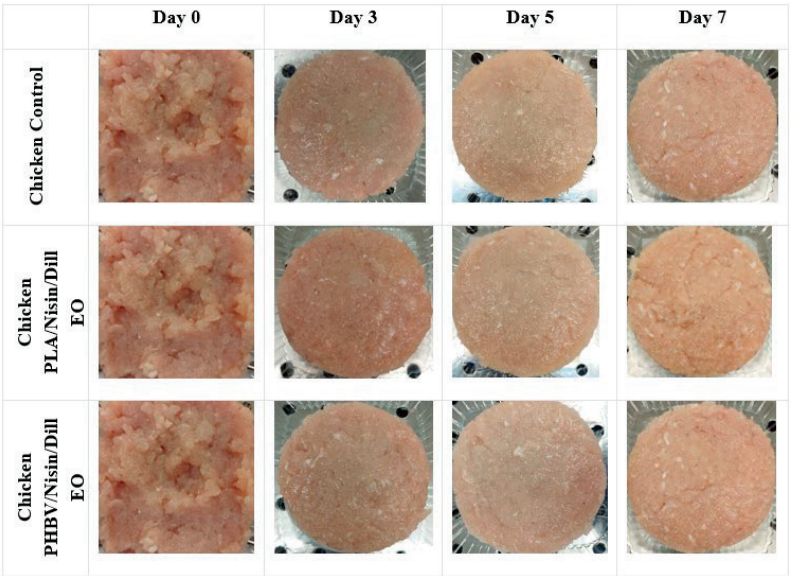


Figure 2. Appearance of minced chicken meat samples during the refrigerated storage period

Regarding the microbiological analysis, the evolution of NTG values, as shown in Table 5, shows an increase of approximately 1 logarithmic cycle during the storage period. However, at the end of the analysis period, the samples of minced chicken meat packaged in the presence of the tested films obtained lower values of this parameter compared to the control sample, which leads to the conclusion that the films used to package the product reduced the microbial activity within the samples.

Table 5. TVC values of fresh chicken meat during storage

Sample / Moment of analysis	Day 0	Day 3	Day 5	Day 7
	Total viable count (lgCFU)			
Chicken Control	4.060	4.91	5.21	5.57
Chicken PLA/Nisin/Dill EO	4.060	4.85	5.12	5.27
Chicken PHBV/Nisin/Dill EO	4.060	5.16	5.03	5.46

Table 6 shows both the presence of Enterobacteriaceae and *E. coli*/Coliforms in all analyzed samples, regardless of the packaging method. However, a microbial reduction can be observed in the samples packaged in the presence of films based on PLA/Nisin and PHBV/Nisin, compared to the control sample, during the refrigerated storage period at a temperature of 4±0.5°C.

Table 6. The values obtained following the determination of *E. coli*/Coliforms and Enterobacteriaceae

Sample / Moment of analysis	Enterobacteriaceae*				*E. coli*/Coliforms*			
	Day 0	Day 3	Day 5	Day 7	Day 0	Day 3	Day 5	Day 7
Chicken Control	+++	++	++	++	+/+++	+/+	+/+	+/+
Chicken PLA/Nisin/Dill EO	+++	++	+	+	+/+++	-/+	-/+	-/+
Chicken PHBV/Nisin/Dill EO	+++	++	+	+	+/+++	-/+	-/+	-/+

* - no CFU were identified + under 50 CFU ++ over 50 CFU +++ over 100 CFU

CONCLUSIONS

According to the results obtained, the minced chicken meat samples packed in the presence of the two studied films (PLA/Nisin/Dill EO and PHBV/Nisin/Dill EO) and stored at 4°C demonstrated a good behavior for 7 days, maintaining their quality, while the control sample started the process of degradation after only 5 days from packaging. The microbiological analyzes showed that the microbial load of the tested samples had a continuous decrease during the refrigeration period for all the analyzed samples. However, the samples packaged in the presence of PLA/Nisin/Dill EO and PHBV/Nisin/Dill EO films presented lower values of the microbial load, compared to the Control sample during the storage period, demonstrating that these materials have the potential to slow down the development of microorganisms in fresh minced chicken meat.

