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Faculty of Biotechnology

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SUMMARY

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DEVELOPMENT OF A BLUNT-END CLONING STRATEGY TO CONFIRM THE *Rvi2* GENE IN APPLE VARIETIES CULTIVATED IN ROMANIA

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Abstract

The aim of this study was to construct a bacterial artificial chromosome (BAC) library to confirm the presence and structure of the Rvi2 gene, which provides resistance to apple scab caused by Venturia inaequalis, in several apple cultivars developed in Romania. This approach is intended to aid in detailed genetic analysis to improve apple breeding programs. The clones were sequenced and the results were compared with the sequence of the Rvi2 gene registered in the "National Center for Biotechnology Information" database (locus "AY626824"). BLAST analysis confirmed the presence of the Rvi2 gene in all 20 varieties: 'Alex', 'Bistrițean', 'Cezar', 'Ciprian', 'Dany', 'Discoprim', 'Delicios de Voinesti', 'Estival', 'Ionaprim', 'Luca', 'Pomona', 'Redix', 'Remar',', 'Romus 3', 'Romus 4', 'Romus 5', 'Salva', 'Starkprim', 'Voinea' and 'Voincel'. Query identity ranged from 94.6% to 100% for the forward primer and from 95.39% to 100% for the reverse primer, with query coverage ranging from 85% to 92% for the forward primer and 80% to 93% for the reverse primer. The results confirm the presence of the Rvi2 gene, demonstrating high sequence identity and coverage.

Key words*: breeding, competent cells, bacterial artificial chromosome, BLAST, resistance, Rvi2.*

INTRODUCTION

The SCAR amplification technique is technically simple and easy to perform. The main limitation is that the sequence data in the RAPD polymorphic fragment are necessary to design SCAR primers. This requirement of prior knowledge of sequence information hinders using the SCAR technique, as the costs for developing primers are high. Compared to RAPD primers, SCAR primers are longer, and the low reproducibility constraints associated with RAPD analysis are overcome in SCAR. The reason for improved reproducibility is that the PCR reaction is less sensitive to reaction conditions (Cheng et al., 2016; Abdin et al., 2012). In addition, being a molecular marker-based only on PCR, without restriction enzymes, a smaller amount of DNA is required for SCAR analysis compared to RFLP and AFLP techniques, where the concentration of DNA should not be less than 300 ng/µl. Due to their low acquisition cost and high reproducibility, SCAR markers have proven to be a valuable tool and a practical way to screen numerous samples simultaneously (Li et al., 2012; Kiran et al., 2010). $OPL19_{433}$ SCAR was developed by cloning the 550 bp fragment from the RAPD marker $OPL19_{550}$, located near the *Rvi2* gene, at a distance of 2.5 cM (Gardiner et al., 1999; Bus et al., 2000). The SCAR marker was also mapped at 1 cM (Bus et al., 2005b close to *Rvi2* (*Vh2*) in differential host 2 ('TSR34T15'). Still, it is also close to the *Rvi8* gene (*Vh8*) in differential host 8 (*Malus sieversii* W193B), being considered very useful for identifying varieties carrying the Vr gene (*Rvi2* or *Rvi8*), as demonstrated by Bus et al. (2005a). The identified sequences of the 433 bp fragments confirmed that the two genes belong to the same locus; in other words, they have homologous loci. The OPL19 marker has been used successfully in many research studies. Molecular studies in Greece on 20 domestic apple

cultivars cultivated in various regions led to identifying the *Rvi8* gene for nine varieties out of 20 (Karapetsi et al., 2020). Khankishiyeva (2020) used the OPL19 marker in a molecular screening for five, respectively eight apple varieties selected from the fruit gardens of the Fruit and Tea Growing Research Institute of Azerbaijan and twenty donors reference apple varieties from Julius Kühn-Institut (JKI) Dresden, Germany. Nurtaza et al. (2022) selected specimens of the species *M. niedzwetzkyana*, as follows: 17 specimens from the collection of the Astana Botanical Garden, three specimens from the Talgar Pomological Garden and three specimens from the Tscherkesay Canyon. Following the study, the OPL19 marker amplified the 433 bp fragment on all 23 samples. Using the OPB18 marker, Nurtaza et al. (2022) distinguished between the *Rvi2* gene and the *Rvi8* gene, only five species carrying the *Rvi8* gene, the rest having the *Rvi2* gene. The usefulness of the OPL19 marker can be seen in other research studies: Urbanovich and Kazlovskaya, 2008; Lyzhin and Saveleva, 2018; Dar et al., 2020; Höfer et al., 2021; Uzun et al., 2023; Iancu et al., 2022; 2023a; 2023b). Building a cDNA library is a complicated and laborious technology that involves a series of enzymatic procedures. After removing 3' and 5' heads, vector ligation is possible by treating cDNA with a blunt enzyme or T4 DNA Polymerase. The recombinant construct (cDNA-vector) is introduced into a host (*Escherichia coli*) to clone and build the BAC library (Cseke et al., 2011). The genomic region of interest is identified by sequencing clones and analyzing sequencing results (Bandara, 2016). Plasmid DNA was first isolated by Radloff et al. (1967), using a method that is based on differentiated absorption of ethidium bromide. Alkaline lysis Birnboim and Doly (1979) and boiling lysis Holmes and Quigley (1981) have become the most classical methods used in molecular biology. The aim of this work is to build a bacterial artificial chromosome (BAC) library to confirm the presence and structure of the *Rvi2* gene, known to confer resistance to the apple root rot (*Venturia inaequalis*), in certain apple cultivars developed in Romania. This approach will facilitate detailed genetic analysis and marker-assisted selection to improve apple breeding programs.

MATERIALS AND METHODS

Biological material studied

The 20 genotypes were created at Research Station for Fruit Growing Voinești ('Cezar', 'Ciprian', 'Discoprim', 'Delicios de Voinesti', 'Luca', 'Pomona', 'Redix', 'Remar', 'Voinea', 'Voinicel'), Research Station for Fruit Growing Bistrița ('Alex', 'Bistrițean', 'Dany', 'Ionaprim', 'Salva', 'Starkprim'), Research Station for Fruit Growing Cluj ('Estival') and registered by the Research Institute for Fruit Growing Pitesti ('Romus 3', 'Romus 4', 'Romus 5').

Chemical material

The fragment of interest was inserted in the vector using the "CloneJET PCR Cloning" kit from Thermo Scientific, the vector "pJET1.2" being of blunt type. Also included in this kit are two enzymes, "DNA Blunting Enzyme" and "T4 DNA Ligase", but also the buffer for the reactions to be initiated, "2X Reaction Buffer". The competent cells "DH10B Competently Cells" attached to the cloning kit were used for transformation. Also, the SOC medium was included in the competent cell kit. The LB medium (Luria Bertani) was prepared in the laboratory, having the following ingredients at 1 liter: 10 g tryptone, 5 g yeast extract, 10 g NaCl, 15 g agar and ampicillin 1 ml (100 mg/ml). The ampicillin stock solution was prepared by dissolving 5 g of ampicillin in 50 ml of ultra-pure water. The "Alkaline lysis method" was used to isolate plasmid DNA. This method involves preparing three solutions: alkaline solution I (glucose 50 mM, Tris-HCl 25 mM, EDTA 10 mM) at pH 8.0, Alkaline Lysis Solution II (0.2 N NaOH, 1% (g/v) SDS) and Solution III (potassium acetate 5 M and glacial acetic acid 2M). The Stock solutions were prepared at a volume of 100 ml in ultra-pure water.

PCR amplification

Has been worked in a reaction volume of 15 µl, including the following components in the final concentration: 11.5 µl "MyTaq™ Red Mix" from Meridian Bioscience; 0.1 µl for each of the F and R primers (0.6 µM in the final volume of reaction), 3 µl DNA (75 ng / µl) and 0.3 µl ultrapure water, under the following reaction conditions: initial denaturation at 95°C for 2.45

min.; 40 cycles of 1 min. at 94°C, 1 min. at 58°C and 2 min. at 72°C; final extension of 10 min. to 72°C.

Excise the fragment of interest

The amplified DNA fragment was excised from the gel using Sigma Aldrich's horizontal transilluminator, "MyView Compact UV Transilluminator", model 3100-E, producer Accuris Instruments, USA (Figure 1 and Figure 2).

Figure 1. Horizontal Transilluminator for excision of fragments of interest from agarose gel

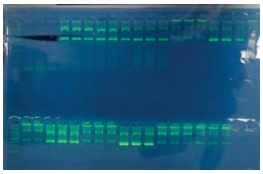


Figure 2. Excision of the fragment of interest from agarose gel

Purification of the excised fragment

It was made using the Geneaid "Gel/PCR DNA Fragments Extraction Kit", according to the manufacturer's instructions.

Blunting protocol

Using the DNA Blunting Enzyme included in the cloning kit, pipetting on the ice at the laminar flow hood, easy vortexing and incubation at 70^0C for five min., sticky ends have been converted to blunt ends. At the end of incubation, the samples were moved directly to the ice. The reaction mixture was: 2X Reaction Buffer (10 µl), excised and purified fragment (25 ng/µl), DNA Blunting Enzyme (1 µl), ultrapure water (5 µl), the total volume being 18 µl.

Blunt ligation reactions. Plasmid construction

The DNA fragment was ligated to the vector by adding the cloning vector and the binding enzyme over the components of the blunt reaction, also on ice. The mixture was vortexed and incubated at room temperature for five minutes. The volume of one reaction was 20 µl: 2x Reaction Buffer (10 µl), pJET 1.2/blunt Cloning Vector (1 µl), T4 DNA Ligase (1 µl), purified PCR product (1 µl), and 7 µl ultrapure water.

Transformation of competent cells

Initially stored at -80^0C, the competent cells were thawed slowly on wet ice. After thawing, each competent cell tube was aliquoted in volumes of 50 µL into Eppendorf tubes. Over the aliquot, 5 µl plasmid DNA volumes were added for each sample. After easy mixing, the plasmid DNA and competent cells were incubated on ice for 30 minutes. To relax the wall of competent cells, the cells received a brief heat shock for 30 seconds at 42^0C and were placed immediately on the ice for 2 minutes without mixing or shaking the tubes. In the end, it was pipetted 250 µl SOC medium over each sample and then incubated (at 37°C) for an hour, in a slightly vertical position, in a Thermoshake Incubator Shaker (225 rpm), producer Gerhardt, Germany (Figure 3).

Figure 3. The thermoshaker for incubation of transformed cells

Incubate bacterial culture on LB medium

The resulting samples from the transformation stage were diffused on sterile Petri plates and then incubated overnight at 37^0C. Only colonies containing the plasmid DNA of interest will grow, the colonies without plasmid being inhibited by the antibiotic (Figure 4).

Growing transformed cells overnight

To obtain a good plasmid DNA concentration, 10 colonies were picked from every petri plate and put in the liquid LB environment with antibiotic, then incubated (at 37°C) overnight in flat bottom glass balloons, in a volume of 100 ml (1 colony per 10 ml). A thermoshaker was used for incubation (Figure 5).

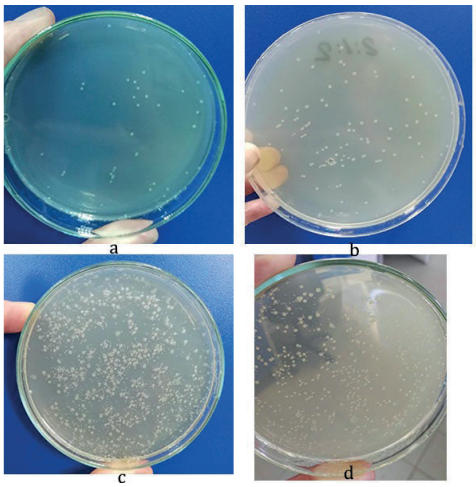


Figure 4. *E. coli* colonies containing plasmid DNA: a. 40 colonies; b. 100 colonies; c. > 500 colonies; d > 300 colonies

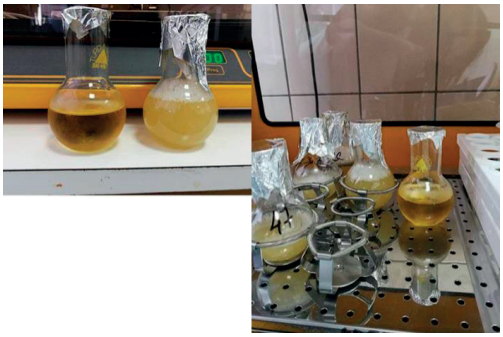


Figure 5. Growing *Escherichia coli* in liquid LB medium

Isolation of Plasmid DNA by Alkaline Lysis

Plasmids were purified using the classic alkaline lysis protocol (Birnboim and Doly, 1979; Sambrook et al., 1989). The working method involved the following steps:

Precipitation of crop grown overnight*:* 1 ml of *E. coli* culture, grown overnight, was pipetted into a 2 ml tube. After centrifuging the cell culture at 12,000 rpm for 5 minutes and discarding the supernatant, the cells were pelleted to remove them from the growth medium. This stage was repeated five times (Figure 6).

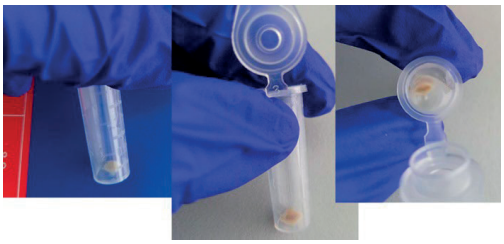


Figure 6. Bacterial pellet sedimentation

Resuspension bacterial cells: the bacterial pellet was resuspended in 350 µl alkaline solution I. At this stage, 10 µl de RNase A were also added. The mixture was left to incubate for 5 minutes at room temperature, with a slight inverse of the tube from time to time to eliminate bacteria through hyperlytic osmosis and release total DNA and other contents (Figure 7).

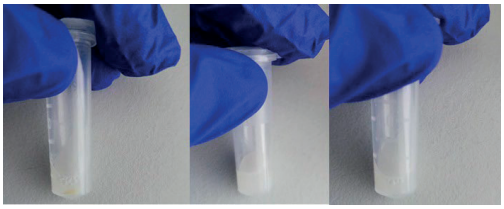


Figure 7. Bacterial pellet resuspension

Bacterial lysis*:* to denature the plasmid, chromosomal DNA and proteins, it was added, over alkaline solution I, the alkaline lysis solution II (400 µl), followed by tube inversion 6 times and placed on ice for 10 minutes.

Neutralization. Selective renaturation of plasmid DNA*:* selective renaturation of plasmid DNA with its resuspension was done by adding the acid III solution (500 µl) over alkaline solutions I and II used in previous stages. This was followed by incubation on ice for 10 minutes and centrifugation at 12.000 rpm for 30 minutes. The chromosomal DNA and proteins were denatured and precipitated by centrifugation, and the plasmid DNA, due to its smaller size, was renatured, diffusing in the supernatant (Figure 8).

Figure 8. Selective renaturation of plasmid DNA

Precipitation, purification and elution of plasmid DNA: plasmid DNA, recovered from the supernatant, was isolated and purified following the instructions recommended by "ISOLATE II Plant DNA Kit" from Meridian Bioscience, USA.

Evaluation of the results

Fragments amplified by PCR reaction and plasmid DNA were loaded into 3% agarose gel and migrated using a horizontal electrophoresis system, from Thistle Scientific, UK. The gel was read with the "Essential V6" imaging platform, producer Uvitec Cambridge, UK. Plasmid DNA sequencing was outsourced to Macrogen Europe, USA. The query in the NCBI database was done using the BLAST program, which will search equal-length segments. When aligned to one another, without gaps, they have maximal aggregate scores. For Query Identity % (the number of exact matches in the alignment relative to the query length) and Query Coverage % (the alignment length, including mismatches, relative to the query) was used blastn algorithm. This algorithm compares a nucleotide query sequence with nucleotide sequence database (Altschul, 2005) and was described by Altschul et al. (1990). For interpretation of its results, BLAST provides three related pieces of information in the form of the raw scores, bit scores and E-values. BLAST uses statistical theory to produce a bit score and expect value (E-value) for each alignment pair (query to hit) (Madden, 2002; Gibas and Jambeck, 2001). In the case of blastn, the scores are assigned to a simple matching matrix /non-matches for nucleotides, using mathematical formulas: $S= \sum(match\ score) - \sum(\text{gap penalties})$ for raw score and $S= \frac{\lambda S - \ln(k)}{ln2}$ for bit score (Gertz, 2005). For a single distinct alignment, BLAST programs convert scores to "E-values" using the equation: $E=Kmn\,e^{-\lambda S}$, where K and λ are Karlin-Altschul parameters, m effective length of the query sequence, and n the length of the database to which it is compared (Karlin and Altschul, 1990; Altschul, 2005). E-value indicates the likelihood that the match is due to chance, and a lower value means the match is significant. An E-value greater than 1 indicates that the query sequence has been aligned to a sequence in the database to which it is not related. Biological significance is typically represented for E-values less than 0.1 or 0.05 (Madden, 2002; Pertsemlidis and Fondon, 2001). The dendrogram was constructed using Minitab 18, which calculates the genetic distance based on the Euclidean matrix.

RESULTS AND DISCUSSIONS

The OPL19 marker amplified the corresponding 433 bp fragment of the dominant allele *Vr* gene (*Rvi2* or *Rvi8*) in all 20 genotypes (Figure 9). The integrity and presence of plasmid DNA was checked on the agarose gel (Figure 10). Blastn used as a search algorithm in our query, compares the nucleotide query sequence (sequences of the 20 samples) with sequence of the *Rvi2* gene, the result being displayed in the alignment block. The sequence associated with the gene of interest is recorded in the "National Center for Biotechnology Information" database, the locus name being under the name "AY626824" (Figure 11). These results confirm the presence of the *Rvi2* gene in all 20 analyzed genotypes, demonstrating the efficiency of the OPL19 marker in identifying this gene. Amplification of the 433 bp fragment, verification of plasmid DNA integrity and blastn analysis with the reference sequence from the NCBI database (locus AY626824) support the validity of the methodology used. These findings are relevant to marker-assisted selection in apple breeding programs. Each alignment block consists of three lines: the query sequence, the matching sequence, and the subject sequence; these alignments are sorted by "score", "E-value", "percent identity", "query start position" and "subject start position". The matching sequence is represented by bases that match, indicated by vertical lines, and non-matched bases, marked by empty spaces. The blastn algorithm results for the 20 clones showed a matching percentage, ranging from [94.6% to 100%] for the forward primer and

[95.39% to 100%] for the reverse primer. The percentage of the cover between the query sequence and the subject sequence was in the range [85-92%] for the forward primer and [80-93%] for the reverse primer (Table 1). A large query cover (90-100%) indicates that the alignment covers almost the entire query sequence, while a small percentage means that only a small part of the sequence aligns. If the E-value of the query is = 0, it does not matter if the query coverage is <100%. These results indicate a high degree of similarity between the query sequences and the reference *Rvi2* gene, confirming the reliability of the blastn analysis in identifying and validating the presence of the target gene in the studied apple genotypes. The highest percentage of identity was for the variety 'Pomona' (forward and reverse primer), and the most significant cover was for the variety 'Salva' (reverse primer) (Figure 12 and Figure 13). The results: 2e-155 ($2x10^{-155}$), 2e-176 ($2x10^{-176}$), 5e-158 ($5x10^{-158}$) and 7e-163 ($7x10^{-163}$) for E-value are negligible, and the likely that the match is due to chance is zero. Using the Minitab 18 program, the dendrogram was made, both for sequencing with the forward primer and for sequencing with the reverse primer, taking into account the values from the blastn analysis (percent identity, query coverage, max score and total score). These findings confirm the strong genetic similarity between the studied apple varieties and the *Rvi2* gene, with 'Pomona' showing the highest identity and 'Salva' the most significant coverage. The extremely low E-values indicate that the matches are highly significant and not due to chance. The dendrogram analysis further supports the genetic relationships among the genotypes, reinforcing the accuracy of the blastn results. The dendrogram analysis identified 19 clusters and two main groups, A and B, which reflect the genetic diversity among the apple cultivars (Figure 14 and Figure 15). As shown in Tables 2 and 3, the highest similarity between the query sequences and the reference sequence was found in the cultivars 'Remar' and 'Starkprim' for the forward primer, and 'Dany' and 'Jonaprim' for the reverse primer. In contrast, the lowest similarity was observed in the cultivars 'Alex' and 'Cezar' for both primers. These results help to better understand the genetic relationships among the studied genotypes, which is valuable for future breeding programs.