ACKNOWLEDGEMENTS

This work was supported by a grant of the Romanian Ministry of Education and Research, CCCDI - UEFISCDI, project number PN-III-P2-2.1-PED-2019-2878, within PNCDI III, contract no. 379PED/2020, acronym BIOMATFOOD

REFERENCES

Barbiroli, A., Musatti, A., Capretti, G., Iametti, S. & Rollini, M. (2017). Sakacin-A antimicrobial packaging for decreasing *Listeria* contamination in thin-cut meat: preliminary assessment. *Journal of the Science of Food and Agriculture*, *97*(3), 1042-1047.

Chaichi, M., Mohammadi, A., Badii, F. & Hashemi, M. (2021). Triple synergistic essential oils prevent pathogenic and spoilage bacteria growth in the refrigerated chicken breast meat. *Biocatalysis and Agricultural Biotechnology*, *32*, 101926.

Dourou, D., Doulgeraki, A.I., Vitsou-Anastasiou, S., Argyri, A.A., Chorianopoulos, N.G., Nychas, G.J.E. & Tassou, C.C. (2023). Deciphering the growth responses and genotypic diversity of bioluminescent

Photobacterium phosphoreum on chicken meat during aerobic refrigerated storage. *International Journal of Food Microbiology*, *405*, 110334.

Guo, M., Jin, T.Z., Wang, L., Scullen, O.J. & Sommers, C.H. (2014). Antimicrobial films and coatings for inactivation of *Listeria innocua* on ready-to-eat deli turkey meat. *Food Control*, *40*, 64-70.

Hrustemović, E., Caklovica, F. & Dedibegovic, J. (2022). Food pathogen of chicken meat *Campylobacter* spp.: a review. *AgroLife Scientific Journal*, *11*(1), 80-85.

Karaca, I.M., Haskaraca, G., Ayhan, Z. & Gultekin, E. (2023). Development of real time-pH sensitive intelligent indicators for monitoring chicken breast freshness/spoilage using real packaging practices. *Food Research International*, *173*, 113261.

Katiyo, W., de Kock, H.L., Coorey, R. & Buys, E.M. (2020). Sensory implications of chicken meat spoilage in relation to microbial and physicochemical characteristics during refrigerated storage. *LWT - Food Science and Technology*, *128*, 109468.

Mohamed-Noor, S.E., Shuaib, Y.A., Suliman, S.E. & Abdalla, M.A. (2012). Study of microbial contamination of broilers in modern abattoirs in Khartoum state. *The Annals of the University Dunarea de Jos of Galati Fascicle VI – Food Technology*, *36*(1), 74-80.

Mulla, M., Ahmed, J., Al-Attar, H., Castro-Aguire, E., Arfat, Y.A. & Auras, R. (2017). Antimicrobial efficacy of clove essential oil infused into chemically modified LLDPE film for chicken meat packaging. *Food Control*, *73*, 663-671.

Shao, L., Tian, Y., Chen, S., Xu, X. & Wang, H. (2022). Characterization of the spoilage heterogeneity of Aeromonas isolated from chilled chicken meat: *in vitro* and *in situ*. *LWT - Food Science and Technology*, *162*, 113470.

Takma, D.K. & Korel, F. (2019). Active packaging films as a carrier of black cumin essential oil: Development and effect on quality and shelf-life of chicken breast meat. *Food Packaging and Shelf Life*, *19*, 210-217.

Tsafrakidou, P., Sameli, N., Bosnea, L., Chorianopoulos, N. & Samelis, J. (2021). Assessment of the spoilage microbiota in minced free-range chicken meat during storage at 4°C in retail modified atmosphere packages. *Food Microbiology*, *99*, 103822.

Wang, Y., Tang, M., Ma, Y. & Xu, B. (2023). Isolation, identification and spoilage capability of dominant spoilage bacteria on Dezhou-braised chicken with different packaging. *LWT - Food Science and Technology*, *182*, 114710.