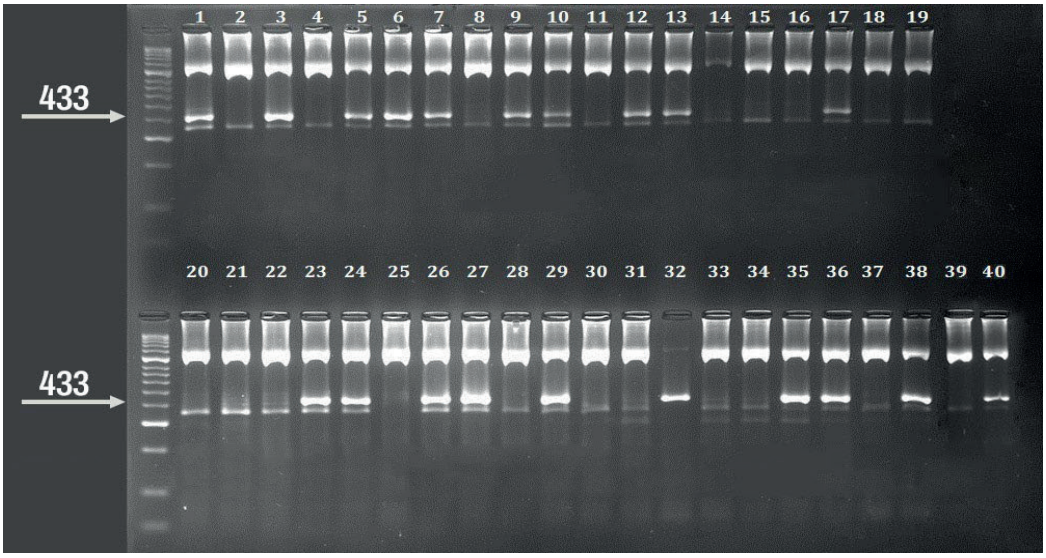


Figure 9. Electrophoretic profile obtained with OPL19 markers, the amplified fragment size being 433 bp.
1. 'Estival', 2. 'Rebra', 3. 'Bistriţean', 4. 'Aura', 5. 'Romus 3', 6. 'Dany', 7. 'Romus 5', 8. 'Productiv de Cluj', 9. 'Luca', 10. 'Ciprian', 11. 'Irisem', 12. 'Slava', 13. 'Ionaprim', 14. 'Rustic', 15. 'Precoce de Ardeal', 16. 'Iris', 17. 'Starkprim', 18. 'Auriu de Cluj', 19. 'Generos', 20. 'Colonade', 21.'Nicol', 22. 'Colmar', 23. 'Delicios de Voineşti', 24. 'Romus 4', 25. 'Remus', 26. Redix'', 27. 'Alex', 28. 'Doina', 29. 'Voinicel', 30. 'Inedit', 31. 'Dacian', 32. 'Voinea', 33. 'Valery', 34. 'Real', 35. 'Discoprim', 36. 'Cezar', 37. 'Frumos de Voineşti', 38. 'Pomona', 39. 'Revidar', 40. 'Remar'

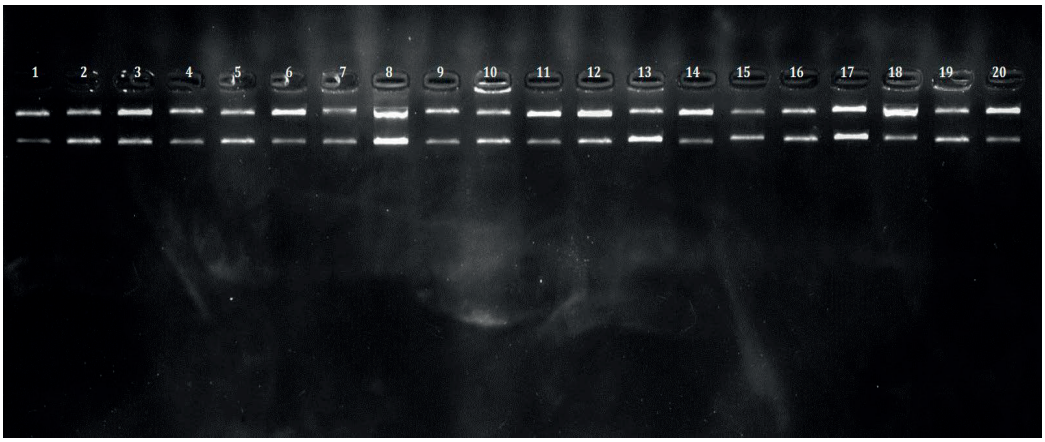
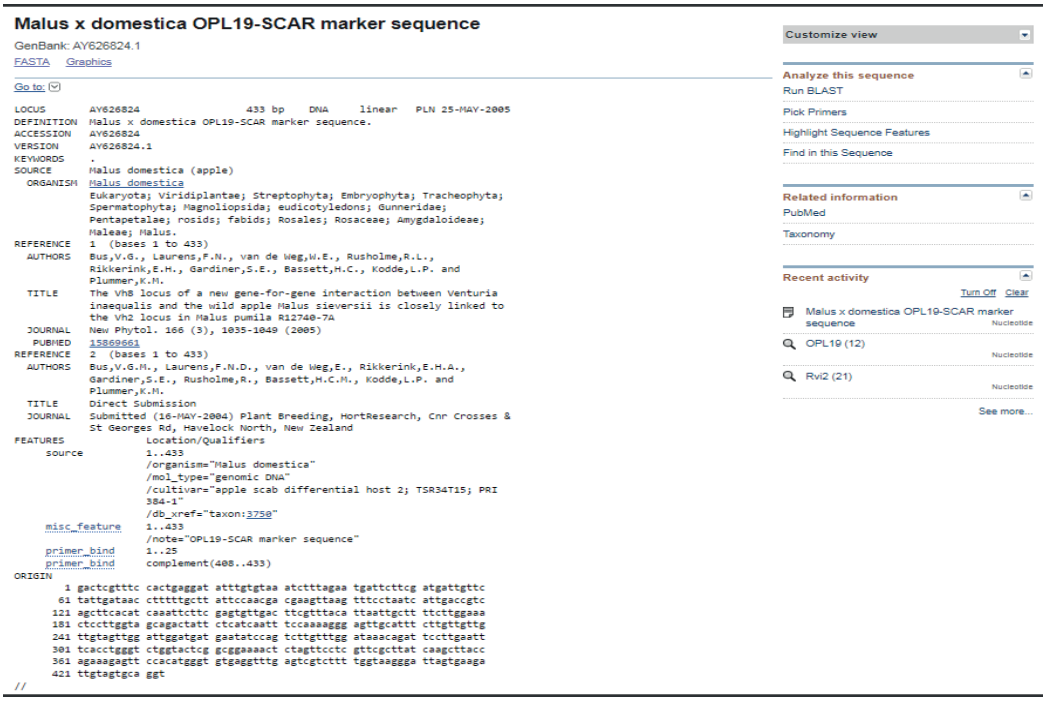


Figure 10. Electrophoretic profile for plasmid ADN
1. 'Alex', 2. 'Bistriţean', 3. 'Cezar', 4. 'Ciprian', 5. 'Dany', 6. 'Discoprim', 7. 'Delicios de Voineşti', 8. 'Estival', 9. 'Jonaprim', 10. 'Luca', 11. 'Pomona', 12. 'Redix', 13. 'Remar', 14. 'Romus 3', 15. 'Romus 4', 16. 'Romus 5', 17. 'Salva', 18. 'Starkprim', 19. 'Voinea', 20. 'Voinicel'

Table 1. Blastn analysis results for forward and reverse primer

Cultivar	Percent identity		Query coverage		Max score		Total score		E-value	
	Forward	Reverse	Forward	Reverse	Forward	Reverse	Forward	Reverse	Forward	Reverse
Alex	97.46%	96.78%	90%	91%	645	643	645	643	0.0	0.0
Bistriţean	96.49%	97.74%	91%	91%	649	664	649	664	0.0	0.0
Cezar	97.97%	97.13%	91%	80%	673	558	673	558	0.0	7e-163
Ciprian	97.98%	97.20%	90%	89%	666	645	666	645	0.0	0.0
Dany	96.74%	98.23%	91%	91%	649	673	649	673	0.0	0.0
Discoprim	98.45%	97.26%	89%	91%	663	654	663	654	0.0	0.0
Delicios de Voineşti	96.98%	95.39%	90%	80%	643	533	643	533	0.0	2e-155
Estival	97.26%	97.68%	92%	89%	665	649	665	649	0.0	0.0
Jonaprim	96.94%	97.78%	89%	92%	638	673	638	673	0.0	0.0
Luca	98.65%	97.97%	85%	90%	642	660	642	660	0.0	0.0
Pomona	100%	100%	86%	81%	673	686	699	726	0.0	0.0
Redix	94.6%	98.68%	90%	87%	604	656	604	656	2e-176	0.0
Remar	99.23%	97.69%	90%	89%	689	650	689	650	0.0	0.0
Romus 3	97.38%	96.98%	87%	91%	639	646	639	646	0.0	0.0
Romus 4	97.16%	89.71%	88%	89%	637	662	637	662	0.0	0.0
Romus 5	97.69%	98.26%	88%	92%	652	682	652	682	0.0	0.0
Salva	95.75%	97.49%	91%	93%	627	659	627	659	0.0	0.0
Starkprim	99.23%	99.23%	90%	90%	689	689	689	689	0.0	0.0
Voinea	97.93%	95.69%	88%	80%	651	543	651	543	0.0	5e-158
Voinicel	98.24%	98.24%	91%	91%	678	679	678	679	0.0	0.0



Malus x domestica OPL19-SCAR marker sequence

GenBank: AY626824.1

FASTA Graphics

Go to:

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LOCUS       AY626824               433 bp    DNA     linear   PLN 25-MAY-2005
DEFINITION  Malus x domestica OPL19-SCAR marker sequence.
ACCESSION   AY626824
VERSION     AY626824.1
KEYWORDS    .
SOURCE      Malus domestica (apple)
  ORGANISM  Malus domestica
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            Maleae; Malus.
REFERENCE   1  (bases 1 to 433)
  AUTHORS   Bus,V.G., Laurens,F.N., van de Weg,W.E., Rusholme,R.L.,
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            Plummer,K.M.
  TITLE     The Vh8 locus of a new gene-for-gene interaction between Venturia
            inaequalis and the wild apple Malus sieversii is closely linked to
            the Vh2 locus in Malus pumila R12740-7A
  JOURNAL   New Phytol. 166 (3), 1035-1049 (2005)
   PUBMED   15869661
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            Plummer,K.M.
  TITLE     Direct Submission
  JOURNAL   Submitted (16-MAY-2004) Plant Breeding, HortResearch, Cnr Crosses &
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      421 ttgtagtgca ggt
//
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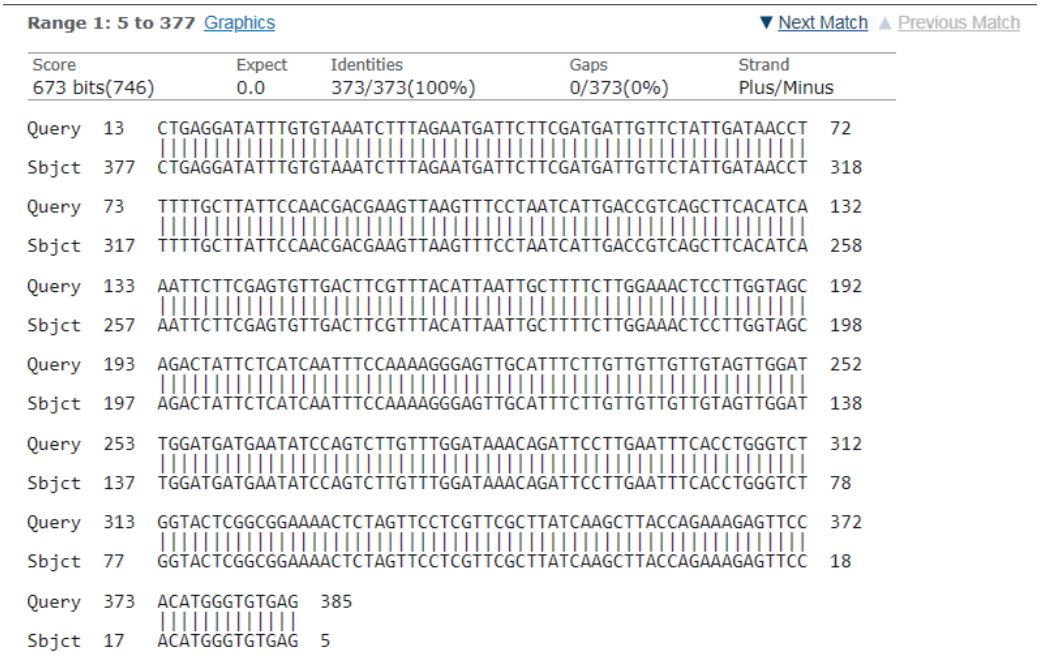
Customize view

Analyze this sequence
Run BLAST
Pick Primers
Highlight Sequence Features
Find in this Sequence

Related information
PubMed
Taxonomy

Recent activity
Turn Off Clear
Malus x domestica OPL19-SCAR marker sequence — Nucleotide
OPL19 (12) — Nucleotide
Rvi2 (21) — Nucleotide
See more...

Figure 11. The *Rvi2* gene recorded in the National Center for Biotechnology Information database



Range 1: 5 to 377 Graphics ▼ Next Match ▲ Previous Match

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Query  373  ACATGGGTGTGAG  385
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Sbjct  17   ACATGGGTGTGAG  5
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Figure 12. Alignment section from a BLAST report showing pair-wise sequence alignment between the query sequence for cultivar 'Pomona' and the sequence of the *Rvi2*

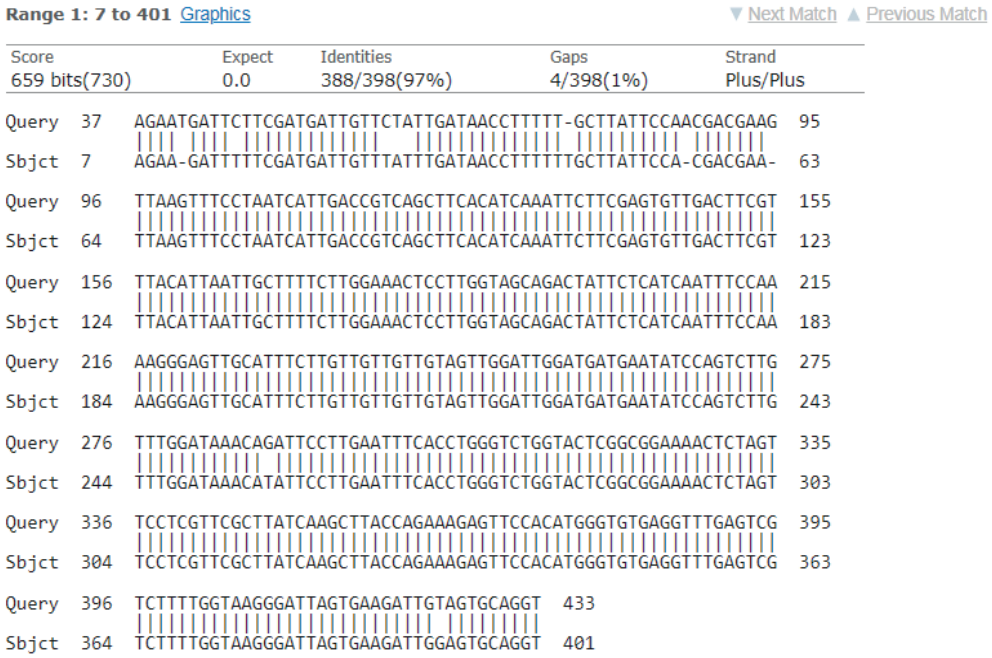

Figure 13. Alignment section from a BLAST report showing pair-wise sequence alignment between the query sequence for cultivar 'Salva' and the sequence of the *Rvi2*

Table 2. Amalgamation Steps

Step	Number of clusters	Similarity level	Distance level	Clusters joined		New cluster	Number of obs. in new cluster
1	19	100,000	0,000	13	18	13	2
2	18	99,998	0,002	2	5	2	2
3	17	98,824	1,414	16	19	16	2
4	16	98,823	1,414	9	15	9	2
5	15	98,823	1,414	4	8	4	2
6	14	98,823	1,415	7	10	7	2
7	13	97,647	2,828	9	14	9	3
8	12	96,471	4,243	4	6	4	3
9	11	96,470	4,243	2	16	2	4
10	10	96,470	4,243	1	7	1	3
11	9	94,118	7,071	3	20	3	2
12	8	90,588	11,314	1	9	1	6
13	7	84,304	18,868	11	13	11	3
14	6	82,353	21,213	1	2	1	10
15	5	82,353	21,213	3	4	3	5
16	4	72,941	32,527	12	17	12	2
17	3	68,918	37,363	3	11	3	8
18	2	43,529	67,882	1	12	1	12
19	1	0,000	120,208	1	3	1	20

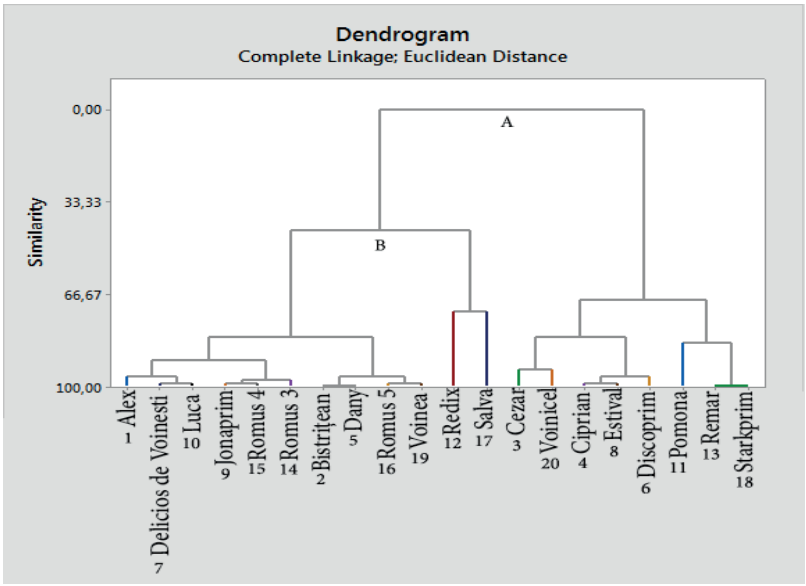

Figure 14. The level of similarity shown in relation to BLAST analysis for the forward primer

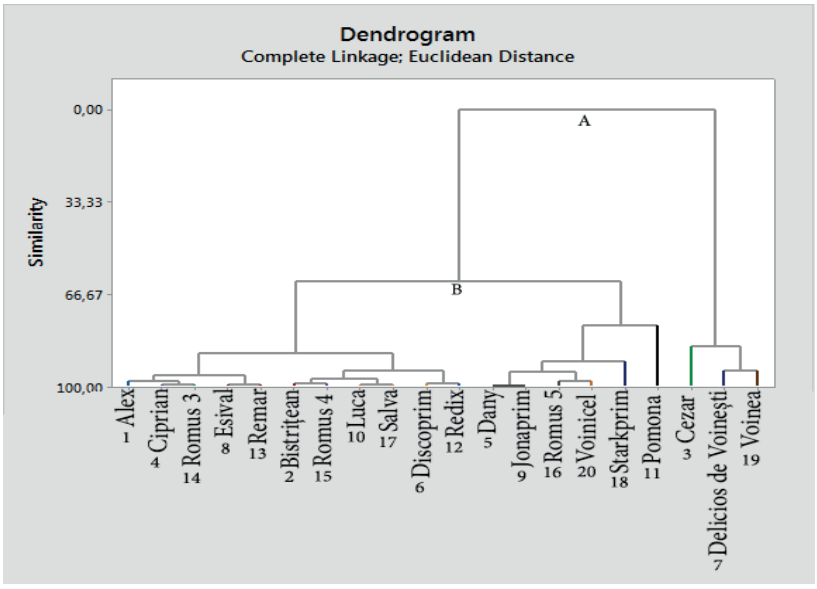

Figure 15. The level of similarity shown in relation to BLAST analysis for the reverse primer

Table 3. Amalgamation Steps

Step	Number of clusters	Similarity level	Distance level	Clusters joined		New cluster	Number of observation in new cluster
1	19	99,9955	0,011	5	9	5	2
2	18	99,4258	1,414	8	13	8	2
3	17	99,4257	1,414	4	14	4	2
4	16	99,4257	1,415	10	17	10	2
5	15	98,8515	2,829	6	12	6	2
6	14	98,8511	2,830	2	15	2	2
7	13	98,2774	4,243	1	4	1	3
8	12	98,2774	4,243	16	20	16	2
9	11	97,1289	7,071	2	10	2	4
10	10	95,9805	9,900	1	8	1	5
11	9	94,8321	12,728	5	16	5	4
12	8	94,2579	14,142	7	19	7	2
13	7	94,2579	14,142	2	6	2	6
14	6	90,8126	22,627	5	18	5	5
15	5	87,9416	29,698	1	2	1	11
16	4	85,6447	35,355	3	7	3	3
17	3	77,8426	54,571	5	11	5	6
18	2	62,0456	93,477	1	5	1	17
19	1	0,0000	246,288	1	3	1	20

CONCLUSIONS

In conclusion, the OPL19 marker has proven to be effective for molecular screening to highlight the *Vr* gene (*Rvi2* or *Rvi8*), confirming its utility in the genetic analysis of apple varieties. The results of the BLAST analysis identified the gene sequence in all 20 varieties and revealed intraspecific differences based on the matching sequence. The method developed in this study will be extended to other molecular screenings to build a database that will support the construction of genetic maps. Furthermore, in an extended study using the BLAST program, intraspecific differences can be identified by comparing the nucleotide sequences of the varieties, offering valuable insights for future research and breeding programs.

ACKNOWLEDGEMENTS

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INFLUENCE OF NATURAL AND SYNTHETIC SWEETENERS ON FLAVOPROTEINS FOUND IN BLACK TEA

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Abstract

Black tea is a very popular drink and is consumed worldwide for its flavor and health benefits. Due to its powerful antioxidant compounds, drinking black tea has health benefits for the consumer. Flavoproteins are one of the most widely studied families of enzymes - proteins that contain a nucleic acid derivative of riboflavin. Flavoproteins have either FMN (flavin mononucleotide) or FAD (flavin adenine dinucleotide) as a protein group or cofactor. The majority of flavoproteins, carry out redox reactions. This work paper aims to study the sweetener-induced changes in flavoproteins. The results showed very significant changes induced by honey (V_3) and saccharin (V_4) on the concentrations of the active forms of flavoprotein in black tea.

Key words*: antioxidant, black tea, FAD, flavoproteins, FMN.*

INTRODUCTION

Tea, made from the leaves of the *Camellia sinensis* plant, is one of the most popular and widely consumed beverages in the world. It has many health benefits, with a specific taste and flavor. Black tea has a very valuable chemical composition, and it has several chemical compounds with a powerful antioxidant effect (Ye et al., 2021).

The notion of black tea as 'living food' is based on its chemical composition, which is extremely valuable, and its biodynamic properties, which allow concentration changes in nanoseconds (Savescu, 2017b).

Consumer demand for dietary supplements is growing, especially those with high antioxidant potential due to their ability to combat free radicals (Belitz et al., 2009; Savescu, 2017c).

Riboflavin is an alloxazine derivative consisting of an isoalloxazine nucleus and a ribitol side chain.

Riboflavin is known as the 'growth vitamin' and plays an important role in the body's oxygen reactions. It works with other substances called NAD-dependent oxidoreductases to help the body grow and absorb vitamin B3 (Savescu, 2017b). Vitamin B2 is found in the structure of its coenzyme forms, FMN and FAD (Savescu, 2020 b). It is converted to a monophosphoric ester (FMN) in all the body's cells when acted upon by a flavo-kinase. FAD-dependent oxidoreductases are another type of enzyme that utilizes vitamin B2 (riboflavin or 7,8-dimethyl-10-dimethyl-10-ribiethyl-isoaloxazine) (Savescu, 2017a, 2017b). These enzymes are found in the aerobic dehydrogenase group. Flavin enzymes (FMN, FAD) are involved in reactions involving the transfer of electrons and protons, which are mediated by the isoalloxazine nucleus (Savescu, 2021).