Zhou, H., Xie, Y., Liu, H., Jin, J., Duan, H. & Zhang, H. (2015). Effects of Two Application Methods of Plantaricin BM-1 on Control of *Listeria monocytogenes* and Background Spoilage Bacteria in Sliced Vacuum-Packaged Cooked Ham Stored at 4°C. *Journal of Food Protection*, *78*(10), 1835-1841.

Scientific Bulletin. Series F. Biotechnologies, Vol. XXVII, No. 2, 2023
ISSN 2285-1364, CD-ROM ISSN 2285-5521, ISSN Online 2285-1372, ISSN-L 2285-1364

OPTIMIZATION OF A qPCR METHOD FOR THE DETECTION OF VIABLE *Saccharomyces* AND non-*Saccharomyces* CELLS DURING WINEMAKING

Virgil VRĂJMAȘU[1], Raluca-Ștefania RĂDOI-ENCEA[1, 2], Oana-Alina BOIU-SICUIA[1], Camelia Filofteia DIGUȚĂ[1], Florentina MATEI[1, 2]

[1]University of Agronomic Sciences and Veterinary Medicine of Bucharest, Faculty of Biotechnology, 59 Mărăşti Blvd, District 1, Bucharest, Romania
[2]Transylvania University of Brașov, Faculty of Food Industry and Tourism, 140 Castelului Street, Brașov, Romania

Corresponding author email: camifilo@yahoo.com

Abstract

*The use of Saccharomyces and non-Saccharomyces (NS) during wine making is a new concept to keep the wines' local specificity. Different molecular tools were developed to quantify Saccharomyces yeast during winemaking, but for the NS several limitations were detected. In this regard, our work focused on the development of a qPCR method employing propidium monoazide (PMA) for the detection of NS viable cells. Very good correlation parameters and standard curves were obtained during the optimisation method for Saccharomyces reference strain versus NS belonging to Candida stellata and Torulaspora delbrueckii. The detection limit varied from 38 fg/µL to 49 fg/µL which corresponds to quantification limits of 70 CFU/mL to 1.03*10^2 CFU/mL. The optimised PMA-qPCR method can be considered as a rapid and suitable method for assessing the viable microbial count for both NS yeast species.*

Key words*: alcoholic fermentation, yeast, Saccharomyces, non-Saccharomyces, PMA, qPCR.*

INTRODUCTION

Spontaneous fermentation is an uncontrolled process, in which the alteration microorganisms could rapidly multiply and reach high levels quickly, which may negatively impact the quality of the finished product. The conventional practice of producing wines on an industrial scale with the use of *Saccharomyces* species involves controlled fermentation from all points of view, but, for a greater specificity, a possible direction might be the use of local yeasts from each geographical region, in addition to using grapes harvested from those areas (Radoi-Encea et al., 2023). Knight et al. (2015) delivered the concept of "microbial terroir", which implies that the microbial consortia that include *Saccharomyces* and non-*Saccharomyces* (NS) yeast, in a certain wine-growing area are specific to that certain area and are producing flavors typical of the area. NS yeasts, also known as wild yeasts, include diverse species, most of which belong to the genera *Hanseniaspora, Candida, Lachancea, Metschnikowia, Pichia, Torulaspora,* and *Zygosaccharomyces*. Although there are several PCR-based fingerprinting methods for typing *S. cerevisiae* strains, there are not so many available for different NS yeasts, and the main limitation has been the lack of sufficient genomic sequence data (Nisiotou et al., 2022). Meanwhile, the main disadvantage of DNA-based quantification methods is their inability to distinguish between viable and non-viable cells, as dead cells may also retain a significant amount of DNA.