Flavin enzymes are different from NAD^+ and $NADP^+$ because they can either accept one electron or a pair of electrons. Flavine enzymes have standard redox potentials Eo between +0.19V (stronger oxidizers than NAD^+) and -0.49V (stronger reducing agent than NADH). (Savescu, 2017b).

This shows that their redox properties can vary a lot depending on the environment and the substrate. Some flavin enzymes contain a metal (molybdenum or iron) in their molecule. These enzymes can stabilize the semiquinone form by pairing the lone electron with the unpaired electrons existing in the metal ions. The metal can also help the flavin enzymes to transport electrons (Savescu, 2021).

Succinate dehydrogenase is an enzyme that works by removing two hydrogen atoms from succinic acid and transferring them to the FAD group (Skoog, 2007; Savescu, 2016a).

Many of these flavin enzymes also contain a metal that is essential for the function of metal-flavoprotein enzymes. FAD is also involved in the process of converting fatty acids to water, which is the first step in the process of oxidation (Savescu et al., 2019).

As demonstrated by Nobel Laureate Szent-Györgyi, flavoproteins function as electron carriers between dehydrogenase and iron-containing cytochrome enzymes. The electron transport chain segments were reconstituted in vitro using purified components (Isenberg et al., 1958).

Vitamin B3 (niacin, vitamin PP) is represented by two forms: nicotinic acid (niacin) and nicotinic acid amide (niacin amide). This vitamin functions in oxidoreduction reactions within the body, in the form of two coenzymes: NAD^+ (nicotinamide adenine dinucleotide) and $NADP^+$ (nicotinamide adenine dinucleotide phosphate). The process of electron collection from various substrates by NAD^+-dependent dehydrogenase enzymes is known as the 'NADH+H^+' process. These electrons are subsequently transferred along the respiratory chain via flavoproteins and NADH-dehydrogenases (Savescu, 2017c; Tita et al., 2021).

Two discrete categories of flavoproteins possess dehydrogenase activity. L-amino acid oxidase is localized in the endoplasmic reticulum, whereas D-amino acid oxidase is found in the microsomes of liver cells (Savescu, 2016b). Some parts of the oxidases ($FADH_2$ and $FMNH_2$) react directly with molecular oxygen, generating H_2O_2. After this, catalase catalyzes the decomposition of H_2O_2 into water and oxygen. This process takes place in the peroxisomes of liver cells (Savescu et al., 2019).

Flavin mononucleotide (FMN) is a prosthetic group of flavoprotein. It is similar in structure to flavin adenine dinucleotide (FAD), but lacks adenine as a nucleotide (Savescu, 2017b). FMN (like FAD) can accept two electrons and two protons to yield $FMNH_2$. When bound to the active site of some enzymes, FMN can accept electrons, converting to a semiquinone radical (Savescu et al., 2020a).

It is estimated that approximately 90% of flavoproteins are classified as oxidoreductases. These enzymes are involved in a wide range of cellular processes. Flavins are a type of molecule that can change color and are found in many different proteins (Figure 1). Flavins come from vitamin B2 and are found in proteins such as flavin adenine dinucleotide (FAD) or flavin mononucleotide (FMN). (Toogood et al., 2020; Zhuang et al., 2022).

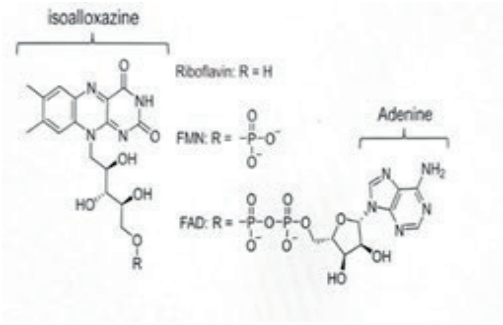

Figure 1. Molecular structure of flavins (Zhuang et al., 2022)

MATERIALS AND METHODS

The effect of various natural and synthetic sweeteners on the chemical composition of black tea has been studied in the laboratory.

Figure 2. Experimental variants

In order to obtain the control variant (V_0) of unsweetened black tea, 10 grams of black tea leaves were added to 1 liter of water in a suitable container. The tea was then subjected to a heating, cooling, and filtration process to yield the experimental variant from which the other sweetened black tea variants were derived (Figure 2).

The production of ten sweetened tea variants was undertaken from this experimental variant. The following variants were obtained, and are highlighted below:

V_0: a variety of unsweetened black tea;

V_1: a variety of black tea sweetened with white sugar;
V_2: a variety of black tea sweetened with brown sugar;
V_3: a variety of black tea sweetened with honey;
V_4: a variety of black tea sweetened with saccharin;
V_5: a variety of black tea sweetened with Sucrazit;
V_6: a variety of black tea sweetened with Diamond;
V_7: a variety of black tea sweetened with fructose;
V_8: a variety of black tea sweetened with xylitol;
V_9: a variety of black tea sweetened with sorbitol;
V_{10}: a variety of black tea sweetened with stevia.
The experimental version Sucrazit (V_5) is the trade name of a synthetic sweetener containing 25% saccharin, citric acid, whose acidity has been buffered with sodium bicarbonate.
The experimental variant (V_6) Diamond is the trade name for a synthetic sweetener containing a combination of sodium cyclamate and sodium saccharin.
For this research, Certified Reference Materials (Pure Analysis substances) were used, with the help of which calibration scales with different concentrations were made, which helped to find the wavelength at which the maximum molecular absorption spectra for flavoproteins and riboflavin are recorded.
As demonstrated in our previous study, a dilution of 2% was employed to enable the transmission of the incident light beam through the black tea solution. This dilution was obtained by passing the black tea through filter paper and its subsequent transfer to the spectrophotometer cuvettes for analysis (Butoi et al., 2023).
A UV-VIS T92 Plus spectrophotometer from PG Instruments (UK) was used to obtain the molecular absorption spectra of the experimental variants. The spectrophotometer has been configured to operate with a wavelength bandwidth of one centimeter. This configuration permits the measurement of nanometers of molecular absorption in the ultraviolet (UV) range of 190-400 nanometers and in the visual (VIS) range of 400-700 nanometers (Butoi et al., 2023).
Absorption measurements were conducted utilizing UV cuvettes composed of quartz and exhibiting a parallelepiped configuration, with a square side measuring 1 cm. Before analysis, the raw material was tested using A.A.S. (Atomic Absorption Spectroscopy) to check for any contamination such as residues or heavy metals (L'vov, 1990). The effect of sweeteners on the base chemical composition was investigated by utilizing UV-VIS optical spectrometry and mathematical statistics (Savescu, 2017b; Butoi et al., 2023).
The initial phase of sample preparation for atomic absorption spectrometry entailed mineralization, which involved microwave digestion (Savescu, 2017c).
Savescu showed in his paper that the process of microwave digestion constitutes a technique that has been utilized to prepare samples originating from a variety of sources (e.g., plants, soil, foodstuffs, pharmaceuticals) for elemental analysis by ICP, ICP-MS, or AA (Savescu, 2017c). The process necessitates the dissolution of the sample before its introduction into the analyzer (Savescu, 2017c). The sample matrix is subject to acid digestion, a process that serves to disintegrate it, thereby leaving the elements of interest in the solution. After all these operations, the experimental variants are ready for analysis (Savescu, 2017c). The efficiency of microwave electrolytic matrix digestion systems in rapidly digesting a wide variety of sample matrices has been well documented. The analysis of these samples subsequently reveals the analytes of interest in a clear solution (Savescu, 2017c; L'vov, 2005)
The experiment used a 1200 W MARS mineralized microwave EMF system. The MARS EMF system is a multimodal platform equipped with a magnetic stirring plate and a rotor that allows multiple vessels per batch to be processed in parallel (Savescu, 2017b). The experiments were conducted using two different vessels: the first was an HP-500 (Teflon insert - TFA) with an 80-ml vessel volume, a maximum pressure of 350 psi, and a maximum temperature of 210°C; the second was a Chem (borosilicate glass insert) with an 80-ml vessel volume, a maximum pressure of 200 psi, and a maximum temperature of 200°C. Both vessels were based on a fourteen-position rotor (Savescu, 2017b).

The system delivers continuous power between 0 and 1200 W. The temperature is controlled internally by a fiber optic probe in a control reference vessel (Savescu, 2017b).
In summary, 10 g of dry substance tea was measured with analytical precision. For the mineralization step, 10 g of product (black tea), 6 mL of concentrated nitric acid, and 3 mL of 30% hydrogen peroxide were added to each digestion cartridge (Savescu, 2017b).
A digestion cartridge containing no product, only reagents, was used as a control. The AAS method was conducted using a Varian SpectrAA 220Z atomic absorption spectrometer furnace system, equipped with a Varian SpectrAA 220Z autosampler, a Varian GTA 110Z furnace, and a Varian UltrAA. The stages of microwave digestion are described in Table 1.

Table 1. Stages of microwave digestion

Power, W	Time, min	Stirring	Comments
300	4	yes	For cartridge protection
0	1.5	no	To help the sedimentation process on the downside of vessels
400	2.5	yes	
600	2.5	yes	
200	1	no	
800	2.5	yes	
1000	4	yes	
0	10	no	Cooling for opening cartridges

The analysis was facilitated by related Windows interface software (Savescu, 2017a). The results are expressed as the mean ± standard error of the mean (SEM), except some plants with high oil content can be used, which is expressed as the mean ± standard deviation (SD). For better statistical analysis, Kruskal-Wallis two-way ANOVA followed by Dunn's post-hoc test can be used (Savescu, 2017c). All calculations were performed utilizing the ORIGIN PRO 2020 software.

RESULTS AND DISCUSSIONS

When developing innovative black tea supplements, it is important to ensure that the sweeteners used preserve the active forms of flavoproteins and riboflavin.

The results of this stage of the research showed the best sweetening option for the black tea food supplement. The product that was made and chosen to be used (as a raw material) in the next stages of the process had a similar chemical makeup to the unsweetened one, but also much better sensory properties (Savescu, 2017b).
The use of the AAS technique revealed high concentrations of potassium, magnesium, and calcium in the parts of the tea plant used, as shown in Table 2.

Table 2. Cations composition of black tea obtained by AAS technique

Indicator/ Constituent	BLACK TEA	
	Dry matter ppm (mg/kg)*	Watery extract 1:10 ppm (mg/L) Average value
Na^{+}	1012±3.56	153.04
K^{+}	16720.2±3.26	285.21
Ca^{2+}	742±2.46	7.98
Mg^{2+}	462±1.64	7.93
Zn^{2+}	48±2.48	1.79
Mn^{2+}	84.5±1.89	0.23
Fe^{2+}	74.24±1.84	0.68
Al^{3+}	10.42±0.67	0.49
Cu^{2+}	10.46±0.66	0.35
Pb^{2+}	0.036±0.001	missing

In the experimental V_0, the control variant, a peak in NAD^{+} was observed, accompanied by a decrease in tocopherol and a high theobromine content.
When white sugar is added to the experimental variant V_1, minor alterations in pigments and a marginal decline in specific chemicals, notably theobromine, can be discerned (Butoi et al., 2023). Levels of flavoprotein concentrations when using white sugar are relatively low compared to other sweeteners.
The modifying tendency of flavoproteins is also observed when brown sugar is used in V_2, but to a greater extent in terms of protecting oxidized and reduced forms. The concentrations of oxidized and reduced forms of riboflavin are higher in brown sugar than in white sugar, making brown sugar a better option.
Honey used as a sweetener in the V_3 variant showed the highest concentrations of flavoproteins, as well as the highest concentrations of oxidized and reduced forms of riboflavin, as demonstrated in the accompanying graphs.

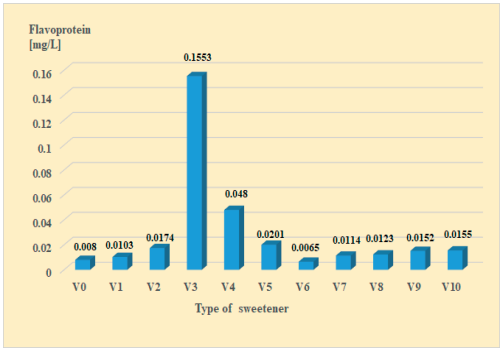

Figure 3. Black tea flavoprotein concentration

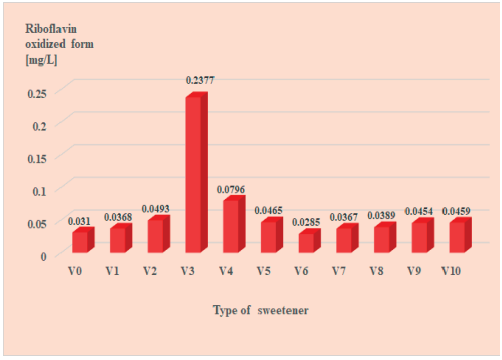

Figure 4. Concentration of the oxidized form of riboflavin in black tea

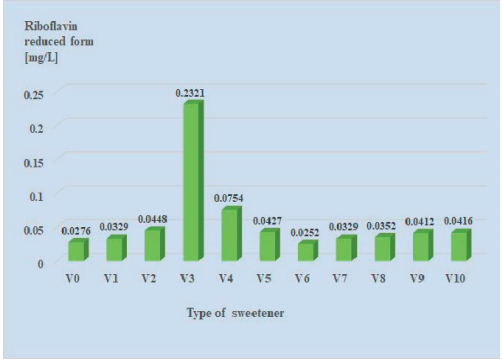

Figure 5. Concentration of the reduced form of riboflavin in black tea

The graphs show that V4 (sweetened with saccharin) has high levels of flavoprotein. The version of the experiment that used saccharin as a sweetener protects oxidized and reduced forms of riboflavin.

Sucrazit (V_5) exerts a significant influence on the concentration of the primary active compounds present in black tea.

The use of Diamond V6 leads to the lowest concentrations of flavoprotein forms, as can be seen in the graph in Figure 3.

Studies have shown that using a mixture of sodium cyclamate and saccharin can help to keep the concentration of specific oxidoreductase coenzymes stable. This also keeps them from losing their strong antioxidant properties (Butoi et al., 2023).

In the case of variant V_7, fructose was utilized. Notably, this does not have a significant effect on the color intensity in the UV and visible range, thereby ensuring the maintenance of the color tone. The levels of oxidized and reduced forms of riboflavin and flavoproteins are maintained at low levels.

The use of sweeteners such as xylitol (V_8), sorbitol (V_9), and stevia sweetener (V_{10}) is mandatory to maintain the levels of oxidized and reduced forms of riboflavin at a consistent level. Sorbitol and stevioside prove similar effects on flavoprotein concentrations.

There is a similar situation regarding the influence of sweeteners on the concentrations of reduced (Figure 4) and oxidized (Figure 5) forms of riboflavin.

CONCLUSIONS

The following conclusions can be drawn from the results:

As evidenced by the obtained results, the honey utilized in the experimental version V_3 induces a considerable influence on black tea flavoproteins. The honey-sweetened (variant V_3) exhibited elevated concentrations of oxidized and reduced forms of riboflavin in black tea.

A similar trend is observed in flavoprotein concentrations. It is evident that V_3, sweetened with honey, shows the highest concentrations of flavoproteins.

When sodium cyclamate was used in combination with saccharin (V_6) it led to the lowest flavoprotein concentrations.

The saccharin used as a sweetener (V_4) in black tea has relatively high flavoprotein concentrations, after honey, which makes it a good sweetener option.

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OPTIMIZATION OF *Agrobacterium rhizogenes* - MEDIATED HAIRY ROOT INDUCTION IN *Salvia officinalis*

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Abstract

Sage (Salvia sp.) is a key medicinal plant from the Lamiaceae family. Recently, the mass production of hairy roots has become important for producing plant secondary metabolites, offering fast growth and stable bioactive compound production, making them ideal for transgenic research. In this study, the optimization of hairy root induction conditions by Agrobacterium rhizogenes in Salvia officinalis, has been evaluated. For this purpose, the effect of 3 different times of bacterial co-culture (24, 48, 72 h) with explants (whole seedling, leaf, stem and petiole) was investigated. The 48h co-culturing interval was considered the most suitable time. The highest rate of success in hairy root production (88.4 %) belonged to the leaf explants. In order to evaluate the stability and quantity of hairy roots, liquid 0.5X MS medium with 3 treatments of IBA (0, 0.5, 1 mg/L) were used. The highest percentage of growth was observed in liquid 0.5X MS and at the hormone level of 1 mg/L. Transgenic hairy roots produced were confirmed by PCR.

Key words*: hairy root, secondary metabolites, Salvia officinalis, Agrobacterium rhizogenes, PCR.*

INTRODUCTION

The history of treating diseases with medicinal plants is as old as the history of human life on Earth. Humans have always treated themselves with the help of medicinal plants, based on experience, science, and according to their needs. Medicinal plants are a crucial resource for both traditional medicine and the pharmaceutical industry. In modern times, the interest in using herbal remedies for disease treatment and prevention has grown significantly. As a result, the incorporation of medicinal plants into the production of pharmaceutical, food, cosmetic, and health products is expanding rapidly, raising their value and significance in the global market (Cunningham, 1993; Angelova-Teneva & Beshkova, 2015).

Some bioactive compounds, essential in the pharmaceutical industry, cannot be synthesized in the lab and can only be extracted from plants. These compounds either have unknown chemical structures or possess such complex structures that their synthetic production is challenging and costly, which is why pharmaceutical companies show great interest in plant-based medicines (Omid Beygi, 2010).

Salvia officinalis L. is one of the best-known species of the mint family (Lamiaceae), native to the Mediterranean regions but distributed throughout the world (Hojati, 2024; Rahmanifar et al., 2023). It has a long-standing history in traditional medicine for treating conditions such as fever, rheumatism, bronchitis, and various mental disorders. Numerous studies have highlighted its beneficial effects, including anti-diabetic (Christensen et al., 2010), anti-inflammatory (Rodrigues et al., 2011), antimicrobial (Garcia et al., 2012), antioxidant (Generalić et al., 2012; Mihai et al., 2021), neurodegenerative disease prevention (Takano et al., 2011), and anti-tumor properties (Al Barzanji et al., 2013).

To enhance the production of biochemical substances, various biotechnological strategies have been developed (Asghari, 2006; Zayova et al., 2016). Hairy root cultures, generated by the soil bacterium *Agrobacterium rhizogenes*, are an effective and efficient method for mass-producing secondary metabolites. These cultures grow rapidly, are easy to maintain, and

can synthesize a diverse array of beneficial compounds (Shanks & Morgan, 1999). The bacterium induces the development of hairy roots by transferring a T-DNA fragment from its root-inducing plasmid (Ri) into the plant's genome, leading to the expression of stable *rol* genes (*rol A*, *rol B*, *rol C*, and *rol D*) in the plant cells (Chaudhuri et al., 2005). The efficiency of this transformation process depends on factors such as plant species, tissue age, physiological state, bacterial strain, bacterial concentration, and co-culture duration (Kumar et al., 2006). Additionally, the culture medium's composition, especially its hormonal components like auxins, plays a significant role in the growth rate and secondary metabolite production (Washida et al., 2004; Pakdin Parizi et al., 2014).

Although hairy root induction using *A. rhizogenes* is a well-known process, there have been few studies focused on inducing hairy roots in sage. This research seeks to optimize of *A. rhizogenes*-mediated hairy root induction in *S. officinalis* and investigate the factors that influence this process.

MATERIALS AND METHODS

Preparation of sterile seedlings

Sage seeds were obtained from the National Agricultural Research and Development Institute. To prepare sterile seedlings, seeds were surface disinfected with 75% ethanol for 30 seconds, followed by a 10-minute treatment with a 5% sodium hypochlorite solution. Seeds were abundantly rinsed with distilled water and then cultured in hormone-free MS medium supplemented with 3% sucrose (Murashige & Skoog, 1962) (Table 1).

The pH of the medium was adjusted to 5.6-5.8. The cultures were maintained under a 16-hour light/8-hour dark photoperiod at 25°C.

Bacterial strain and growth conditions

The reference strain *A. rhizogenes* ATCC 15834 was used for hairy root transformation in *S. officinale*.

The bacterial strain was reactivated from lyophilized stock on Nutrient Agar medium (purchased VWR International BVBA, Leuven, Belgium). The refreshed, pure strain was then grown in Nutrient Broth (purchased VWR International BVBA, Leuven, Belgium) for 3 days at 26°C and 150 rpm shaking. Cells were then harvested by centrifugation at 6000 rpm, for 10 min. The obtained pellet was washed with sterile 0.5X MS medium and resuspended in the same nutritional solution, at 10^8 CFU/ml.

Table 1. MS culture medium substances

Category of substances	Substance	Concentration (mg/L)
Macroelements	NH_4NO_3	1650.0
	KNO_3	1900.0
	$CaCl_2.2H_2O$	440.0
	KH_2PO_4	170.0
	$MgSO_4.7H_2O$	370.0
Microelements	$ZnSO_4.7H_2O$	8.6
	$MnSO_4.4H_2O$	22.3
	$CuSO_4.5H_2O$	0.025
	H_3BO_3	6.2
	KI	0.83
	$Na_2MoO_4.2H_2O$	0.25
	$CoCl_2.6H_2O$	0.025
Chelater iron	$FeSO_4.7H_20$	27.8
	Na_2EDTA	37.3
Organic supplements	Nicotinic acid	0.5
	Pyridoxine (B_6)	0.5
	Thiamine (B_1)	0.1
	Glycine	2.0
	Myoinositol	100.0

Inoculation of explants

To inoculate the explants with bacteria, leaves, stems, petioles and whole seedlings were used. The explants were divided into 2 cm pieces and shaken with bacterial suspension for 20 minutes, to allow the transfer of the rooting genes within the vegetal tissue. The explants were then placed on 0.5X MS-NH_4 solid culture medium without hormones and transferred to a growth room at 25°C under dark conditions. After 24, 48 and 72 hours of inoculation, the explants were washed and transferred to 0.5X MS-NH4 medium supplemented with 500 ppm of Cefotaxime. This process was then repeated weekly, each time decreasing the antibiotic amount with a 100 ppm. After complete removal of the bacteria, the explants were placed in antibiotic-free medium. Then, daily observations were made to check the appearance of hairy roots in the explants inoculated with bacteria. After one month of hairy root growth, various characteristics such as T-DNA transfer success

and the rate of hairy root production were evaluated.

Development and maintenance of hairy roots

Hairy roots longer than 2 cm were isolated and transferred into Erlenmeyer flasks containing 0.5X MS liquid culture medium. A shaker was used at 120 rpm in the dark at 25°C to maintain the hairy roots and they were subcultured every two weeks. To investigate the stability and growth rate of the hairy roots, three concentrations of IBA hormone (0, 0.5 and 1) were used in 0.5X MS liquid culture medium, and finally, after 60 days, their wet and dry weights were measured.

Molecular analysis

Plant DNA was extracted using a classical cetyltrimethylammonium bromide (CTAB) isolation protocol (Ciucă et al., 2020). Briefly, fresh roots from untreated plants and bacterial inoculated explants were aseptically mortared. Then 100 mg of root paste was treated with 1 ml CTAB lysis buffer, containing 2% CTAB, 1.42M NaCl, 200 mM EDTA, 100 mM Tris, 1% sodium thiosulphate, and 2% β-mercaptoethanol in sterile deionized water. After 1h of water bath incubation at 65°C, with periodic stirring, the samples were centrifuged at 10,000 ×g for 10 min. The supernatant was treated with an equal volume of chloroform: isoamyl alcohol 24:1 (v/v), and the mixture was centrifuged at 10,000 ×g for 10 min. The aqueous phase was then collected and treated with 1.5 ml RN-ase solution (10 mg/ml) for 45 min at 37°C. Samples were then treated with 60 µl 3M sodium acetate (pH 5.5), 60 µl 10 mM ammonium acetate, and 0.7 volumes of 2-propanol, and incubated overnight at -20°C. Samples were then centrifuged for 6 min at 12,000 × g, and the translucent pellet was hydrated in TE (1M Tris, 0.5M EDTA, pH 8) and stored at -20°C.

Bacterial DNA was extracted from fresh cultures obtained in NB. The DNA was isolated using the ZR Fungal/Bacterial DNA MiniPrep™ (Zymo Research, SUA), according to the manufacturer recommendations.

The PCR analysis was conducted to confirm the sage transformation by the *A. rhizogenes.* The targeted genes were *rolB* and *rolC* (Li et al., 2015). The oligonucleotide primer pairs used to amplify these genes were 5′- GCT CTT GCA GTG CTA GAT TT -3′ and 5′- GAA GGT GCA AGC TAC CTC TC -3′ for *rolB*, 5′- CTC CTG ACA TCA AAC TCG TC -3′ and 5′- TGC TTC GAG TTA TGG GTA CA -3′ for *rolC* (Grzegorczyk et al., 2006).

Each reaction was performed with 2 µl of template DNA, 1X Dream Taq buffer, 10 mM dNTPs, 10 µM primers, 0.5U Dream Taq and up to 25 µl of MilliQ water. The PCR was performed with Eppendorf Mastercycler Gradient (Eppendorf, Hamburg, Germany) and began with a 3 min initial denaturation step at 94°C, followed by 35 cycles of denaturation at 94°C for 30 sec, annealing at 55°C for 1 min, extension at 72°C for 1 min, and finally 7 min at 72°C. The PCR products migrated to 1.2% agarose gel, in 0.5X TBE, stained with ethidium bromide. The expected amplification products should have 383 bp for *rolB* and 585 bp for *rolC* (Grzegorczyk et al., 2006).

Statistical analysis

Statistical calculations, analysis of variance, and graph drawing were performed using SPSS software ver. 22, and comparison of means was performed using Duncan's test at a confidence level of 95%.

RESULTS AND DISCUSSIONS

After 2-3 weeks after *A. rhizogenes* inoculation of leaf, stem, petiole and seedlings explants, hairy roots appeared (Figure 1).

Figure 1. *Salvia* plant grown *in vitro* condition (a); Hairy roots grown after inoculation (b)

The highest percentage of hairy root induction and the largest number of hairy roots were observed at the wounded areas of *A. rhizogenes* treated leaf explants, with a success rate of

88.4%. In contrast, stem explants showed the lowest success in hairy root formations (10.4%). Following stem explants, petiole explants had the second-lowest induction rate (12.4%), while whole seedlings demonstrated an average ability of 32.4% to form hairy roots. Additionally, all control explants lacked hairy roots. These suggest that the type of explant plays a significant influence in the succeeding rate of *A. rhizogenes* transformation and hairy roots formation. Differences in the rate of hairy root production due to the use of different types of explants have been noted in other previous studies. Several research groups also reported leaf explants to have the highest efficiency in producing hairy roots (Mirzaie Delbari et al., 2022; Zare et al., 2022; Kardoost & Mahmoodnia, 2023). In only a few cases, leaf explants were the only ones capable of developing hairy roots, while to the rest of the explants the transformation was ineffective. When studying *A. rhizogenes* transformation in sage, Norouzi et al. (2017) were able to observe hairy roots formation only on bacterial treated leaf explants, while on petiole and stem explants no hairy roots were developed (Norouzi et al., 2017). However, there are also cases in which leaf explants produced fewer hairy roots than other treated explants (Lee at al., 2007; Brijwal & Tamta, 2015). Differences in physiological status, DNA synthesis, and cell division of various tissues may account for their competence to produce hairy roots (Pirian et al., 2012).

On the other hand, in the present study, petiole and stem explants were very sensitive to displacement and quickly turned brown. There losses caused by necrosis occurred during the subcultures in which the cefotaxime concentration was reduced. And although sage stem and petiole explants formed callus and hairy roots, they eventually died due to the browning and necrosis (Figure 2).

Infection of plant tissues by different strains of *A. rhizogenes* in many cases causes necrosis and browning of plant tissues. One of the reasons for the browning of explants after infection by *A. rhizogenes* is the presence of phenolic compounds and their oxidation by the enzyme polyphenol oxidase. This enzyme is responsible for their browning in plant tissue and plays an important role in activating programmed cell death (Shakeran & Keyhanfar, 2017). Hypersensitivity reactions are considered one of the plant defense responses, which are generally characterized by features such as localized and rapid cell death and the accumulation of antimicrobial agents *in situ* (Richter & Ronald, 2000). This is a series of events that cause necrosis and cell destruction. This can occur in the cell layer where the T-DNA was introduced or it may prevent the regeneration of transgenic cells isolated from such necrotic tissues. Thus, the yield of clones with transgenic cells is reduced (Shakeran & Keyhanfar, 2017).

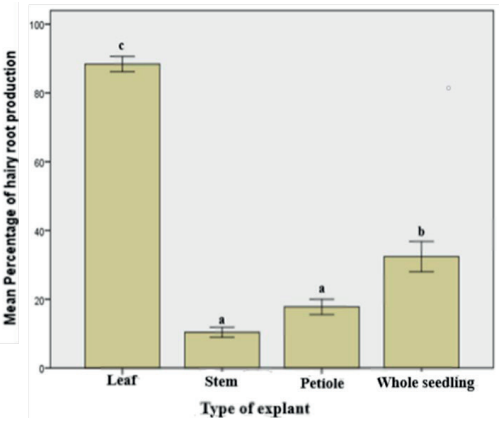

Figure 2. The hairy roots production on different explants. The data are presented as average values, while bars reflect the standard deviation. Different letters of columns indicate significant differences based on Duncan's test ($p < 0.05$)

In several studies, different types of explants from various plant species exhibit differing percentages of browning after being infected with *A. rhizogenes*, highlighting the influence of both plant genotype and bacterial strain on the rate of browning (Putalun et al., 2007; Zolala et al., 2007; Rahnama et al., 2008; Shakeran & Keyhanfar, 2017). In addition, the current study shows that in most cases, before hairy roots formation on the explants, callus formed at the site of cuts and wounds, and then hairy roots appeared. In some other studies, callus formation in explants has been reported after induction with *A. rhizogenus* and during the process of hairy root formation (Li et al., 2018; Sohrabinejad et al., 2018).

In this study, the rate of hairy root induction in a bacterial co-culture system was evaluated at

three different time points: 24, 48, and 72 h. The results revealed a significant variation in induction efficiency across the time intervals. The lowest percentage of hairy root induction occurred within explants from the 24 h treatment, with only 12.4% successful hairy root formation. In contrast, the highest induction rate was observed at 48 h, with a substantial 80% of the cultures successfully forming hairy roots. However, after the 72 h treatment, excessive explants colonization with *A. rhizogenes* made difficult the removal of the bacteria, and despite the antibiotic treatment, a complete bacterial elimination was not always achieved, leading to explant destruction. As a result, the transformation rate dropped significantly to 43.4% (Figure 3).

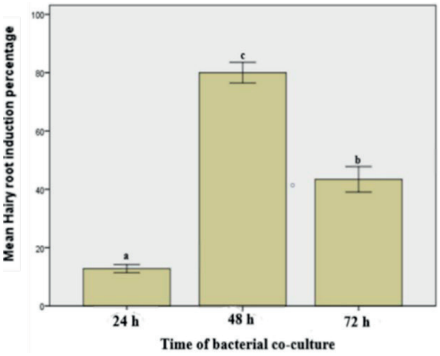

Figure 3. The rate of hairy roots induction after different times of co-culturing the explants with the bacteria. The data are presented as average values, while bars reflect the standard deviation. Different letters of columns indicate significant differences based on Duncan's test ($p < 0.05$)

Similar studies have also identified 48 hours as the ideal duration for bacterial co-culture and T-DNA transfer, supporting the findings of this research (Shakeran & Keyhanfar, 2017; Mirzaie Delbari et al., 2022; Kardoost Parizi & Mahmoodnia Meimand, 2023).

To confirm the transgenic nature of the hairy root lines and verify the presence of the *rolB* and *rolC* genes, polymerase chain reaction (PCR) was conducted using specific primers for these genes. The PCR results confirmed the presence of T-DNA, validating that the hairy roots had been successfully transformed by *A. rhizogenes* (Figure 4).

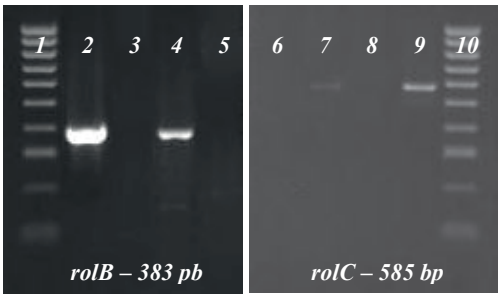

Figure 4. PCR amplification of *rol B* and *rolC* oncogenes in *A. rhizogenes* transformed roots and *A. rhizogenes* ATCC 15834 strain. The corresponding lanes are as follows: 1 and 10 = Molecular marker of 100bp, 2 and 9 = transformed sage roots, 3 and 8 = untreated sage roots, 4 and 7 = *A. rhizogenes* ATCC 15834 strain, 5 and 6 = negative control (MilliQ water)

The variance analysis table (Table 2) in this study indicated that the application of the auxin hormone IBA significantly affected the growth characteristics of the hairy roots, including both fresh and dry biomass. However, a treatment of 0.5 mg/l of IBA had no significant effect on hairy root growth. The highest growth rate was observed at a concentration of 1 mg of IBA hormone, where sage roots had 10.65 g fresh weight, and 0.36 g dry weight (Figure 5).

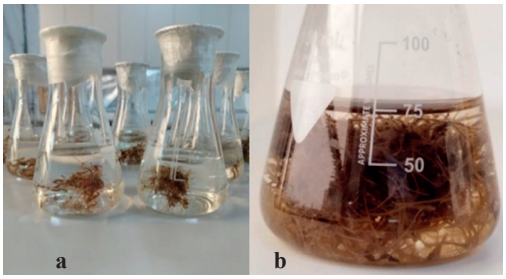

Figure 5. Sage hairy roots:
a. Hairy roots on the first day of hormone treatment;
b. Hairy roots after 2 months of treatment with 1 mg IBA/L

These results are consistent with the findings of Sohrabinejad et al. (2018) about production of hairy root in *Calendula officinalis*. Their study showed that among treatments with IAA concentrations of 0, 0.5, and 1 mg/l, the best root growth occurred at the 1 mg/l concentration in 0.5X B5 medium (Sohrabinejad et al., 2018).

Table 2. Results of analysis of variance, the effect of hormone concentration on hairy root growth

Tests of Between-Subjects Effects						
Source	Dependent variable	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected model	Wet weight (g)	112.963[a]	2	56.482	76.642	0.000
	Dry weight (g)	0.065[b]	2	0.033	12.933	0.001
Intercept	Wet weight (g)	499.278	1	499.278	677.493	0.000
	Dry weight (g)	0.541	1	0.541	214.032	0.000
IBA	Wet weight (g)	112.963	2	56.482	76.642	0.000
	Dry weight (g)	0.065	2	0.033	12.933	0.001
Error	Wet weight (g)	8.843	12	0.737		
	Dry weight (g)	0.030	12	0.003		
Total	Wet weight (g)	621.085	15			
	Dry weight (g)	0.637	15			
Corrected Total	Wet weight (g)	121.807	14			
	Dry weight (g)	0.096	14			

[a]R Squared = 0.927 (Adjusted R Squared = 0.915)
[b]R Squared = 0.683 (Adjusted R Squared = 0.630)

Furthermore, studies on secondary metabolite production in *Cichorium intybus* L. through hairy root induction, in the presence of four concentrations of naphthyl acetic acid (0, 0.5, 1, and 1.5 mg NAA/l), showed that combination of 1.5 mg NAA/l with 3% and 4% sucrose produced higher increases in both fresh and dry weight, as well as other growth parameters (Fathi et al., 2019).

CONCLUSIONS

Hairy roots induction on medicinal plants to produce high-value therapeutic compounds has become increasingly important in recent years. This raised interest is driven by the substantial demand for plant-derived metabolites, which are often found in low quantities in natural plant sources. As a result, there is a growing emphasis on exploring methods that can enhance the production of such bioactive compounds.

Hairy roots are known to generate plants secondary metabolites more effectively, rapidly, and consistently than other plant tissues such as aerial parts or callus cultures. Therefore, advancing *in vitro* plant propagation techniques, particularly for generating and producing hairy roots, is a key research objective. This study focuses on *Salvia officinalis*, a plant of significant medicinal value, and examines the production of hairy roots within this species.

Different plant explants exhibited varying capacities for hairy root formation, and factors such as the duration of bacterial co-culture and the concentration of plant hormones were found to significantly influence both the induction and growth of the hairy roots. Co-inoculation on sage leaves for 48 h in *A. rhizogenes* ATCC15834 suspension, as well as 1 mg of IBA hormone treatment revealed to be the most promising way for increasing the biomass production of hairy roots in this medicinal plant species. Although research on hairy root production in sage species has been limited, the results of this study suggest that inducing hairy roots in this plant holds great potential for producing valuable medicinal compounds.

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ECOLOGICAL CONTROL OF POWDERY MILDEW OF CUCUMBER BY POTASSIUM PHOSPHATE AND NEEM OIL AND THEIR EFFECTS ON CONTENTS OF CHLOROPHYLLS IN LEAVES

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Abstract

Powdery mildew, caused by Sphaerotheca fuliginea is a widespread disease affecting cucumber plants causing yield losses worldwide. A greenhouse experiment was carried out to evaluate the effects of the ecological plant protection products of monopotasium phosphate, dipotasium phosphate and Neem oil on powdery mildew disease severity. The study, also, evaluated the changes of photosynthetic pigments contents in leaves in relation to treatments. All treatments resulted in a significantly lower mildew infection development than untreated control plants (water spray). The application of Neem oil emulsion alone recorded the lowest efficacy rate. The most significant disease severity reduction was registered in the treatment with integrated use of dipotasium phosphate (1%) and Neem oil (0.5%) compared to the other treatments. The foliar application of the tested compounds improved the status of photosynthetic pigments in leaves and no phytotoxicity was observed on the plants. This research demonstrated that the integrated application of potassium phosphates and Neem oil had fungicidal activity against powdery mildew.

Key words: *chlorophylls, cucumber, Neem oil, potassium phosphates, powdery mildew.*

INTRODUCTION

Cucumber (*Cucumis sativus* L.) plays a pivotal role in organic production of legumes. There is a body of information indicating that a major constraint of sustainable vegetable production is the high sensitivity of crops to different kinds of diseases. Among these diseases, powdery mildew of cucumber, caused by the fungus S*phaerotheca fuliginea*, is a serious problem for both legume producers and researchers (Amtmann et al., 2008; Elad et al., 2021). This pathogen is widely spread in many countries, including the Republic of Moldova and it attacks cucurbit vegetable crops under both field and greenhouse conditions, significantly reducing crop quality and yield (Rur et al., 2018). Powdery mildew infects all organs in cucumber plants which have negative repercussions on the physiological and metabolic processes of the plants (Cheah et al., 1996). In particular, powdery mildew reduces photosynthetic parameters and chlorophylls content in leaves of melon (Tian et al., 2024). As a rule, conventional agricultural practices rely on synthetic fungicides to control plant diseases. However, the application of these fungicides has raised concerns regarding human and environmental health for the long-term (Morishita et al., 2003). Likewise, public awareness has increased regarding the contamination of the natural resources and vegetables food products with pesticides. In organic production of vegetables**,** various ecological products are used to protect against fungus disease. However, their efficacy to control powdery mildew in legume crops is low. Therefore, it is necessary to look for more effective ecological plant protection products and find out new approaches of their application in order to sustainably manage powdery mildew in crops, especially cucumber plants. One of these compounds with antifungal activity is mineral salts and essential oils of different plants. It has been demonstrated that applications of potassium phosphates (Reuveni et al., 1995; Deliopoulos et al., 2010) as well as neem oil (Mostafa et al., 2021; Raveau et al., 2020; Viradiya & Gangwar, 2022) have a potential in controlling plant diseases, particularly cucumber powdery mildew. Many researchers revealed that separate use of these ecological products does not provide sufficient

efficiency to combat the powdery mildew (El-Sharkaway et al., 2014; Rur et al., 2018).
The purpose of this research was to evaluate the efficacy of monopotassium phosphate, dipotassium phosphate applied alone or in combination with emulsion of Neem oil to control powdery mildew disease of cucumber. In addition, in this study we examined the treatments effects on chlorophylls contents in cucumber leaves.

MATERIALS AND METHODS

Cucumber plants were grown from seed sown in soil under greenhouse conditions. A cucumber cultivar Iulian was used in this study. This cucumber variety has moderate susceptibility to the powdery mildew pathogen S*phaerotheca fuliginea*. The experimental design included six treatments with three replicates of each treatment. The applied treatments were as follows: 1. Control, untreated plants; 2. Monopotasium phosphate 1%; 3. Dipotassium phosphate 1%; 4. Neem oil 0.5%; 5. Monopotasium phosphate 1% + Neem oil 0.5%; 6. Dipotassium phosphate 1% + Neem oil 0.5%. The plants to be treated had five fully expanded leaves at 4 weeks after transplanting, and sprays were made at weekly intervals. Plants sprayed with water served as control. The grown plants were artificially inoculated by shaking naturally infected cucumber leaves three days after the first spraying with the tested compounds. Disease severity was assessed three times after the second spray. A powdery mildew severity scale from 0 to 5 A powdery mildew severity scale from 0 to 5 according to Paulus et al. (1969) was used to assess the disease. The percentage of treatment efficiency in the reduction of powdery mildew severity was calculated according to the method published in the literature of specialty (Viradiya & Gangwar, 2022). The chlorophyll content in leaves was determined according to the method proposed by Arnon (1949). The contents of Chl. *a* and *b* were measured by absorption using a spectrophotometer at 663nmand 645nm. The data were analyzed using Statistic Program 7. Means were compared by least significant differences (LSD) at $p \leq 0.05$.

RESULTS AND DISCUSSIONS

In organic farming, the application of ecological plant protection products has been increasingly accepted, particularly for vegetables such as cucumber. Unfortunately, limited studies have compared the influence of potassium phosphates and Neem oil applied individually or alternately on powdery mildew development in cucumber. Our study's results showed that the application of monopotassium phosphate, dipotassium phosphate and Neem oil individually or in alternation, reduced the disease severity of powdery mildew. The data of the first evaluation of the disease severity are presented in Figure 1.

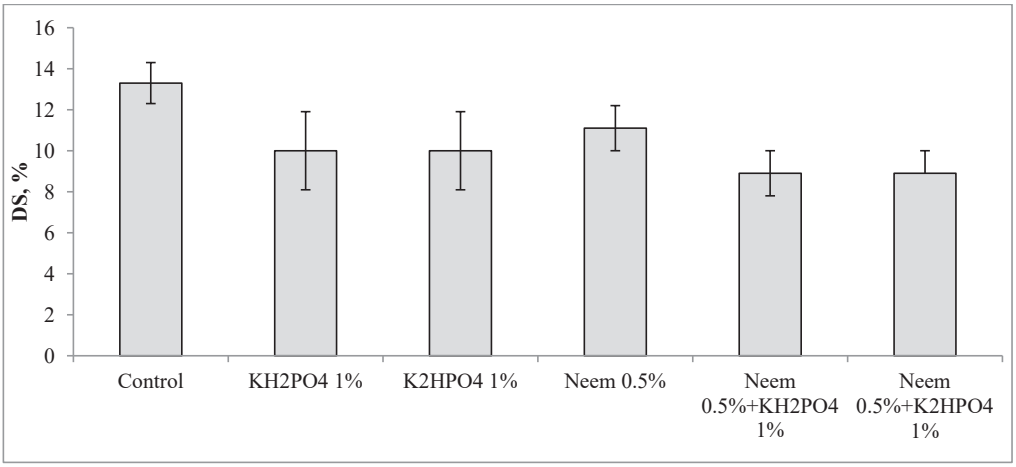

Figure 1. Effect of treatments of potassium salts and Neem oil on powdery mildew disease severity (DS, %) of cucumber plants. Data presented as the means of three replicates ±SE. (Ist disease rate 28.06.2024)

The highest disease development was observed on leaves of control plants receiving four weekly applications of water. The treatments displayed different levels of effectiveness in reducing disease severity. According to experimental results, the most effective treatment was the alternate use of dipotassium phosphate (1%) and Neem oil (0.5%). In general, the tested products applied alone or in alternation, reduced the disease severity of powdery mildew under greenhouse conditions in all disease evaluations (Figures 1, 2 and 3). Therefore, the results recorded at the first evaluation of the disease severity found that the intensity of the powdery mildew in the control variant was the highest and constituted 13%. However, the application of potassium phosphates salts decreased the disease severity. It has been observed that the application of monopotassium phosphate and dipotassium phosphate separately had the same effect on the spread of the disease (first disease assessment). On the other hand, the plant treatments with emulsion of Neem oil alone had a low effect compared to the application of other treatments. The lowest intensity of the disease was registered in the treatment of combined application of potassium phosphate and Neem oil. It is important to note that no significant difference was established between treatments of monopotassium phosphate+Neem oil (variant 5) and dipotassium phosphate+Neem oil (variant 6) at the first assessment of disease severity (Figure 1). Experimental data revealed that disease severity in these variants reached lower indices, being 31% lower than the value registered in control variant. This suggests that monopotassium phosphate, dipotassium phosphate, and Neem oil exhibit fungicidal properties, whether applied separately or together, in managing powdery mildew. However, these treatments had different grades of powdery mildew disease suppression. The use of these ecological protection products to manage powdery mildew was moderately effective in minimizing the danger of this disease at second and third evaluation of the disease severity (Figures 2 and 3).