By the use of the conventional cultivation-based methods wine-associated NS species can often be underestimated, due to variable growing rates of the different microorganisms in culture media or to the presence of viable but non-cultivable (VBNC) microorganisms. In this regard, cell viability could be defined in different ways, but it is mainly based on the presence of an intact membrane or to some metabolic activities. Some methods are taking advantage of dyes, such as propidium monoazide (PMA) or ethidiummonoazide (EMA), which are able to penetrate in membrane-compromised cells or in the dead cells and covalently bind to nucleic acid after photoactivation. Because the DNA that is covalently bound to

these dyes cannot be PCR amplified, only the DNA from viable cells, including the VBNC, can be detected and the application of quantitative PCR (qPCR) could specifically reveal viable cells (Rizzotti et al., 2015).
Our trials focused on the development of a PMA-qPCR method for the detection of NS viable cells during wine alcoholic fermentation, focusing on less studies NS yeast like *Wickerhamomyces anomalus*, *Candida stellata* and *Torulaspora delbrueckii*

MATERIALS AND METHODS

Microorganisms and yeast sample preparation

In this study were targeted one strain of *Saccharomyces cerevisiae* (EC 1118) and three non- *Saccharomyces* (non-S) isolated from local grapes and identified by molecular tool as *Wickerhamomyces anomalus* (synonym *Pichia anomala*) (MI 201), *Candida stellata* (MI 202) and *Torulaspora delbrueckii* (MI 203). These are stored in the microbial collection of USAMV of Bucharest, Faculty of Biotechnology. Their preparation follows several steps. After 24 hours cultivation in PDB medium (Difco Laboratoires, Franța) at 25ºC the viable cells were counted optical microscopy tool and verified by plating.
To prepare the dead yeast cells, 5 mL of fresh culture were kept for 1 hour at 80ºC and the viability was checked in the end.
For the qPCR test 1 mL of viable and dead yeast cells was centrifuged at 4000 rpm for 10 minutes at 4ºC; the sediment was re-suspended in 400 µL distilled water. PMA (propidium monoazide) was added under dark conditions, respectively 1 µL (20 mM stock solution) in 400 µL of sample, because PMA is know as a photo-reactive DNA-binding dye used in viability PCR. The incubation time was 10 minutes at room temperature, followed by twice photolysis treatment for 10 minutes and a centrifugation at 5000 rpm for 10 minutes; after that, the DNA extraction was performed.

DNA preparation

The DNA extraction followe the steps of the kit ZR Fungal/Bacterial MiniPrep™ (Zymo Research, SUA), under small modifications. The extracted DNA purity was measured by SpectraMax® QuickDrop ™ (Molecular Devices, SUA). Before the manipulation, the extracted DNA was stored a -20ºC.

Yeast quantification by qPCR

The quantification was performed in a RT-PCR (thermocycler) Rotor-Gene 6000 (Corbett Life Science, Sydney, Australia).
The employed primers are described below. For *W.anomalus* Wu et al. (2017): W-CAR1-F (GCAATAGGGTTCCAAAAGTG) and W-CAR1-R (AGCCATTTCCACAACTTGA). For *C.stellata* Garcia et al. (2017).: CS1-F (AGTAACGGCGAGTGAACAGG) and CS1-R (GGCTATCACCCTCTATGGCG). For *T. delbrueckii* (Zott et al., 2010): Tods L2 (CAAAGTCATCCAAGCCAGC) and Tods R (TTCTCAAACAATCATGTTTGGTAG). For *S. cerevisiae* (Zott et al., 2010): SC (GAAAACTCCACAGTGTGTTG) and SC2 (GCTTAAGTGCGCGGTCTTG).
The reaction mix (final volume 25 µL) included the following: 12.5 µL Maxima SYBR Green qPCR Master Mix (2x), 0.125 µL Primer F (100 µM), 0.125 µL Primer R (100 µM), 7.25 µL water free Rnase and 5 µL DNA.
The amplification program for Tods L2/Tods R2 and SC1/SC2 primers, according to Zott et al. (2010) was the following: initial denaturation at 95°C/3 min; 40 cycles of denaturation at 95°C/15 sec + hybridisation at 60°C/60 sec + elongation at 72°C/30 sec.
The amplification program for W-CAR1-F/W-CAR1-R primers, according to Wu et al. (2017) was the following: initial denaturation at 95°C/3 min; 40 cycles of denaturation at 95°C/30 sec + hybridisation at 50°C/30 sec + elongation at 72°C/40 sec.
The amplification program for CS1-F/CS1-R primers, according to Garcia et al. (2017) was the following: initial denaturation at 95°C/3 min; 40 cycles of denaturation at 95°C/30 sec + hybridisation at 50°C/30 sec + elongation at 72°C/40 sec.
In the end, the temperature was raised with 1ºC at each 10 seconds, from 65°C to 95°C to get the right melting temperature (Tm) fo each specific qPCR.

The threshold cycle (Ct) calculation

The Ct was automatically defined by the use of Rotor-Gene Q Series software. According to the provider, to measure if the PMS has adequately inhibited the dead cells DNA amplification a ΔCt was calculated for each reference yeast strain, according to the following formula:

$\Delta Ct \text{ viable} = Ct_{\text{(viable, PMA treated)}} - Ct_{\text{(viable, non-PMA treated)}}$

$\Delta Ct \text{ dead} = Ct_{\text{(dead, PMA treated)}} - Ct_{\text{(dead, non PMA treated)}}$

The extracted DNA treated with PMA was decimally diluted to generate the standard curve. The PCR efficiency (E%) was calculated according to the following formula:

$E = [10^{-1/slope}-1] \times 100$, and the slope value was extracted form the standard curve according to $C_T = f(\log Q)$, where Q is equal with the DNA quantity for each reference strain (Figure 1). The DNA quantity for each yeast reference strain it was obtained by the extrapolation of the Ct value established on the standard curve.

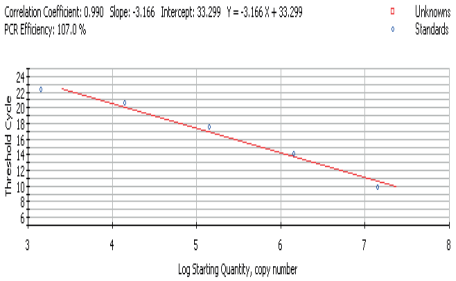


Figure 1. The slope calculation (CT versus log DNA)

RESULTS AND DISCUSSIONS

In a first step of the qPCR method optimisation, the thermic treatment efficiency was determined.

The thermic treatment efficiency was confirmed by CFU (colony formic unit) technique on PDA medium, by comparing the treated cell growth absence (thermal and thermal + PMA) with the viable cells growth treated and non-PMA treated. It was noticed o slight viability decrease after the use of the PMA on the viable cells (Table 1).

The fungal DNA extracted from the viable and dead cells, treated and non-PMA treated it was isolated by a simple and efficient method, using a routine commercial kit.

Table 1. The yeast cells viability treated and non-PMA treated

Samples	Log CFU/mL
W. anomalus viable, non-PMA	7.4
W. anomalus viable, PMA	7.3
W. anomalus dead, non-PMA	0
W. anomalus dead, PMA	0
C. stellata viable, non-PMA	7.6
C. stellata viable, PMA	7.0
C. stellata dead, non-PMA	0
C. stellata dead, PMA	0
T. delbrueckii viable, non-PMA	7.5
T.delbrueckii viable, PMA	7.3
T. delbrueckii dead, non-PMA	0
T. delbrueckii dead, PMA	0
S. cerevisiae viable, non-PMA	7.5
S. cerevisiae viable, PMA	7.1
S. cerevisiae dead, non-PMA	0
S. cerevisiae dead, PMA	0

The DNA quality and concentration were tested in comparison by two different tools: a SpectraMax® QuickDrop ™ (Molecular Devices, SUA) and agarose gel electrophoresis. The results are presented in Table 2; good quality and purity DNA was obtained, ready for the qPCR reactions.