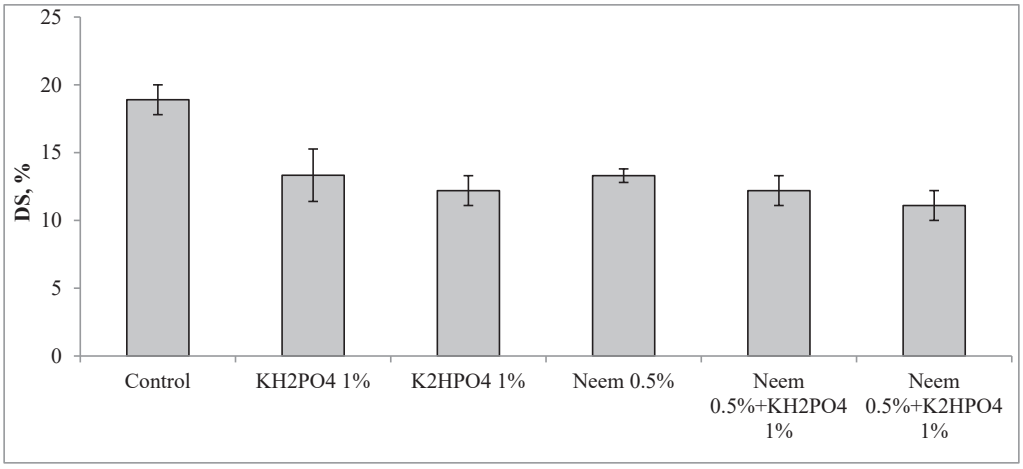

Figure 2. Effect of treatments of potassium salts and Neem oil on powdery mildew disease severity (DS, %) of cucumber plants. Data presented as the means of three replicates ± SE (II-nd rate 5.07.2024)

This study observed that as cucumber plants progressed in their growth cycle, powdery mildew severity increased in all variants, including treated plants. Analysis of these data demonstrates that the separate application of potassium phosphate and Neem oil had a beneficial effect in reducing the spread of the cucumber powdery mildew. From the experimental results of second assessment of disease severity (Figure 2) it can be observed that the application of dipotassium phosphate showed a better effect than the variant with the use of Neem oil alone, in terms of reducing the severity of powdery mildew. Nevertheless, the integrated application of Neem oil and dipotassium phosphate ensured the best

biological efficacy in combating powdery mildew compared to the other experimental variants. Treatments with alternate use of dipotassium phosphate and Neem oil resulted in the highest decrease of in the powdery mildew with a disease severity of 11%. The effects of treatments with potassium phosphate and emulsion of Neem oil at the third evaluation of the disease severity rate highlighted increased differences between the control variant (untreated plants) and the treatments with biorational protection products (Figure 3).

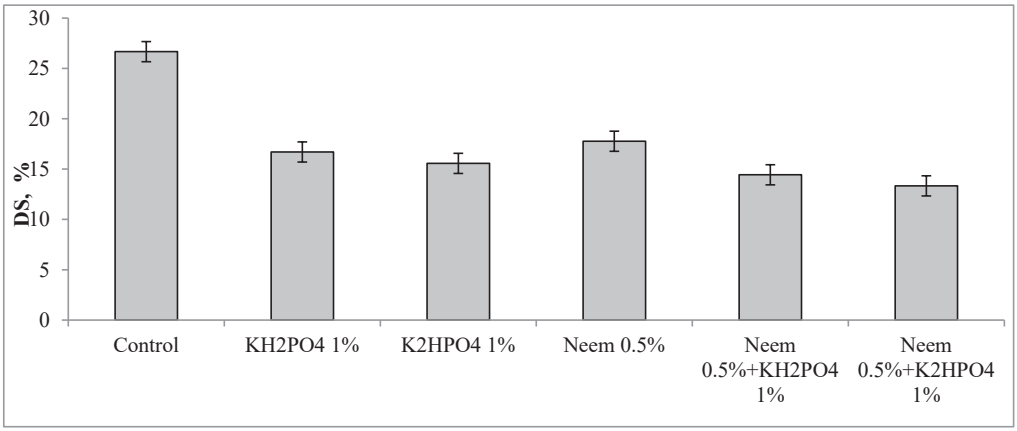

Figure 3. Effect of treatments of potassium salts and Neem oil on powdery mildew disease severity (DS, %) of cucumber plants. Data presented as the means of three replicates ± SE (III-rd rate12.07.2024)

Similar to the previous two estimations of the disease development the highest severity of powdery mildew disease was established in the control variant where this value registered the level of 27%, and the lowest intensity was observed in the sixth variant, where treatments were made with dipotassium phosphate and Neem oil (the disease severity recorded 13.3%). Therefore, the disease development at this growth stage of cucumber reached lower indices due to the applied four treatments. Hence, the disease severity was almost two times lower than those recorded in the control. Perhaps, the obtained results could be explained by an increased resistance of plants against powdery mildew due to higher activity of enzymes and accumulation of antioxidants. In this respect researchers Irving & Kuc (1990) found out that induced resistance in cucumber plants with dipotassium phosphate increased the activity of peroxidase and chitinase enzymes.

One of the most crucial metabolic processes in crops is photosynthesis. The foliage treatments have a direct impact on the chlorophylls status of crop leaves. Besides evaluating the disease severity of cucumber, we examined the effects of monopotassium phosphate, dipotassium phosphate and Neem oil applied individually or alternately on changes in contents of chlorophylls *a* and *b* in leaves. Experimental data revealed that powdery mildew infection reduced chlorophylls (Chl.) contents in leaves. Results showed that treatments alleviate the adverse effect of powdery mildew on chlorophylls status in cucumber leaves.

In the present study, there was a moderate increase in the content of Chl. *a* and Chl. *b* in leaves due to the application of the dipotassium phosphate alone or in conjunction with Neem oil compared to the untreated plants (Figure 4 and 5). Determination of chlorophylls revealed that the concentration of Chl. *a* was low (1.17 $mg \cdot g^{-1}$ FW) in the control variant and this parameter increased with dipotassium phosphate and Neem oil to 1.35 $mg \cdot g^{-1}$ FW. We suggest that plants treated with mineral salts and Neem oil have a protective effect on chlorophylls against pathogen attack in cucumber leaves.

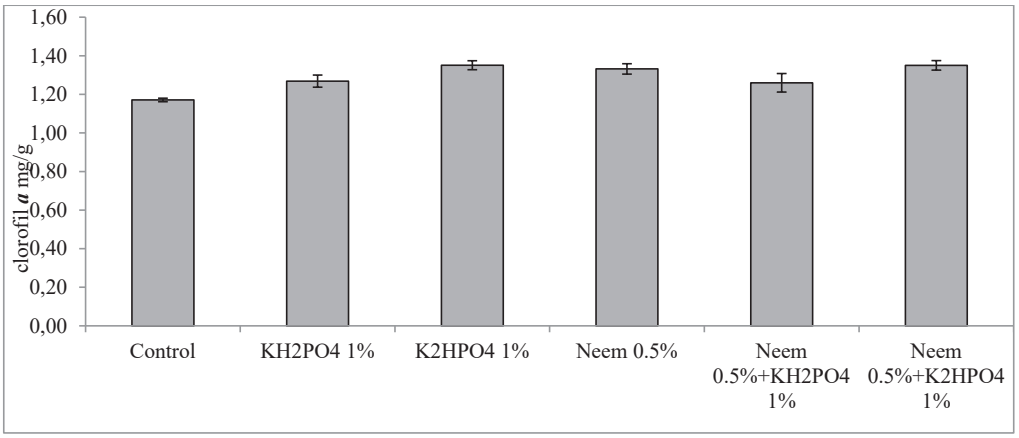

Figure 4. Effect of potassium phosphates and Neem oil on chlorophyll ***a*** contents (mg/g of fresh weight) in the cucumber leaves. Mean ± standard error (n = 3)

Figure 5 demonstrates the changes in Chl. *b* in leaves after four treatments. The data revealed that the chlorophyll content significantly changed in relation to application of dipotassium phosphate alone or in combination with Neem oil. The lowest value of Chl. *b* has established in the control variant. Meanwhile, the highest value of Chl. *b* content was registered (0.58 mg·g^{-1} FW) as a result of the combined application of dipotassium phosphate and Neem oil. The untreated plants showed the lowest concentration of the photosynthetic pigment of Chl. *b* (0.49 mg·g^{-1} FW). Therefore, results of the study showed that pathogen infection decreased the photosynthetic pigments concentrations in cucumber plants, but this adverse effect was at some level attenuated by the integrated use of potassium phosphates and Neem oil emulsion.

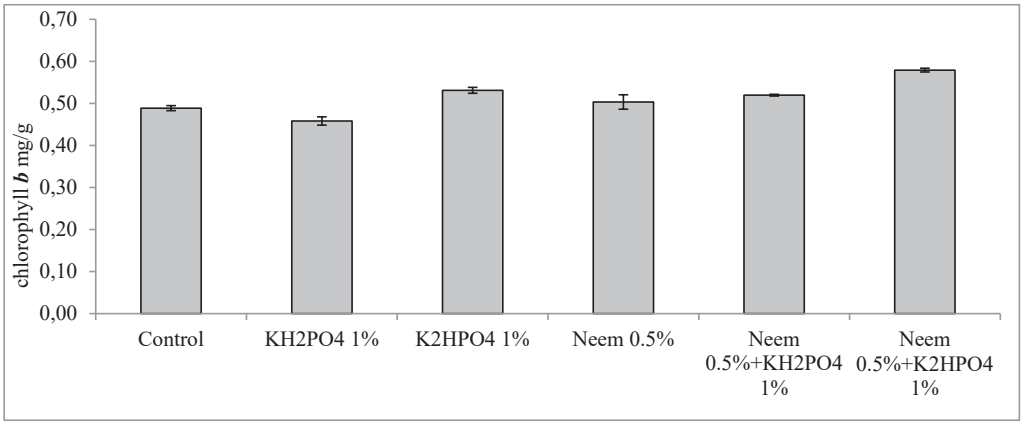

Figure 5. Effect of potassium phosphates and Neem oil on chlorophyll ***b*** contents (mg/g of fresh weight) in the cucumber leaves. Vertical bars represent the mean of three replicates ± standard error. Mean ± standard error

On the other hand, it is worth noting that four treatments with these mineral salts of potassium phosphates of course contributed to a certain extent to the improvement of mineral nutrition of cucumber plants, increasing the photosynthetic activity, which undoubtedly had positive repercussions on the improved development of plants and the increase in fruit productivity. Hence, our study showed that all treatments led to an increase in the content of Chl. *a*, and had less impact on Chl. *b* modifications. These findings are consistent

with those of Ahmed et al. (2021), who found that celery treated with biological control agents showed a significant increase in chlorophylls and carotenoids pigments in leaves of celery. Overall, the results of the current research indicate that treatments with potassium phosphate in conjunction with Neem oil application had a beneficial impact on pathogen tolerance, protecting the functions of the photosynthetic apparatus by improving the status of chlorophylls in cucumber leaves. Hence, based on the experimental findings we can suggest that the integrated use of the tested biorational products could offer an ecologically alternative to the use of synthetic fungicides in suppressing powdery mildew in cucumber plants.

CONCLUSIONS

Results of the study demonstrated that application of monopotassium phosphate and dipotassium phosphate, applied alone or alternated with the Neem oil displayed fungicidal activity against cucumber powdery mildew.

The alternate use of dipotassium phosphate and Neem oil was the most effective for controlling powdery mildew disease compared to individual treatments. The concentrations of photosynthetic pigments increased after treatments with potassium phosphate in combination with treatments of Neem oil emulsion.

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SUSTAINABLE AGRICULTURAL SOLUTIONS APPLIED TO BUCKWHEAT COVER CROP IN 2024

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Abstract

In the context of the climate challenges of 2025, characterized by prolonged drought, buckwheat stands out as a sustainable solution for agriculture in Stupina Cornetu in Ilfov County, Romania. This plant, used as a cover crop, offers multiple benefits, including improving soil quality, reducing water loss and supporting biodiversity, while also being a valuable source of pollen for bees. Buckwheat sowing was carried out in June, in drought conditions, by using seeds of an adapted variety (Fagopyrum esculentum L. 'Zita'), characterized by a short vegetation period and high tolerance to water deficit. The cultivation scheme included the use of technological links of minimal soil preparation in order to conserve moisture. The results obtained contributed to the creation of a sustainable agricultural ecosystem through the cultivation of buckwheat plants. This culture can thus become a key component in adapting to climate change and supporting a sustainable agriculture in Romania.

Key words*: cover-crop plants, climate change, agricultural ecosystem, honey base, sustainable agriculture.*

INTRODUCTION

Buckwheat has proven a high melliferous potential, providing a constant source of pollen and nectar for bees, during the essential period in the context of climate change. The current study explores the mellifer potential values of the buckwheat plant grown in Stupina Cornetu and its contribution to a sustainable agricultural ecosystem.

Buckwheat plants (*Fagopyrum esculentum* L.) of the 'Zita' variety, represent a promising agricultural solution in areas affected by prolonged drought, having importance both for improving the soil and for beekeeping, according to previous studies (Drăgan et al., 2022; 2023).

At present, several studies have highlighted the benefits of using cover crops in agricultural production, often focusing on the soil's physical, chemical, or biological properties (Păceşilă et al., 2022; Adetunji et al., 2020; Saleem et al., 2020).

The outlook of research conducted on buckwheat cultivation (https://agrolifejournal.usamv.ro/index.php/agrolife/article) has been shaped by variations in domestication conditions and the limited advancements in improving modern varieties. Trotsenko et al. (2020) primarily aimed to examine a buckwheat collection to identify suitable samples for summer sowing in the Northern Forest-Steppe region of Ukraine.

At the same time, Saleem et al. (2020) concluded from their research that additional growth of cover crop roots can provide benefits to the soil, including increasing soil organic carbon content, available nutrients, as well as soil aggregation. In addition, cover crops have been shown to improve nutrient cycling, soil physicochemical and biological properties, as well as soil pest management and weed suppression.

According to Sharma et al. (2018), cover crops help to reduce soil water evaporation, thus preserving soil moisture for the next crop. Although this has been observed in some environments (Basche et al., 2018), under water-limited conditions without irrigation, cover crops have frequently been found to limit soil

moisture for subsequent crops (Nielsen et al., 2020; 2015).

Between June and October 2024, Ilfov County, along with other regions in southern Romania, was severely affected by severe drought, combined with record hot temperatures. During this period, the average temperature was 2-3.5°C above normal, according to data from the EU's MARS System, marking the summer of 2024 as one of the warmest on record. The soil showed an extreme lack of moisture, with very severe pedological drought, which seriously compromised agricultural crops and affected extensive areas, including in Ilfov, where the main economic spring crops suffered significant losses, sometimes 100% (https://joint-research-centre.ec.europa.eu/monitoring-agricultural-resources-mars_en).

In Figure 1, an estimate of the average annual temperature for the Cornetu region is graphically represented. The dotted blue line represents the linear trend of climate change. The trend line is upward from left to right, the temperature trend is positive warming in Cornetu due to climate change.

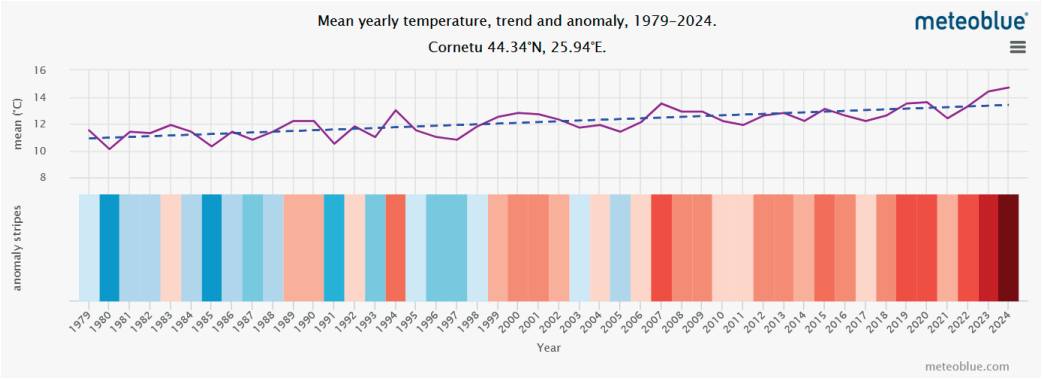

Figure 1. Estimation of the average annual temperature for the Cornetu region, Ilfov county.
Legend: https://www.meteoblue.com/en/climate-change/cornetu_romania_680754

In the lower part of the graph, the heating streaks are highlighted. Each colored band represents the average temperature for a year - blue for colder years and red for warmer years (https://www.meteoblue.com/ro/climate-change/cornetu_romania_680754).

Since 2019, the temperature warming situation in the Cornetu region, Ilfov county (Figure 2) has become worrying, this situation has highlighted the urgency of adopting sustainable measures in agriculture, such as the buckwheat plant (cover-crop plant) following the research carried out by Drăgan et al. (2024), for its resistance to water stress and for the ability to conserve soil moisture. Such an approach can help reduce the impact of climate change on agriculture.

Islam et al. (2021) as well as Gutema et al. (2023), demonstrated that management practices that integrate soil conservation works with cover crops could help recover, preserve and build soil quality (SQ) to address food security issues. The study in this paper is based on the conclusion of some objectives pursued, during the years 2021-2024, for the implementation of the project financed by the Agency for the Financing of Rural Investments - Romania, and the Regional Center for the Financing of Rural Investments 8 Bucharest - Ilfov, Project code: CRFIR-BUCURESTI-ILFOV, with the acronym AGROAPIS (https://agroapis.polenizare.ro/).

In collaboration with the Faculty of Biotechnologies of the University of Agronomic Sciences and Veterinary Medicine in Bucharest, with the main objective of identifying some ground cover plants and their valorization in order to sustainably develop honeydew crops and incorporate the plant mass through mechanical plowing in the field after the flowering period in November 2023.

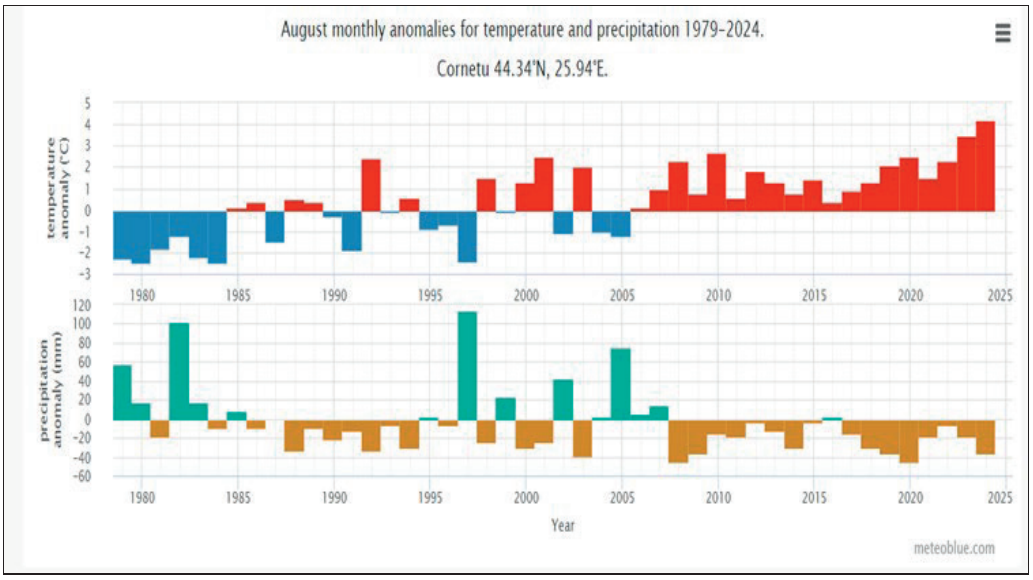

Figure 2. Climate changes – in the town of Cornetu; Temperature and precipitation anomaly in August 2024
Legend: www.meteoblue.com

Consequently, the work integrates the proposals and development objectives proposed to the parties involved in the project in the apiary of Cornetu, county Ilfov - Romania in order to ensure the territorial balance.

MATERIALS AND METHODS

Buckwheat (*Fagopyrum esculentum* L.) the 'Zita' variety selected for its high tolerance to drought was sown on 01.06.2024 in the apiary in Cornetu, the soil being prepared by light tillage.

The increase or decrease of the water deficit (drought) in the soil depends on the duration of the drought in the atmosphere and on the concrete properties of the soil: texture; structure; compaction (porosity; organic matter content, etc.). Through various agrotechnical, agrochemical and phytotechnical actions, it is possible to make the plants use the available water reserves from the soil as efficiently as possible, first of all, the easily accessible ones and to increase the volume of agricultural production.

In the case of buckwheat cultivation, this methodology makes it an attractive crop for water-scarce areas, at the same time offering multiple ecological benefits, including pollinator support and soil regeneration (Drăgan et al., 2024).

The experiment was carried out on the buckwheat culture in the town of Cornetu, on August 5, 2024.

The following technological links were applied for the establishment of the culture.

Sowing depth: Buckwheat seeds were sown at a depth of 2-3 cm, enough to keep optimal contact with the soil and retain the moisture necessary for germination.

Minimal fertilization: For this purpose, modern technologies of embedding the plant mass in the soil were used in order to obtain a sustainable ecological agriculture of increasing the quality of soil humus in the Cornetu apiary by cultivating honeydew cover crops (Drăgan et al., 2024).

After the end of the flowering period, all these crops were incorporated into the soil by mechanized plowing in order to increase the amount of humus, thus ensuring an ecological method of sustainability through cover crops. (Figure 3).

The determinations regarding the secretion of nectar (mg), the content in soluble carbohydrates and the carbohydrate index (mg/flower) were performed by the capillary method (Drăgan et al., 2023; Ion et al., 2007) in 3 series of 10 samples each (30 repetitions) from the buckwheat culture (*Fagopyrum esculentum* L.) variety 'Zita'.

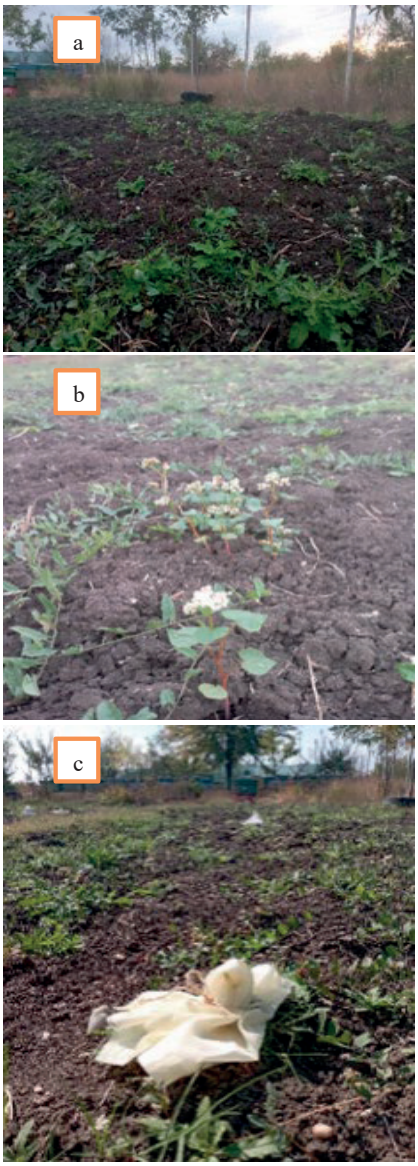

Figure 3. Buckwheat cover-crop culture established at the Cornetu apiary in 2024: a) sprouted buckwheat plants in the furrow; b) buckwheat plants at the beginning of flowering; c) buckwheat flowers isolated with gauze during 24 hours in order to monitor the nectar

The evaluation methodology of the productive parameters referred to those of the mellifer potential determined during the flowering period of the buckwheat plants (Figure 4).

Thus, data were collected regarding the carbohydrate index (mg/flower), the daily honeydew potential (kg/ha/day) and the honeydew potential per crop (kg/ha) at three time intervals (9:00, 12:30, 16:30) using the method of Ion et al. (2007).

Figure 4. Evaluation of honeydew potential determined during the flowering period of buckwheat plants: a) nectar collection by the capillary method; b) overview of the buckwheat plant at 9 o'clock in the Cornetu apiary

The carbohydrate analysis was carried out by standardized methods, and the honey values were calculated based on the available nectar quantities.

RESULTS AND DISCUSSIONS

According to the collected data which are represented in Table 1, the following results were obtained for the following analyzed parameters:

The carbohydrate index varied between 0.0016 mg/flower (at 16:30) and 0.003 mg/flower (at 9:00), with an average value of 0.0022 mg/flower. These values demonstrate a constant availability of carbohydrates, essential for bees.

Daily honeydew potential was highest at 9:00 AM (1.75 kg/ha/day), gradually decreasing to 0.94 kg/ha/day at 4:30 PM. This variation can be correlated with air temperature and humidity.

The honeydew potential/per crop reached an average of 33.26 kg/ha, highlighting the importance of the buckwheat plant as a honeydew base in drought conditions (Table 1). These results suggest that buckwheat is an adaptable crop that supports biodiversity and contributes to the resilience of local beekeeping.

Table 1. The values recorded at the level of the samples collected from the conventional buckwheat culture for the evaluation of the mellifer potential, evaluation date August 5, 2024, at Cornetu apiary (Ilfov county, in S-E Romania)

SERIES	Average number (flowers /ha)	Glycemic index (mg/ flower)	The entire flowering period (number of days)	Daily honeydew potential (kg/ha/days)	Honeybee buckwheat potential (kg/ha)
SERIES 1 9:00 a.m.	475.200.000	0.0030000	26	1.75	45.56
SERIES 2 12:30 p.m.	475.200.000	0.0020000	26	1.15	29.83
SERIES 3 16:30 p.m.	475.200.000	0.0016000	26	0.94	24.41
Average values	**475.200.000**	**0.0022000**	**26**	**1.28**	**33.26**

(*with variation limits between 230.400.000 and 720.000.000)

The high values of honeydew potential prove that it is a viable solution for diversifying pollinators' food resources (Figure 5), especially in the early part of the day when atmospheric moisture contributes to the nectar secretion of plants.

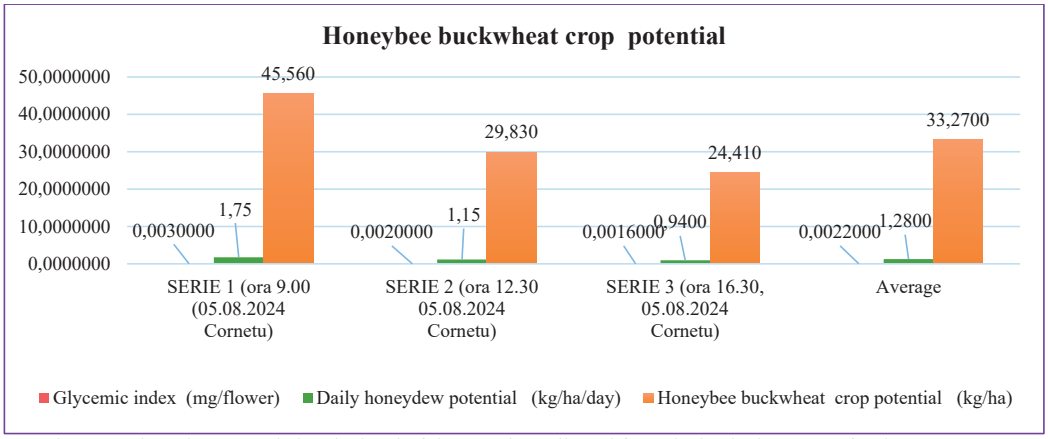

Figure 5. The values recorded at the level of the samples collected from the buckwheat crop, for the assessment of honeydew potential - assessment date August 5, 2024, SERIES 3 - 9 am - 12.30 pm - 4.30 pm, at Cornetu apiary (Ilfov county, in S-E Romania)

CONCLUSIONS

Buckwheat is proving to be an essential crop for maintaining the health of agricultural ecosystems and supporting beekeeping, especially in drought conditions.

The high values of honeydew potential underline the importance of using this plant in sustainable regenerative agriculture strategies.

The high temperatures of 2024 negatively influenced nectar secretion during the day, at the level of buckwheat flowers.

Thus, the results obtained in 2024 confirmed the fact that the flowers of the buckwheat plants of the studied variety 'Zita' accumulated the best honey activity in the first part of the day (morning), and as a result the bees were active during that period, in order to maximize honey production.

The parameters of honey potential values were within the optimal parameters, but their decrease occurred in the afternoon, which proves a clear indicator of thermal stress on the culture.

To optimize the results, it is recommended to expand the areas cultivated with buckwheat and continue studies on its adaptation to various climatic conditions.

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RESPONSE OF THE REPRODUCTIVE SYSTEM MORPHOLOGY TO CHANGES IN THE PITUITARY-OVARIAN AXIS CONTROL IN DIFFERENTLY SELECTED HEN STRAINS DURING EGG-LAYING CYCLE

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Abstract

The biotechnology of producing egg-laying hen hybrids requires a high genetic selection pressure upon the origin inbred strains. The selection may affect the response of the reproductive system to the pituitary-ovarian axis control. In this work, we investigated the changes of ovarian and oviductal histomorphology response to the control exerted by the pituitary-ovarian axis in Lohmann and Leghorn hybrids, versus Sussex hens that has not been subjected to any selection pressure. Determinations were performed on 32-week-old hens, a key-age during the laying cycle. The results revealed differences between the hybrid hens and Sussex hens regarding the blood levels of gonadotropin follicle-stimulating hormone, luteinizing hormones ($P<0.01$), estrogen ($P<0.05$), triiodothyronine and tetraiodothyronine, and higher values of egg-laying frequency in hybrids versus Sussex. The number of different types of ovarian follicles (white follicles, small yellow and large yellow follicles), the structure of the follicle perivitelline membrane, and structure of the yolk was also modified in hybrids versus Sussex. Differences in epithelium histology of the infundibulum, magnum, isthmus and shell gland as well as histochemical particularities of these structures were also identified.

Key words*: egg-laying hybrid hen, pituitary control, ovary histology, oviduct histology.*

INTRODUCTION

The histological structure, development, and the physiology of the ovary and oviduct in birds are hormonal controlled, either directly by the pituitary gonadotropic hormones FSH and LH in the case of the ovary, or by estrogens and progesterone in the case of the oviduct. Accordingly, functional changes in the pituitary-ovarian axis generate changes in ovarian and oviduct structure and function (Dojană, 2009). The long-term biotechnology programs of selection and improvement of inbred strains, which are used to obtain performative egg-laying hen hybrids have modified the pattern of control exercised by the pituitary-ovarian axis on the structure and physiology of the ovary and oviduct in the hen. Hanlon et al. (2021) reported that the ovary and oviduct of Lohmann laying hens were significantly heavier than those of unselected Shaver and Smoky Joe hen breeds ($P < 0.05$), suggesting major changes on the control exerted by the hypothalamic-pituitary-ovarian axis on ovarian weight and structure during the biotechnological process of selection.

Researches on the changes induced by gene-tic selection on the control of hypothalamic-pituitary-ovarian axis on the ovary and oviduct in chicken were conducted by Mimura (1937), Joseph et al. (2014), Hanlon et al. (2021) and Bunaciu et al. (2009). Bunaciu and Dojană (1982) incriminate the degree of homozygoty among factors which changed the control of pituitary-ovarian axis upon the ovary and oviduct physiology in inbred strains of chickens and turkeys. On the other hand, according to Sechman (2013), the ovary and oviduct function seems to be under the influence of iodinated thyroid hormones in hen: *in vitro* studies have demonstrated that T3 decreases estrogen secretion from ovary non-hierarchical white follicles and from the theca layer of yellow preovulatory follicles, while, on the other hand, T3 increases progesterone production from the granular layer of these follicles. As the direct effects of progesterone and especially estrogens

on the morphology and physiology of the oviduct are well known, one can conclude about the influence of the thyroid (secretion of iodinated hormones, respectively) on this structure.

The present work investigates the extent to which the long-term selection biotechnology processes of the inbred strains used for the production of Lohmann and Leghorn laying hybrids have altered the control pattern of the pituitary-ovarian axis on the structural and functional response of the ovary and oviduct in these hybrids during laying pick. The comparison is done with the Sussex, a chicken breed that has not been subjected to high selection pressure for egg or meat production.

MATERIALS AND METHODS

Animals and experimental design

To achieve the objectives of this experiment, two groups of 32-week-old hybrid laying hens of 5 cap. each were used: a group of Lohmann hens and a group of Leghorn hens. Comparisons were made with 5 cap. group of 32-week-old Sussex hens. The birds were raised in an industrial system, watered and fed *ad libitum.* The commercial diet recommended by the hybrid supplier was used for feeding. The birds also benefited from a 15-hour light program, from 5:30 to 20:30. Eggs were daily collected for four weeks and the egg-laying frequency was calculated as number of eggs/ cap./week for each group.

Hormone and histology analysis

Blood samples were taken by axillary vein puncture for hormonal determinations. The samples were stored and processed according to the instructions of the kit suppliers for the determination of follicle-stimulating hormone (FSH), luteinizing hormone (LH), estrogen, thyroxine (T4) and triiodothyronine (T3). Dedicated kits were used for hormonal determinations, and the analyses were performed based on sandwich enzyme-linked immuno-sorbent assay (ELISA) technology. For this purpose, standard curves were drawn using serial dilutions of reagents and the optical densities were read at 420 nm. After blood was sampled, the birds were slaughtered for the collection of ovaries and oviducts. The ovaries and oviducts were immediately weighed. The weight of the oviduct was determined after removing the ovum or developing egg (as appropriate) possibly located in its lumen. Then tissue samples were taken from these organs and processed histologically and stained with HEA, toluidine blue and periodic acid Schiff (PAS) according to Radu & Diaconescu (1984) and Cornilă & Manolescu (2014).

The systematization of ovarian follicles was done according to Wang et al. (2017) cited by Holkoe et al. (2022), as it follows:

- pre-hierarchical follicles which include:

a) small white follicles, ø < 5 mm

b) small yellow follicles, 5< ø < 10 mm;

- hierarchical follicles, also known as preovulatory follicles, ø > 10 mm.

Measurements were performed by optical micrometry on a sufficient number of follicles (n ≥ 10) from each category, determining the thickness of the follicular granulosa and the average of the large and small diameters of the follicular yolk.

Statistic processing of the data

The obtained data were statistically processed by determining the mean, standard error of the mean and standard deviation using a GLM procedure of SAS statistical package (1999) program. The one-way ANOVA was used to compare the means between hybrid groups and Sussex as control. The statistic difference between hybrid groups and Sussex was considered significant when the probability of the null hypothesis $P < 0.05$.

RESULTS AND DISCUSSIONS

Analysis of the hormonal values of the pituitary-ovarian axis revealed significant differences between Sussex and Lohmann and Leghorn hybrids for LH and estrogen. FHS did not show significant differences between Sussex and hybrids, but higher values were noted in hybrids compared to Sussex (Table 1). Among the iodinated thyroid hormones, only T3 showed significantly higher values in the two hybrids compared to Sussex. Changes in the secretory activity of the pituitary-ovarian and pituitary-thyroid axes were reported by Hanlon et al. (2021) of Lohmann laying hybrids versus unselected Shaver and Smoky Joe hens: higher serum estrogen values, as well as differences in the gene expression of the mRNA of ovarian

FSHr and LHr receptors were reported. Bentley et al. (2003) reported differences in gonadotropic hormone secretion in song sparrows (*Melospiza melodia*) in different reproductive conditions, versus house sparrows (*Passer domesticus*) relative to chicken-gonadotropin-releasing hormone. The differences in egg laying frequency values can be attributed to a higher ovulation frequency imprinted by higher FSH and LH values to hybrid hens (Liu et al., 2001). Liu et al. (2001) cite papers and authors who showed that administration of exogenous LH in hypophys-ectomized hens (Nakada et al., 1994) or in intact hens (Lang et al., 1984) induced premature and more frequent ovulations, and subsequently eggs, than in controls. Prastiya et al (2022) found that FSH levels were significantly associated with egg laying frequency in ISA Brown hens, which is also verified in our research. On the other hand, according to Stojević et al. (2000) variations in egg production intensity largely follow variations in blood T3 concentration.

Table 1. FSH, LH, estrogen, T3 and T4 values and egg-laying frequency in 32-wk-old Sussex chicken and in the egg-laying Lohmann and Leghorn hybrids

	FSH (ng/mL)	LH (ng/mL)	Estrogen (pg/mL)	T4 (ng/mL)	T3 (ng/mL)	Egg-laying frequency (eggs/cap./week)
Sussex hens	38±4.2	8.22±1.06	760±34	15.5±11.4	1.35±0.11	5.15±1.77
Lohmann hybrid	40±3.7	11.0**±1.04	890**±111	15.6±6.6	5.55*±2.21	6.43±2.09
Leghorn hybrid	42±6.0	12.0**±3.01	980*±144	16±4.9	5.9±1.02	6.09±0.54
P	0.211	0.005	0.003	0.195	0.002	0.052

Note: each value represents the average of 5-6 determinations
Values are expressed as mean ± standard error of mean
* $P < 0.05$; ** $P < 0.01$ versus Sussex as a control

According to the data presented in Table 2, significantly higher values (P = 0.001) of WF are found in the two lines of egg-laying hybrids compared to Sussex. The two hybrids also present higher values of SYF and LYF compared to Sussex. The number of atretic WF follicles was higher than that of WF (normal) but no data were found in the literature regarding the ratio between the two categories of follicles (Joseph et al., 2002). Joseph et al. (2002) attribute the differences in the number of ovarian follicles to the different sensitivity to photoperiod changes, via the hypothalamic-pituitary-ovary axis. The authors also determined the evolution by age of the number of ovarian follicles in two lines of broiler parent hens. The authors did not analyze the differences between the selected broiler lines. However, at 32 weeks of age, the ovaries showed an average of 14 WF, values that are higher than in the case of Lohmann and Leghorn hybrids, but higher than in Sussex. Other values reported by the authors: 9.8 SYF and 6.2 LYF, to which are added 62 atretic WF and 4 atretic SYF. The number of large yellow follicles was maximum at sexual maturity, numbered at 8.78, decreasing with age. The number of small white follicles (2-5 mm ø) and that of small yellow follicles remained relatively constant with the age of the broiler lines analyzed. Also, according to Du et al. (2020) hens with higher FSH levels tend to have a higher number of (developing) ovarian follicles, which is in agreement with our results.

Table 2. The number of different types of ovary follicles in 32-wk-old hybrid and Sussex hens

Group	n	WF	SYF	LYF	Chistic follicles	Atretic follicles	
						WF	SYF
Sussex	5	9.32±2.30	6.63±0.76	4.44±0.65	2.0±0.19	13.9±4.09	2.12±0.55
Lohmann hybrid	5	16.09±2.90**	8.10±1.98	5.85±1.09*	3.3±0.65	22.09±3.67	4.50±0.45
Leghorn hybrid	5	16.96±4.99**	8.21±2.11	6.32±2.11*	3.0±0.98	22.22±3.08	4.41±0.77

n = number of animals
LYF = large yellow follicles (greater than 10 mm in diameter)
SYF =small yellow follicles (5 to 10 mm in diameter)
WF = white follicles (2 to 5 mm in diameter)
*P < 0.05; ** P < 0.01 versus Sussex

The histological and histochemical study of the ovary revealed relevant similarities and differences between Lohmann and Sussex hens (Figure 1). Thus, HE-A staining revealed that the follicular granulosa layer is less organized in the small follicles, an apparently thicker granulosa was found in Sussex but with more ordered nuclei in Lohmann. PAS and toluidine staining revealed a more intense PAS+ and toluidine+ material in the cytoplasm of the granulosa cells of the hierarchical follicles in Lohmann. In the perivitelline theca, aromatase cells and interstitial cells with weak PAS+ or toluidine+ of the cytoplasm were identified in both studied taxonomic entities.

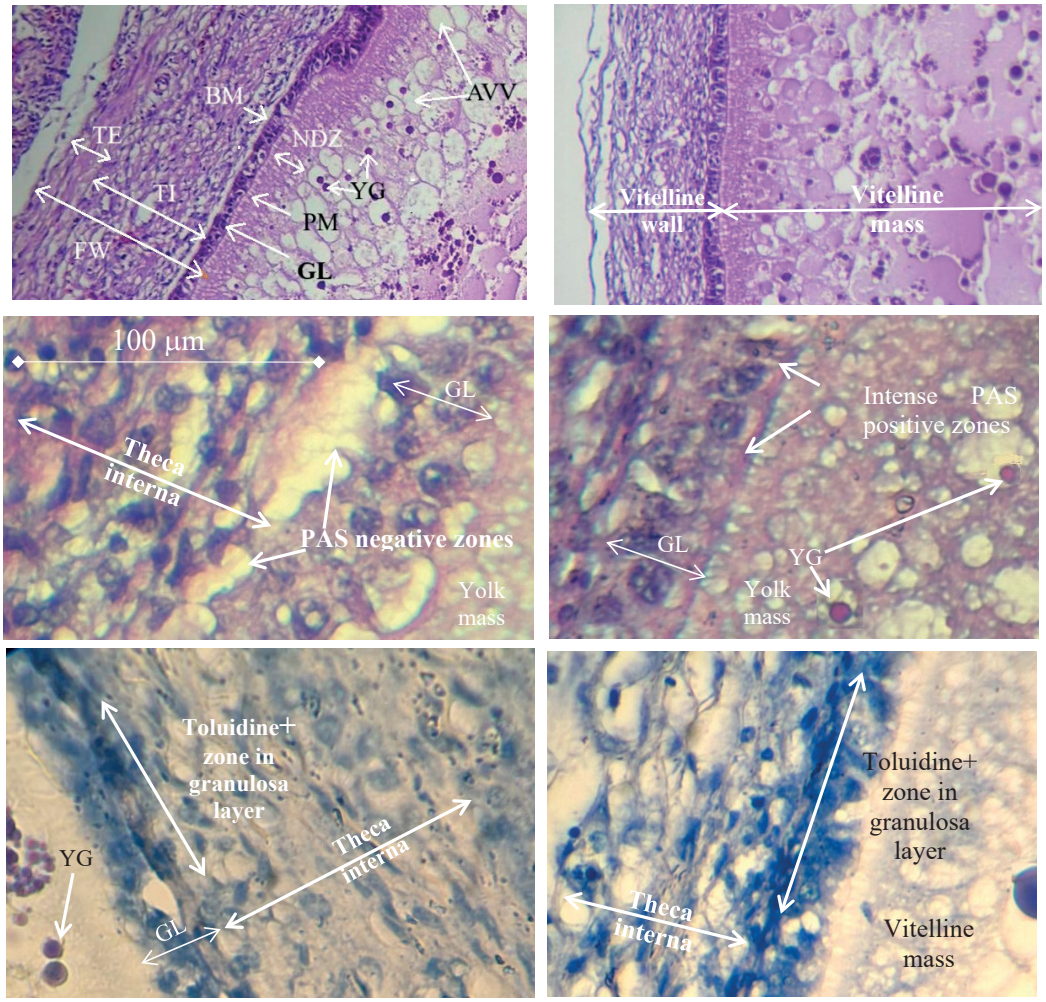

Figure 1. Comparative structural details of ovarian follicles in Sussex (left) and Lohmann hens (right) aged 32 weeks:
Upper left: structural detail of a small yellow follicle. FW - follicular wall; TE - theca externa; TI - theca interna; GL - granuloasa layer; BM - basement membrane; PM - perivitelline membrane; NDZ - undifferentiated zone; YG - yolk granules; AVV- albuminoid vitelline vesicles; ob. 20, HE-A stain;
Upper right: Lohmann follicle of the same size HE-A stained; ob. 20;
Middle left: detail of granulosa, generally negative to PAS-stain; ob. 90;
Middle right: a general PAS-positive reaction is noted, more intense in Lohmann versus Sussex. Two PAS+ yolk granules are present (in red); ob. 90;
Lower left: low concentration of RNA in the cytoplasm of the follicular theca granulosa cells, revealed by toluidine blue stain; ob. 40;
Lower right: high concentration of RNA granules revealed by an intense toluidine stain reaction in granulosa cell cytoplasm; ob. 40