Table 2. The purity and concentration of the exttacted DNA

Samples	Conc. (ng/µL)	A260	A 260 nm/280 nm
S. cerevisiae viable, non-PMA	55	0.042	1.854
S. cerevisiae viable, PMA	45	0.018	1.779
S. cerevisiae dead, non-PMA	13	0.020	1.501
S. cerevisiae dead, PMA	8	0.019	1.751
C. stellata viable, non-PMA	60	0.078	1.636
C. stellata viable, PMA	38	0.056	1.521
C. stellata dead, non-PMA	6	0.16	1.854
C. stellata dead, PMA	4	0.010	1.558
T. delbrueckii viable, non-PMA	55	0.076	1.751
T.delbrueckii viable, PMA	49	0.016	1.824
T. delbrueckii dead, non-PMA	14	0.015	1.752
T. delbrueckii dead, PMA	4	0.019	1.905
W. anomalus viable, non-PMA	51	0.071	1.854
W. anomalus viable, PMA	45	0.068	1.812
W. anomalus dead, non-PMA	14	0.027	1.675
W. anomalus dead, PMA	10	0.018	1.714

The PMA is a photoreactiv compound, extremely selective, which penetrates only the microbial dead cells which have a compromise membrane; this compromise membrane is stable binding thorough covalent strings to the DNA; in this way, the DNA extraction is inhibited, followed by a delayed or inhibited amplification by qPCR.