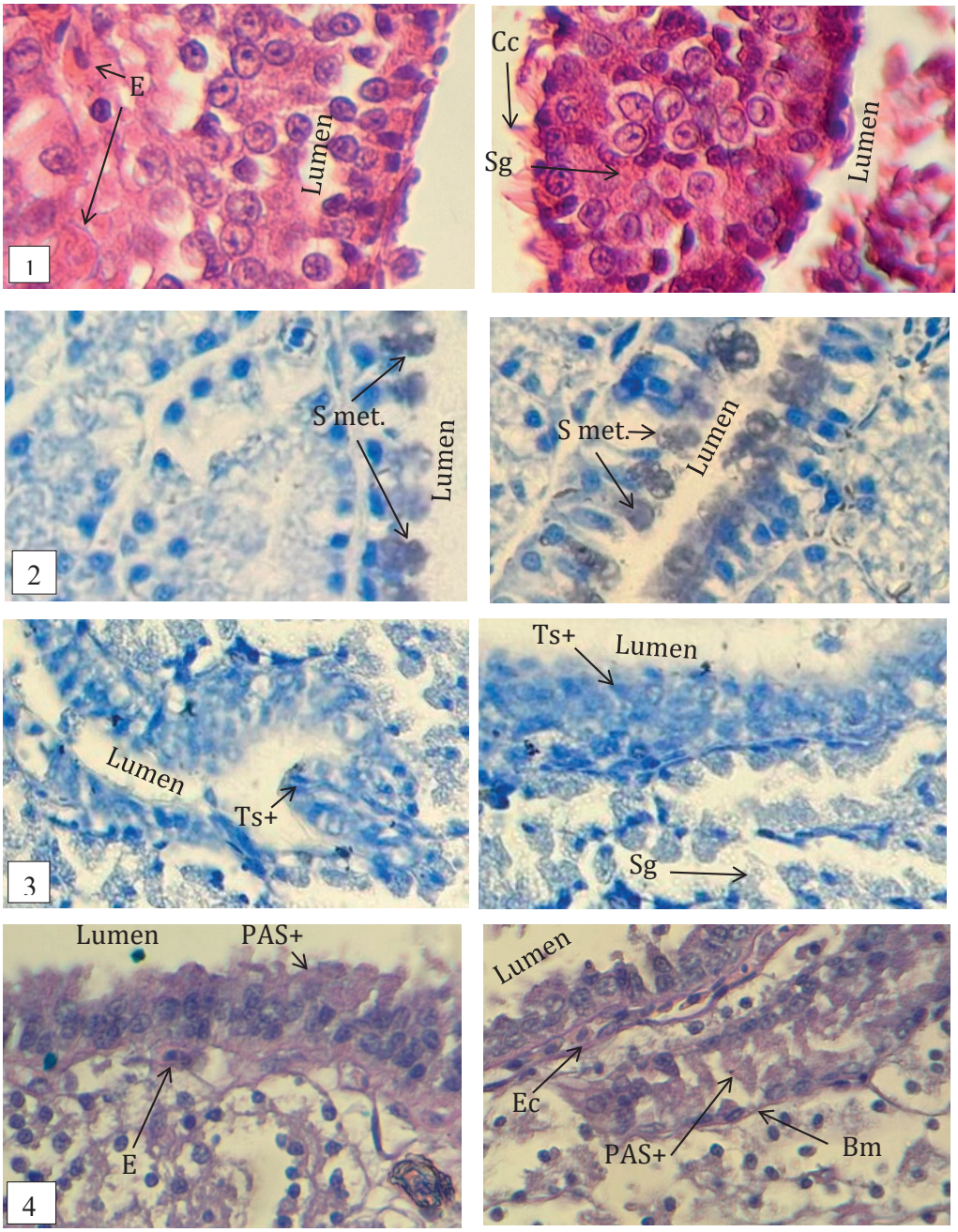

Figure 2. Histology and histochemistry of the oviduct in 32-week-old Sussex (left) and Lohmann (right) hens:
1. HEA stain of infundibulum. E - erythrocytes; Cc - ciliated cells; Sg - groups of pink secretory granules are present in both, hybrid and Sussex hens. Ob. 90;
2. Magnum stained by toluidine blue. Rt+: positive reaction to toluidine; S met - metachromatic (black) stained secretions by toluidine blue. Ob. 40;
3. Isthmus stained by toluidine. Sg: secretion granules; Rt+: positive reaction to toluidine. Ob. 40;
4. Shell gland stained by PAS. PAS+: PAS+ polysaccharide mass; Bm - basal membrane; E - erythrocyte; Ec- erythrocytes ligned in a capillary vessel, under the granular layer. Ob. 40

The comparative histological and histochemical study of the oviduct allowed highlighting the following aspects (Figure 2):
- the infundibulum presents a pseudostratified epithelium, without breed or hybrid differences. The Schiff and toluidine reactions revealed a high density of secretory cells, without breed/ hybrid differences;
- the magnum presents a simple or pseudostratified epithelium, with pink-colored granulations HE-A stained. The PAS reaction revealed denser PAS+ material in Lohmann versus in Sussex epithelium. On the surface of the epithelial cells and in their upper third, a globular secretory material stained metachromatic in black by toluidine and a more abundant PAS+ polysaccharide secretion is noted in Lohmann versus in Sussex;
- in the isthmus, HE-A staining highlights pink spheroidal agglomerations of secretions in/among the glandular cells. The PAS reaction is generally weakly positive, especially in Sussex. The isthmus epithelium in Sussex and Lohmann shows a very positive cytoplasmic reaction to toluidine but the secretions stained metachromatically in black are absent;
- the epithelium and the wall of the shell gland showed a low reactivity, both in PAS and in toluidine blue staining, without structural or tinctorial affinities differences between Sussex and Lohmann.

Data regarding the comparative histochemistry and histology of the ovary and oviduct in birds are relatively few. Dikikh and Pervenetskaya (2022) conducted macro- and microscopic studies on the uterus of Haysex hens (a breed selected for the intensity and persistence of egg laying). The authors describe primary, secondary and tertiary folds of the mucosa, covered with a ciliated epithelium with several rows of cells, of different types, with laterally compressed nuclei located at different levels, and densely arranged tubular glands. Altaey et al. (2023) performed an extensive histological and histochemical study of the oviduct in chickens, turkeys, ducks, and geese. The results showed that the shape, length, width, and weight of the oviduct varied between different bird species. Furthermore, there were signifi-cant differences in anatomical and histological measurements of the oviduct, such as the length of the mucosal folds, the thickness of the oviduct wall tunicae and the distribution of glands. Other authors have conducted studies on the histological features of the oviduct during its ontogenetic development (Blendea et al., 2012) or in different breeds or hybrids (Mimura, 1937; Apperson et al., 2017), but not comparative studies.

CONCLUSIONS

The genetic selection process of hen inbred strains changed the hypothalamo-pituitary and thyroid secretion pattern. LH, estrogen and T3 serum levels in 32-week-old Lochmann and Leghorn hybrids were found significantly increased versus unselected Sussex hens ($P < 0.05$) while FSH and T4 were not significantly modified by genetic selection. Consequently, white and yellow ovary folicle number was found greater in hybrid selected hens versus unselected Sussex. This fact correlates with greater values of laying frequency in hybrid (as egg/cap./week): 6.43 in Lohmann, 6.09 in Leghorn, versus 5.15 in Sussex hens. Changes in histochemical reactions to toluidine and Schiff reagents were found both in the ovarian granulosa tunica and in the the epithelia of the oviductal infundibulum, magnum, isthmus and uterus structures. The thickness, the appearance and density of epithelial cilia, the thickness of the epithelial cell layers of the anatomical elements of the oviduct show differences in relation to the level of the LH, estrogen and iodinated thyroid hormones that control the growth and evolution of these structures.

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IMPORTANCE OF ZINC COMPOUNDS AND ZINC NANOPARTICLES IN DEVELOPMENT OF PLANTS

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Abstract

Global warming, plant diseases and environmental issues such as excessive use of pesticides and fertilizers have affected the production yield in the agricultural sector. Promoted in the last decade or so, nanotechnology has been offering sustainable solutions to conventional agriculture, having a major impact on improving crop productivity and nutrient supply by increasing and regulating the availability of minerals in the soil. Thus, zinc oxide nanoparticles are widely used in agriculture, due to the multiple roles of zinc in the plant cell, such as the involvement in regulating the metabolism of the proteins, lipids, sugars, and nucleic acids, its enzymatic cofactor activity for some enzymes involved in photosynthesis, or being key element in cellular biomembranes. Although the widespread use of nanoparticles can lead to phytotoxicity phenomena, the research direction in recent years is focused on balancing positive and negative effects. Therefore, the aim of this paper is to update the informations about the applications and the effects of zinc compounds and zinc oxide nanoparticles in agricultural practices.

Key words*: agricultural practices, development of plants, nanotechnology, zinc oxide nanoparticles.*

INTRODUCTION

Nanotechnology is a continuously developing technology that can lead to a new change in every field of exact sciences (Rico, 2011). This kind of technology is used in association with optics, electronics and medical and material sciences.

Multiple researches in this field have taken a great and unexpected scale in recent years by providing innovative solutions in different scientific disciplines. Nanotechnology offers with nanoparticles which are molecular or atomic aggregates characterized by the size less than 100nm. They are actually a modified form of basic elements derived by their atomic modification, also as molecular properties of the elements (Daniel, 2004).

Nanoparticles have gained significant interest due to their unusual and fascinating properties, with diverse applications, over their bulk counterparts (Kato, 2011).

Agricultural technologies have changed significantly in recent decades. Nanotechnology has a great influence on agriculture. The direction of research in recent years is moving towards nanoparticles because they increase the availability of nutrients in the soil. Nanoparticles are, in fact, derived from modifying the molecular and atomic properties of basic substances (Moezzi et al., 2012).

Among the nanoparticles used in agriculture, the most common are those based on metal oxides: zinc oxide, cobalt oxide, cadmium oxide, magnesium oxide. Zinc oxide is used in many fields thanks to its specific properties. Many fields, such as the plastics industry, the cosmetics industry, agriculture, use zinc oxide as an additive. Being so used, zinc oxide can reach the environment, where it can accumulate and cause the occurrence of phytotoxicity phenomena. In agriculture, zinc oxide is used in appropriate concentrations to enhance crop production of various plants, zinc having basic functions in plants (Garcia-Lopez et al., 2018).

New agricultural technologies are the fundamental basis of the structure of the third world economy, but unfortunately, nowadays, this sector faces various global challenges, such as: climate change, urbanization, sustainable use of resources and environmental problems, environmental accumulation of pesticides, fertilizers (Sabir et al., 2014).

Taking into account that the world's population is constantly growing and the demand for food is increasing, agriculture is a major concern.

There are more than 3.5 billion people who suffer from micronutrient deficiency, so research is directed towards finding solutions by which the intake of micronutrients can be replaced (Graham et al., 2001)

The agricultural application of nanotechnology has as its main goal a better absorption of substances by plants having a great impact on protecting the environment and balancing the economy.

Nanoparticles have a very high potential in terms of increasing productivity in agriculture, especially in the field of nano-fertilizers. Nano-fertilizers are encapsulated mineral nutrients that are more easily absorbed by plants than conventionally used fertilizers. Most of the time the problem with nutrients is not represented by their lack in the soil, but by how the plants can absorb them and how much they are available (Akanbi-Gada et al., 2019).

Tech-Nano has a big role to play in changing conventional farming practices in the idea of enhancing crop productivity. Many of the agrochemicals applied to crops are lost and do not reach the originally proposed target. Nanoparticles are a better and more controllable way to deliver nutrients where needed and reduce side effects (Sabir et al., 2014).

In the last decade, nanoparticles based on metal oxides, such as ZnO or CoO, have been used on a very large scale in many fields. In particular, the physicochemical properties of cestor nanoparticles allow their use in multiple fields.

There are several types of zinc nanoparticles such as ZnS, ZnSe or CdSe/ZnS nanocrystals. However, probably the most used type of nanoparticle is zinc oxide (nano-ZnO). Nano-ZnO is used in many fields of industry, cosmetics, medicine, being characterized by the following attributes: high electrical conductivity, high stability at high temperatures, antimicrobial, antifungal, catalytic and photochemical effects, a high optical absorption in UV, which gives it significant importance in the area of cosmetics (Moezzi et al., 2012).

Being used daily in a wide range of fields, these nanoparticles end up in the environment where they can cause real hazards. It is estimated that 1100 tons of cosmetics and skin care products containing ZnO are produced annually and approximately 25.5% of these end up being "discharged" into the aquatic environment. The potential toxic effects on plants of nanoparticles include a large number of factors such as: concentration of nanoparticles, their size, surface structure, physicochemical properties, plant species, growth environment, nanoparticle stability (Keller et al., 2010).

THE ROLE OF ZINC IN METABOLISM; CONSENQUENCES OF ITS DEFICIENCY OR EXCESS

Zinc is an fundamental micronutrient for humans and animals and is extensively involved in carbohydrates protein, lipid, nucleic acid metabolism, and gene transcription (McClung et al., 2019). Its role within the human body is extensive in immune function, reproduction, and wound repair. At the microcellular area, it has a role significant effect on the normal functioning of macrophages, neutrophils, natural killer cells, and complement activity (Fallah et al., 2019; Skalny et al., 2018).

Even if iti is one of the most abundant trace elements in the human and animal body, zinc cannot be stored in significant amounts and hence requires regular intake or supplementation. Zinc is found in a lot of foods, including meat, fish, legumes, nuts, and other dietary sources, although its absorption varies by the substrate carrying it.

Zinc deficiency is a major health problem worldwide, especially in developing countries. Hence, it is designated by the World Health Organization as a major disease contributing factor (Narváez-Caicedo et al., 2018; Santos et al., 2017). Zinc deficiency can present with growth impairment, some dysfunction, inflammation, gastrointestinal symptoms, or cutaneous involvement (Sanna et al., 2018).

Since it does not undergo reversible valence changes, the role of zinc in redox processes in plants is less important than that of iron or manganese. Being integrated in the structure of some carbohydrates, zinc also plays a role in the respiratory process. It is involved in the activity of enolase, which participates in carbohydrate metabolism and takes part in the process of chlorophyll synthesis (Palacios et al., 2006).

Zinc plays an essential role in stimulating the activity of auxins. In zinc deficiency,

peroxidases accumulate, causing the oxidation of auxins, which implicitly decrease their amount and activity (Akbar et al., 2021). Zinc also is involved in the metabolism of tryptophan. It activates the enzyme systems which catalyzes the biosynthesis (tryptophan synthetase) and degradation (tryptophan peroxidase) of tryptophan, especially the synthesis of heteroauxin. Stimulates protein biosynthesis, P vitamin biosynthesis, fruit and seed development (Broadley et al., 2012).

Zinc is a cofactor and activator for numerous enzymes (dehydrogenases, phosphatases, phosphodiesterases, carboxypeptidases, carbonic anhydrase, enolase). Zinc influences the stability of ribosomes, their tertiary and quaternary structure, the biosynthesis of cytochrome C (Lilburn et al., 2015).

The action of zinc on phosphodiesterases, including the hydrolysis and denaturation of deoxyribonucleic acid can be substituted by magnesium, cobalt and calcium (Auld, 2005).

Copper, zinc, manganese, iron play an fundamental role in antioxidant defense as an integral part of superoxide dismutase (SOD). Copper, manganese, iron, and of course zinc ions are very interesting because they exert a positive effect on the respiratory activity and the growth rate of *Saccharomyces cerevisiae* (Barbulescu et al., 2010).

Zinc is an essential element for glutamyl reductase and for carbonic anhydrase, alkaline phosphatase, thymidine kinase, lactate dehydrogenase, etc. It stimulates the activity of vitamins B and A (Barker et al., 2015).

Zinc contributes to the binding of cell walls by means of pectic substances and to the union of different cell constituents. More recent research shows that zinc stimulates chlorophyll biosynthesis and function, starch biosynthesis and tree fruiting (Samreen et al., 2017).

In humans, zinc is involved in the formation of red blood cells, stimulates and regulates the functioning of the pancreas, pituitary gland, genital glands and prostate. The daily requirement for an adult is 15 mg. Zinc is a stabilizer of the functions of cell membranes and lysosomes. It inhibits osmotic hemolysis of erythrocytes, as well as lipid peroxidation in the liver. Zinc also interacts with the body's accumulation of vitamin E, vitamin B12, vitamin C (Stoican et al., 2021).

Zinc deficiency in plants causes growth reduction, decreasing of the phytohormones content, especially auxins, and the accumulation of some phenolic substances that are toxic and calcium oxalate (Benglsson et al., 2003).

Zinc deficiency lead to fruit fall, to yellow spots formation on the leaves, to shortening of the internodes in cereals, etc. Corn, citrus fruits, tobacco, fruit trees, beans, peas, onions are sensitive to zinc deficiency. Zinc deficiency is manifested by stopping of plant growth as a result of the lack of auxins. The internodes of the plants remain short, the chlorotic leaves become small and arranged in a rosette (Hafeez et al., 2013). Zinc plays an important role in the fruiting process; when zinc is insufficient, the plants grow weakly and do not fruit or when they do, the seeds are small (Constantinescu et al., 1991).

It seems that zinc is necessary for both growth and normal chlorophyll synthesis. It participates in the synthesis of auxin, contributes to the accumulation of tryptophan and improves water exchange (Benglsson et al., 2003).

Food sources richer in zinc include: meat, yeast, cereal seeds, pollen, pumpkin seeds, eggs, milk, beetroot, spinach, cabbage (Salgueiro et al., 2000).

In humans, zinc deficiency causes growth retardation, changes in taste and smell, loss of appetite, skin lesions, eye disorders, delayed sexual maturity, prostate enlargement, etc. In prostate cancer the zinc content drops a lot. A similar action occurs in liver diseases, alcoholic cirrhosis, ulcers. Component of more than 70 metalloenzymes from the group of dehydrogenases, peptidases and proteinases, activator of enzymes such as: enolase, dipeptidases, zinc is present in the entire animal body and, without a doubt, many deseases are the consequence of the low level of activity of one or more enzymes that contain or are influenced by zinc.

In animal organisms, zinc deficiency causes a reduction of vitamin A in the plasma and an inhibition of retinol protein synthesis, important in the process of vision and adaptation to darkness.

Excess zinc has serious consequences for human health. High zinc concentration inhibits iron absorption and transport. Iron, as a part of hemoglobin structure, is of major importance in

the metabolism of mammals. High dietary zinc affects iron metabolism in two ways: by affecting ferritin to incorporate or release iron from ferritin, and by decreasing iron absorption and decreasing the amount of iron stored in ferritin (Roohani et al., 2013).

ZINC UPTAKE AND TRANSLOCATION IN PLANTS

The absorption of the Zn^{2+} ion proceeds initially as a physical process of ion exchange and diffusion. Zinc absorption is strongly reduced in case of low temperatures, in conditions of anaerobiosis and water stagnation. Absorption increases with increasing zinc concentration in the nutrient solution (Hafeez et al., 2013).

It is necessary to fertilize corn, soybean, bean, pea and potato crops with zinc and organic complexes with zinc, because by the annual use of nitrogen and phosphorus fertilizers in large doses, the zinc content of the soil decreases, with negative repercussions on plant growth, due to the decrease in the biosynthesis of tryptophan and auxins (Rico et al., 2011). The absorption of zinc from the soil solution is greatly influenced by the pH.

The acidic reaction of the soil favors the solubility of zinc compounds and the dissociation of the absorbent zinc-clay complex from the soil. Because of this, acidic forest and forest-steppe soils have a higher content of soluble zinc than neutral and alkaline soils (Alloway, 2009).

In the conditions of areas with strong light intensity and increased temperature, the symptoms of insufficient zinc nutrition appear more frequently. In less intense light, zinc assimilates better. At an acidic pH of the culture medium (5.5-6.5), the availability of zinc for plants decreases. Under the conditions of a neutral or basic culture medium, it can be precipitated in insoluble forms, as zinc phosphate. At a pH above 7.8 zinc becomes somewhat more accessible (Butnariu, 1992).

FERTILIZERS BASED ON ZINC ION

Micronutrients are basic substances for the living world. Zinc, copper, iron, magnesium, cadmium, cobalt are fundamental substances in the case of plants and animals (Gupya & Kalra, 2006).

Only this metal, zinc, is found in all six classes of enzymes: oxidoreductases, transferases, hydrolases, lyases, isomerases, ligases. It is a fundamental element for animals and plants (vegetables and fruits). Zinc is considered to be the fourth most important nutrient limiting crop production (Prasad, 2012; Datcu et al., 2019).

In soil, the amount of total zinc averages between 0.004 and 0.02% of the total atoms in the Earth's crust. It is found mainly in isomorphic forms in augite, hornblende, biotite, magnesite. For normal plant growth, it is necessary that in the soil solution or in the nutrient solution, zinc is found in concentrations of 0.25-3 ppm. In exchangeable form, it is found that Zn^{2+} and $Zn(OH)^{+}$ are found in the absorption process in plants (Alloway, 2009).

Heavy soils and those with a neutral or basic reaction have a higher zinc content than sandy and acidic soils. Humus-rich soils are also rich in zinc. The amount of mobile zinc compared to the amount of total zinc represents on average 1.5-7% in mineral soils and 15-60% in acid peat and forest soils. The presence of zinc, as well as manganese, copper or fluorine increases the resistance of plants to the increased concentration of soluble salts in the soil solution. Supplying plants with zinc increases their resistance to drought and frost (Bengtsson et al., 2003).

A concentration higher than 3 ppm zinc in the nutrient solution can be toxic to certain plants, especially in acidic soil conditions. An excess of zinc hinders the assimilation of iron. For many crops, good results are obtained by applying zinc fertilizers to the soil.