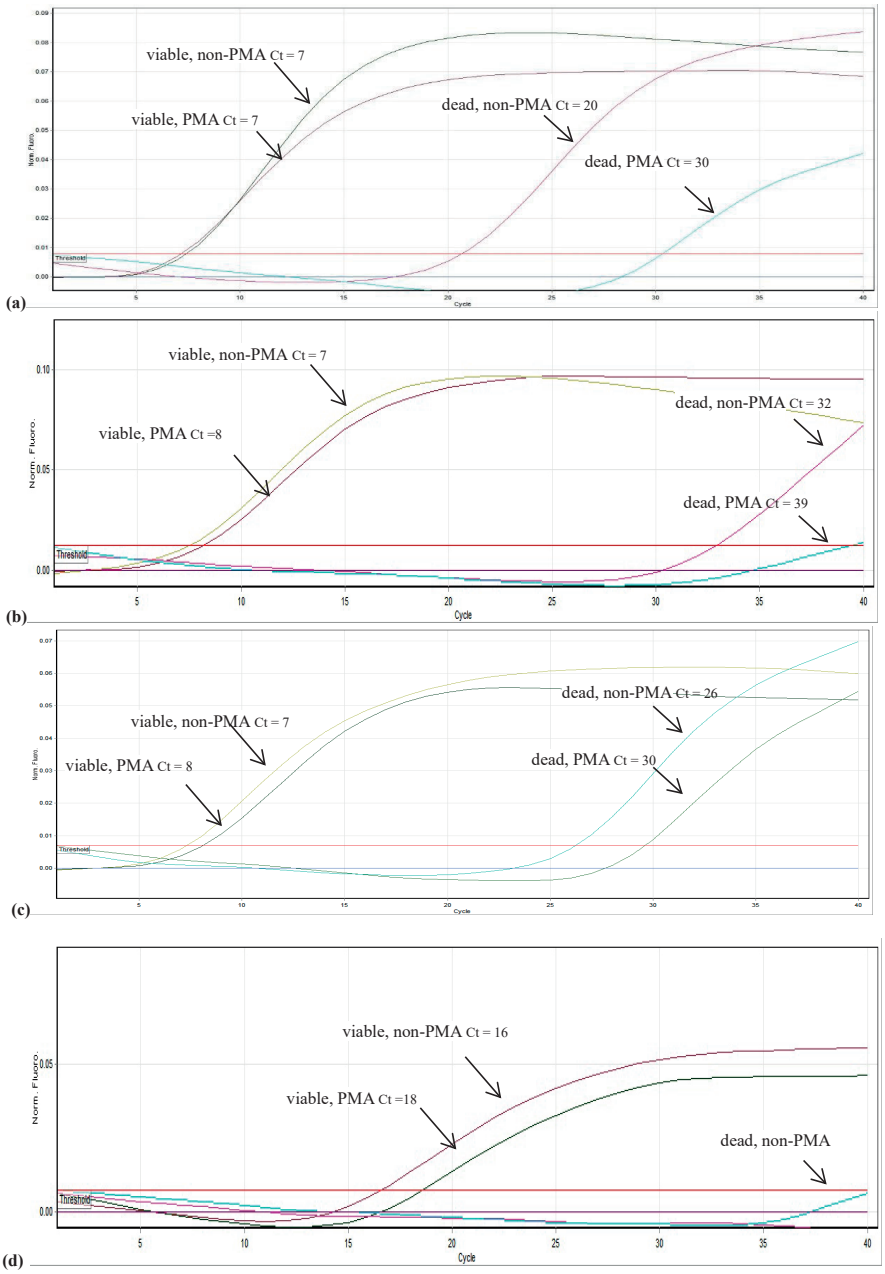


Figure 2. Schematic reprsentation of the PMA treatment in the viable and dead cells for different yeast strains. *S. cerevisiae* (a), *C. stellata* (b), *T. delbrueckii* (c), *W. anomalus* (d)

Depending on the reference strain, it was noticed that the viable PMA treated cells has a delayed Ct comparing to the viable non-PMA treated cells (Figure 2).

Moreover, it was noticed a significant delay of the non-PMA treated dead cells Ct comparing to the non-PMA treated viable cells (Figure 2). Depending on the yeast species, the PMA treated dead cells had a significant reduction of the Ct comparing to the non-PMA treated dead cells. In the case of *W. anomalus*, the Ct of the non-treated and treated dead cells could not be

detected (Figure 2d); much more experiments should be performed for the optimisation.
To figure out if the PMA has inhibited in a adequate manner the DNA amplification of the dead cells, it was calculated the ΔCt for each reference strain (Figure 3). According to the provider recommendations, the expected result for the viable cells is a ΔCt close to zero (+/-1).

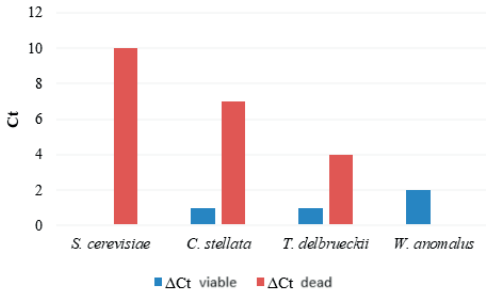


Figure 3. The effect of the PMA treatment on the Ct of the viable and dead cells

In our case, ΔCt for *S. cerevisiae, C. stellata* and *T. delbrueckii* it was 0, respectively 1, which lead to the conclusion that the PMA treatment did not affected the amplification of the viable cells' DNA. In the case of *W. anomalus* it was obtained a higher ΔCt, and new trials need to be performed for the protocol optimisation.In the case of the dead cells ΔCt, it should be higher than 4. In our trials, ΔCt for *S. cerevisiae, C. stellata* and *T. delbrueckii* reached the following values: 10, 7, respectively 4. This indicates that the PMA treatment has inhibited the amplification of the dead cells' DNA.
To generate the standard curve for each reference strain it was used genomic DNA from the PMA treated viable cells. The generated results are enclosed in Table 3. The standard curves' slopes were very similar (close to -3.4 ÷-3.5), which correspond to an amplification efficiency varying between 90.51% to 95.98%. It can be easily noticed that it was obtained a very good linear corelation (R^2), with values from 0.9908 to 0.9959.
Also, good results were obtained in the case of *S. cerevisiae, C. stellata* and *T. delbrueckii* for the detection and quantification limits, meaning that the detection limit varied from 38 fg/µL to 49 fg/µL which corresponds to quantification limits of 70 CFU/mL to $1.03*10^2$ CFU/mL. Similar results for the PMA-qPCR detection limit of *S. cerevisiae* were obtained by different groups (Xu-Cong et al., 2016; Navarro et al., 2020).

Table 3. Parameters of the DNA standard curves obtained by qPCR for *Saccharomyces* and non- *Saccharomyces* reference strains

Yeast species	Slope	Y intersection	R^2	Efficiency (%)	Detection limit	Quantification limit (CFU/mL)
S. cerevisiae viable, PMA	-3.5533	33.866	0.9948	91.17	45 fg/µL	$1.03*10^2$
C. stellata viable, PMA	-3.4221	33.806	0.9959	95.98	38.fg/µL	$9.6*10^1$
T. delbrueckii viable, PMA	-3.5724	35.247	0.9933	90.51	49 fg/µL	$7*10^1$
W. anomalus viable, PMA	-3.5467	33.92	0.9908	91.40	45 pg/µL	$7*10^3$

After the optimisation process, we figured out that the application of PMA-qPCR on wine samples could produce results in one working day, thus presenting a great advantage when compared with the 5-7 days necessary to obtain results from conventional culturing methods.
Similar results were reported by Rizzotti et al, (2015).

CONCLUSIONS

Several NS yeast are of high interest nowadays to obtain distinguished wines, with low alcohol content and specific aromatic profile. In our attempt, we tried to develop a rapid method to be able to supervise the development of *Saccharomyces* versus NS viable cells, which may contribute to the final character of the product. As dye, it was used propidium monoazide (PMA) which proved to be very effective on three put of the four studied species. The detection limit varied from 38 fg/µL to 49 fg/µL which corresponds to quantification limits of 70 CFU/mL to $1.03*10^2$ CFU/mL. In this regard, PMA-qPCR can be considered as a rapid and suitable method for

assessing the viable microbial count for *Candida stellata* and *Torulaspora delbrueckii* versus *Saccharomyces cerevisiae.* Further investigations are requested to optimise the method for other NS species, which are in minority by the end of the wine fermentation.

REFERENCES

García M, Esteve-Zarzoso B, Crespo J, Cabellos JM, Arroyo T. (2017). Yeast Monitoring of Wine Mixed or Sequential Fermentations Made by Native Strains from D.O. "Vinos de Madrid" Using Real-Time Quantitative PCR. *Front Microbiol*., 8:2520.

Knight, S.; Klaere, S.; Fedrizzi, B.; Goddard, M.R. (2015). Regional Microbial Signatures Positively Correlate with Differential Wine Phenotypes: Evidence for a Microbial Aspect to Terroir. *Sci. Rep*., 5, 14233.

Navarro, Y.; Torija, M.-J.; Mas, A.; Beltran, G. (2020). Viability-PCR Allows Monitoring Yeast Population Dynamics in Mixed Fermentations Including Viable but Non-Culturable Yeasts. *Foods*, *9*, 1373. https://doi.org/10.3390/foods9101373

Nisiotou, A.; Gyftogianni, E.; Banilas, G. (2022). Evaluation of Different Molecular Markers for Genotyping Non-*Saccharomyces* Wine Yeast Species. *Microbiol. Res. 13*, 643-654. https://doi.org/10.3390/microbiolres13030046

Rădoi-Encea R.Ş., Pădureanu V., Diguță C.F., Ion M., Brînduşe E., Matei F. (2023). Achievements of Autochthonous Wine Yeast Isolation and Selection in Romania - A Review. *Fermentation*. 9(5): 407. https://doi.org/10.3390/fermentation9050407.

Rizzotti L., Levav N., Fracchetti F., Felis G.E., Torriani 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,* 47, 407-412, https://doi.org/10.1016/j.foodcont.2014.07.052.

Wu Q, Cui K, Lin J, Zhu Y, Xu Y (2017). Urea production by yeasts other than Saccharomyces in food fermentation. *FEMS Yeast Res.*, 17(7):8.

Xu-Cong Lv, Yan Li, Wan-Wei Qiu, Xiao-Qi Wu, Bing-Xin Xu, Yi-Ting Liang, Bin Liu, Shao-Jun Chen, Ping-Fan Rao, Li Ni (2016). Development of propidium monoazide combined with real-time quantitative PCR (PMA-qPCR) assays to quantify viable dominant microorganisms responsible for the traditional brewing of Hong Qu glutinous rice wine, *Food Control*, 66, 69-78, https://doi.org/10.1016/j.foodcont.2016.01.040.

Zott K., Claisse O., Lucas P., Coulon J., Lonvaud-Funel A., Masneuf-Pomarede I. (2010). Characterization of the yeast ecosystem in grape must and wine using real-time PCR. *Food Microbiol*., 27: 559–567.

ISSN 2285 – 1364
ISSN-L 2285 – 1364