The application must be repeated every year and during the same year every 2-3 weeks. Concentrations ranging from 0.015 to 0.3% zinc in the nutrient solution are used (Alloway, 2008).

Traditionally, in agriculture, zinc fertilizers are used as:

- zinc sulfate ($ZnSO_4$ x $7H_2O$) with 22.8% Zn (crystallized salt in the rhombic system, colorless, soluble in water 96.5 mg/100 ml);
- zinc chloride sludge (with a content of 4-6% Zn, zinc chloride particularly soluble in water, 432 mg/100 ml);

- zinc oxide with 78% Zn (white-yellowish, amorphous, hardly soluble in water, 0.16 x 10^{-3} mg/100 ml);
- zinc carbonate, with 52% Zn (colorless, crystallized in the trigonal system, hardly soluble in water, 10-30 mg/100 ml);
- zinc orthophosphate [$Zn_3(PO_4)_2$ x $4H_2O$], with 51% Zn (insoluble in water);
- superphosphate enriched with zinc, with a content of 0.8-1% Zn;
- zinc frit (soluble glass), contains 10-12% Zn;
- zinc lignosulfonate, contains 5% Zn;
- zinc polyflavonoids, contain 10% Zn (Balan, 2003).

EASY SYNTHESIS OF ZN NANOPARTICLES

Zinc nanoparticles can easily be synthesized by numerous more or less elaborate techniques, such as green, metallurgical, solid, liquid, and gaseous. In the metallurgical techniques, the Zinc nanoparticles could be achieved by the roasting of a suitable zinc ore through a direct or indirect process (Zelechowska, 2014). Due to development of the methods of obtaining Zinc nanoparticles that enables precise control of the Zinc nanoparticles size that extensive scientific research is possible today.

Taking into account what has been discussed, the chemical techniques are the most reliable, economical, and environmentally friendly and also offer flexibility for controlling the shape and size of prepared nanoparticles (Espitia et al., 2012).

There are a variety of chemical techniques, for example, the mechanochemical process, the precipitation process, the hydrothermal, physical vapor, solvo-thermal, sol-gel, micro-emulsion process and methods. Recent research on colloids and dispersed systems must also be taken into account (Kołodziejczak-Radzimska, 2014).

NEGATIVE OR TOXIC IMPACTS OF ZINC NANOPARTICLES

The toxic effect of Zn nanoparticles is due to their solubility. Zn nanoparticles dissolve in the extracellular region, which in turn increases the intracellular (Zn^{2+}) level. The mechanism for increased intracellular (Zn^{2+}) level and Zn nanoparticles dissolution in the medium is still unclear (Pandurangan, 2015).

Although the zinc nanoparticles have great commercial importance and are present in various commercial products there is clearly a growing public concern to know about the toxicological and environmental effects of zinc nanoparticles (Sabir, 2014).

To the researchers' dismay, toxicological studies carried out on zinc oxide nanoparticles in the last ten years show that zinc nanoparticles have potential health as well as environmental risks (Franklin et al., 2007).

Zinc nanoparticles can impose serious toxicity to bacteria *Daphnia magna,* freshwater microalga, mice, and even human cells (Sharma, 2008).

*Z*inc nanoparticles are particularly useful in sunscreens because they have intrinsic ability to filter ultraviolet UVA as well as UVB radiations. Thanks to this special and favorable property, they are offering broader protection than any other sunscreen agent. Must be specified that these nanoparticles have ability to penetrate to the skin and to reach viable cells resulting in the potential toxicity exerted by them (Bengalli et al., 2017).

A comparative analysis of dermal penetration between different animal species was performed, rating them in the order rabbit > rat > pig > monkey > man and it is noted that pig and rat skin are up to 4 and 9-11 times more permeable than human skin, respectively (Wang et al., 2008).

Overall, it is observed after many experiments that penetration through compromised skin was likely to be similar to normal skin but more work still needed to improve the understanding about this important safety issue (Brayner, 2006).

Oxidative stress, cytotoxicity, and mitochondrial dysfunction is explained by taking into account the solubility of ZnO nanoparticles and subsequent increase of intracellular (Zn^{2+}) level. Most of the studies were reported that the ZnO nanoparticle cytotoxicity was due to dissolution of ZnO nanoparticle in the extracellular region (Czyżowska et al., 2020).

Whereas, one study reported that ZnO nanoparticles were taken up by the cell and its dissolution occurred in the intracellular region. Induction of oxidative stress is the major

mechanism of ZnO nanoparticle cytotoxicity. Neurological occur result following the systemic exposure of ZnO nanoparticles (Abbasalipourkabir et al., 2015).

CONCLUSIONS

Because of the unique properties of nanoparticles (NPs), nanotechnology offers enormous value in many kinds of fields. One of the most significant uses of nanotechnology is in the field of plant biogenically utilizing various plant extracts.

Because it doesn't produce hazardous byproduct chemicals, the green synthesis of zinc nanoparticles is far safer and more environmentally friendly than chemical synthesis. When these nanoparticles are applied to crops, their yield and growth are increased.

The output of staple crops for food is quite low, despite the fact that the need for food is growing daily. Commercializing metal nanoparticles for environmentally friendly farming is therefore urgently needed.

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ASSESSING THE PHYLOGENY AND GENETIC VARIABILITY OF *Orobanche cumana* Wallr. POPULATIONS USING ISSR MARKERS

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Abstract

This study investigates the genetic differentiation and phylogeny of the parasitic weed Orobanche cumana Wallr. using ISSR markers in 33 populations from Serbia, Moldova, Romania, Bulgaria, Turkey, and China, representing E, G, and H physiological races. Phylogenetic relationships were analysed using Nei's standard genetic distance and the Neighbour-Joining method in POPTREE2. The results of the phylogenetic analysis indicated the presence of three distinct groups. The first clade included populations from Serbia, Turkey, and China. The second clade comprised populations from Moldova and Romania, and the third clade included Bulgarian populations, forming a separate branch. Genetic variation analysis of O. cumana populations at both country and race levels showed that all physiological races and the Moldavian populations exhibited high interpopulation diversity. In contrast, the primary genetic differentiation exhibited by the Bulgarian, Serbian, Turkish, and Chinese populations occurred principally within their respective populations. This genetic diversity is influenced by factors such as the expansion of sunflower production, agricultural development, and climate change. Furthermore, distinct evolutionary adaptations among O. cumana races were evident. The results obtained demonstrate the complex evolution and dispersal mechanisms of this parasitic species and offer crucial insights into effective management strategies for the production of sunflowers.

Key words*: Orobanche cumana, sunflower, genetic differentiation, ISSR, phylogeny*.

INTRODUCTION

Orobanche cumana Wallr. is a non-photosynthetic, obligate, root-parasitic weed that has been identified as having a severe impact on the production of sunflower (*Helianthus annuus* L.). Yield losses can reach as high as 5% to 100% (Kaya, 2014; Miladinovic et al., 2014), depending on the sunflower genotype (Ciuca et al., 2004; Miladinovic et al., 2014), the aggressiveness of the parasite (Guchetl et al., 2014a), and regional environmental conditions (Kaya, 2014). Sunflower broomrape is a pervasive weed that poses a significant agricultural challenge on a global scale (Kaya, 2014; Miladinovic et al., 2014). The emergence of highly virulent races of *O. cumana*, which can effectively overcome the resistance of existing sunflower varieties and hybrids, is influenced by several factors. The intensive exploitation of agricultural land, particularly with large sunflower monocultures that return to the same fields every two to three years, neglects crop rotation, thereby facilitating the proliferation of this parasitic weed (Melero-Vara et al., 2000; Lukomets & Antonova, 2015). Additionally, the introduction of foreign sunflower germplasm has inadvertently heightened local susceptibility to broomrape infestations (Kaya, 2014). The parasite's high reproductive potential, rapid evolutionary dynamics, and climatic changes that favour its expansion further complicate management efforts (Kaya, 2014). Moreover, gene flow between wild and weedy populations has precipitated abrupt molecular alterations within *O. cumana*, thereby accelerating the emergence of novel, more aggressive races (Kaya, 2014; Melero-Vara et al., 2000). The eight races that have been identified are A through H, with the highest levels of virulence being demonstrated by races E, F, G, and H (Kaya, 2014; Duca & Bivol, 2023).

A comprehensive understanding of the evolutionary relationships and genetic differentiation among *O. cumana* populations is crucial to elucidate the adaptations and geographical distribution of its most aggressive races, with direct implications for agricultural management. Molecular phylogenetic approaches employing multilocus analyses facilitate the

reconstruction of species and population histories, elucidating the evolutionary relationships among different plant groups (Benharrat et al., 2002; Hristova et al., 2011). Microsatellite markers, of which a subset may be linked to genes under selection pressure whilst others evolve neutrally, are of particular value for the assessment of genetic variability (Bannikova, 2004). Early molecular studies utilising dominant markers, including Inter Simple Sequence Repeats (ISSRs), have played a crucial role in estimating the genetic diversity of *O. cumana* and related species (Román et al., 2002; Stoyanov et al., 2012; Duca et al., 2020). This has enhanced the understanding of taxonomic and phylogenetic relationships within the genus Orobanche. Furthermore, phylogeographic studies that examine the geographical distribution of genetic lineages provide additional insights into the evolutionary history of *O. cumana* (Avise, 2000). Notwithstanding the significance of *O. cumana*, the understanding of its evolutionary history and phylogeography across disparate geographical regions remains constrained.

This research addresses significant gaps in the field by examining the genetic variation and evolutionary relationships among *O. cumana* populations from different geographical regions and racial groups. Multilocus ISSR markers were utilised in the analysis to facilitate a comprehensive understanding of the population genetics and evolutionary history of *O. cumana.* The results should contribute to the development of improved agricultural management strategies to mitigate the impact of this highly adaptable parasitic weed.

MATERIALS AND METHODS

In the present study, a total of 336 accessions of *O. cumana* were analysed, representing 33 populations from six countries: Serbia (S), Moldova (M), Romania (R), Bulgaria (B), Turkey (T), and China (Ch), across 24 regions. In addition, the samples belonged to three physiological races, E, G and H (Figure 1).

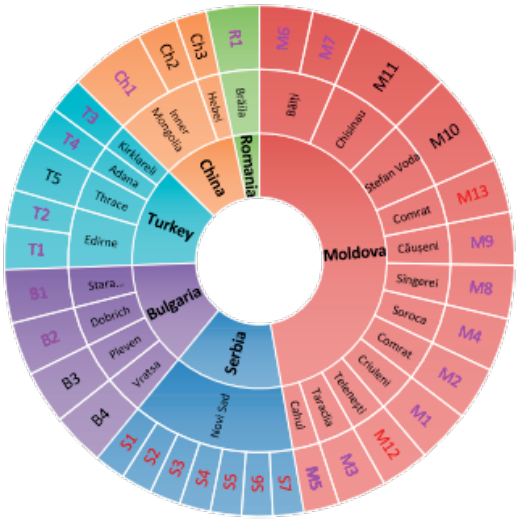

Figure 1. Geographic and racial distribution of *O. cumana* populations sampled for ISSR analysis (red: race E, black: race G, violet: race H)

Total genomic DNA was isolated from frozen samples using the Thermo Scientific GeneJET Plant Genomic DNA Purification Mini Kit #K0791, following the manufacturer's protocol (Thermo Fisher Scientific, USA). The concentration and integrity of the isolated DNA were evaluated by agarose gel electrophoresis (1%) in 1× TAE buffer (40 mM Tris-acetate, pH 8.0; 1 mM EDTA) at a voltage of 2.5 V/cm, complemented by optical density readings obtained with a spectrophotometer (T60 UV-VIS, UK) (Sambrook & Russell, 2001).

Polymerase chain reaction was performed using a set of 13 di-, tri-, and tetranucleotide anchored and non-anchored ISSR primers, as previously reported by Benharrat (Benharrat et al., 2002) (Table 1).

Table 1. ISSR primers used in this study and their specifications

Code	ISSR primers Sequence (5′→ 3′)	NBN	GC, %
BC807	AGAGAGAGAGAGAGAGT	17	47
BC810	GAGAGAGAGAGAGAGAT	17	47
BC835	AGAGAGAGAGAGAGAGYC	18	56
BC841	GAGAGAGAGAGAGAGAYC	18	56
BC857	ACACACACACACACACYG	18	56
$(CAA)_5$	CAACAACAACAACAA	15	33
$(GACA)_4$	GACAGACAGACAGACA	16	50
$(CA)_6RG$	CACACACACACARG	14	57
$(CTC)_4RC$	CTCCTCCTCCTCRC	14	71
$(CAG)_5$	CAGCAGCAGCAGCAG	15	67
$(CT)_8TC$	CTCTCTCTCTCTCTCTTC	18	50
$(CA)_6AC$	CACACACACACAAC	14	50
$(AG)_8YA$	AGAGAGAGAGAGAGAGYA	18	50

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

The PCR reactions were performed on a Genset 9700 thermocycler (Applied Biosystems) under the following conditions: an initial denaturation at 95°C for 5 min; 35 cycles of 95°C for 30 s, 45°C for 45 s, and 72°C for 2 min; followed by a final extension at 72°C for 5 min.

The ISSR amplification products were analysed by electrophoresis in a 2% agarose gel, which had been previously stained with ethidium bromide. The gels were then visualised under UV light (wavelength: λ = 305 nm). The molecular size of the amplicons was estimated using the GeneRuler Express DNA Ladder, ready-to-use SM1553 (Thermo Fisher Scientific, USA), and each gel image was captured with the Doc-Print VX2 gel documentation system (SXT-F20.M, France).

The size of the ISSR fragments was determined using Photo-Capt V. 15.02 software. Bands that exhibited weak staining were excluded from further analysis. The generated data were compiled into a binary matrix indicating each character or locus's presence (1) or absence (0). The allele frequency data was subjected to phylogenetic analysis, with Nei's standard genetic distance and the Neighbor-Joining (NJ) method implemented in the POPTREE2 software for Windows, including bootstrap analysis, employed for this purpose. Furthermore, genetic variation statistics for *O. cumana* populations were analysed using POPGENE v.1.32 software.

RESULTS AND DISCUSSIONS

Thirty-three populations of *O. cumana* from Serbia, Moldova, Romania, Bulgaria, Turkey, and China, comprising three physiological races (E, G, and H), were analysed using ISSR markers to assess genetic diversity and phylogenetic relationships. The results demonstrated that the samples were divided into two major clades based on their microsatellite loci. A notable finding was that the phylogenetic tree revealed that the Bulgarian populations of *O. cumana* occupied a separate branch, distinctly different from other populations (Figure 2A).

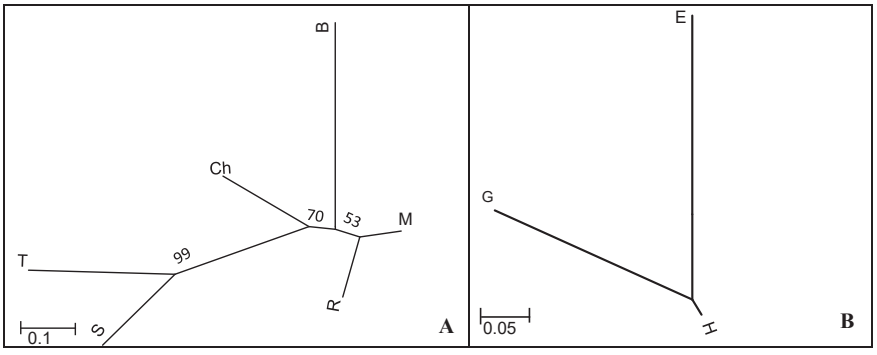

Figure 2. Phylogenetic relationships of *O. cumana* by geographic (A) and racial origins (B). Note: bootstrap values (expressed as percentages of 1000 replications) are given at the nodes

This suggests that Bulgarian populations may have unique genetic characteristics, possibly due to specific adaptive traits influenced by Bulgaria's unique agro-ecological conditions,

different crop management practices, and variations in environmental factors, including soil composition, climate patterns, and localised agricultural policies. Moreover, historical colonisation events may have resulted in genetic admixture, further contributing to the observed genetic distinctness of the Bulgarian populations. Understanding these differences is crucial as it provides insight into the potential for unique adaptations that may influence the parasitic behaviour of *O. cumana* and its impact on sunflower production. The knowledge thus acquired could inform targeted management strategies, taking the specific genetic profiles of local populations into account. The implementation of such management strategies is poised to yield more efficacious agricultural practices.

The analysis of genetic distance coefficients showed that the Bulgarian populations of *O. cumana* were more genetically similar to the Moldavian (0.07), Chinese (0.07), and Romanian (0.11) populations (Table 2). Conversely, the Bulgarian populations were more genetically distinct from the Turkish (0.18) and Serbian (0.19) populations, reflecting greater genetic distances (Table 2).

Table 2. Genetic identity and distance among diverse populations of *O. cumana* based on Nei's measures

Country	S	M	R	B	T	Ch
S	****	0.82	0.79	0.83	0.84	0.82
M	0.20	****	0.93	0.93	0.86	0.94
R	0.24	0.07	****	0.90	0.79	0.89
B	0.19	0.07	0.11	****	0.83	0.93
T	0.17	0.15	0.24	0.18	****	0.87
Ch	0.20	0.06	0.11	0.07	0.14	****

Race	E	G	H
E	****	0.91	0.93
G	0.09	****	0.97
H	0.07	0.03	****

Note: genetic identity is above the diagonal and genetic distance is below the diagonal

The results obtained suggest the presence of a closer genetic relationship between the Bulgarian population and its neighbouring populations. This relationship may be influenced by shared environmental conditions, historical interactions, or genetic exchange. Conversely, the greater genetic distances observed with the Turkish and Serbian populations may indicate more distinct evolutionary trajectories or adaptations to differing ecological conditions.

The total gene diversity (Ht) and the gene diversity within Bulgarian *O. cumana* populations (Hs) were found to be comparatively reduced, at 0.10 and 0.05, respectively (Table 3). These values indicate limited genetic variation within the Bulgarian populations. Additionally, the genetic differentiation (Gst) among these populations was notably elevated at 0.49, while the gene flow (Nm) was measured at 0.51 (Table 3).

Table 3. Population genetic variation statistics of *O. cumana*: country and race comparison

Country		Sample size	Ht	Hs	Gst	Nm (Gst)
S	Mean	49	0.18	0.11	0.36	0.88
	Sd		0.04	0.02		
M	Mean	168	0.17	0.08	0.52	0.47
	Sd		0.03	0.01		
B	Mean	48	0.10	0.05	0.49	0.51
	Sd		0.03	0.01		
T	Mean	41	0.20	0.13	0.36	0.90
	Sd		0.04	0.02		
Ch	Mean	18	0.10	0.07	0.37	0.85
	Sd		0.03	0.01		

Races		Sample size	Ht	Hs	Gst	Nm (Gst)
E	Mean	72	0.22	0.10	0.53	0.45
	Sd		0.04	0.01		
G	Mean	85	0.18	0.08	0.56	0.39
	Sd		0.03	0.01		
H	Mean	179	0.23	0.09	0.62	0.31
	Sd		0.03	0.01		

Note: Ht - total gene diversity; Hs - gene diversity within populations of the group; Gst = (Ht-Hs)/Ht, coefficient of gene differentiation among populations of the group; Nm - gene flow among populations from Gst (Nm = 0.5(1 - Gst)/Gst); Sd - standard deviation

This suggests that 51% of the genetic differentiation is attributable to variation within populations rather than among different populations. These patterns of genetic variability highlight a unique dynamic within the Bulgarian populations, likely shaped by localized environmental pressures and historical introgression events. Elevated Gst

values indicate significant genetic structuring, which can hinder gene flow and increase the potential for local adaptation. Conversely, the relatively elevated Nm value indicates ongoing gene exchange, which may facilitate the introduction of beneficial alleles, thereby enhancing resilience to environmental changes and agricultural practices.

Phylogenetic analysis of the data reveals that three populations from Serbia, Turkey, and China cluster within the first major clade of the tree. The populations from Serbia and Turkey formed one subclade within this clade, while the Chinese populations were grouped into a separate subclade (Figure 2A). Serbia and Turkey demonstrated a notably high bootstrap value of 99, indicating strong confidence in their close genetic relationship, which is further substantiated by a genetic distance value of 0.17 (Figure 2A, Table 2). This suggests a potential shared evolutionary origin influenced by similar geographic conditions, environmental factors, agronomic practices, or a common history of ecological adaptation. In contrast, the Chinese populations, with a bootstrap value of 70, also displayed genetic proximity to the Serbian (0.20) and Turkish (0.14) populations, albeit with less statistical confidence (Figure 2A, Table 2). This lower bootstrap value implies that the Chinese populations may possess distinct genetic characteristics or adaptations that differentiate them from those of Serbia and Turkey.

The analysis of population diversity indicated similar Gst and Nm coefficients among the populations from Serbia (0.36 and 0.88), Turkey (0.36 and 0.89), and China (0.37 and 0.85), respectively (Table 3). These values suggest that all three populations exhibit a high level of intrapopulation genetic differentiation. Additionally, the parameters of total gene diversity Ht for Turkey, Serbia, and China (0.20, 0.18, and 0.10, respectively) and within-population gene diversity Hs (0.13, 0.11, and 0.07, respectively) further support this finding (Table 3). Although genetic differences exist among these populations, they are not as pronounced as those observed in populations with higher Gst values. The analysis indicates that the majority of genetic differentiation occurs within these populations, with 64% for the Serbian and Turkish groups and 63% for the Chinese group. Furthermore, the elevated gene flow values observed among all populations suggest a considerable degree of gene migration, thereby highlighting a dynamic exchange of genetic material. This gene exchange is likely to enhance genetic diversity and reduce the risk of inbreeding among populations. High levels of gene flow suggest that these populations may be more adaptable to environmental change and less susceptible to the negative impacts of genetic isolation. This adaptability may provide greater resilience to external pressures by facilitating the exchange of beneficial genes among populations.

The second separate clade included the Moldavian and Romanian populations of *O. cumana*, indicating the presence of specific local adaptations and limited interpopulation genetic variability. These populations exhibited a close genetic distance of 0.07, accompanied by a relatively low bootstrap value of 53. This suggests a degree of genetic similarity and possibly historical interactions between the two populations (Figure 2A, Table 2). Genetic variation statistics for the Moldavian populations revealed a higher Gst of 0.52 and a lower Nm of 0.47 than the first clade. This suggests a 52% greater genetic differentiation among these populations (Table 3), likely due to distinct ecological conditions, adaptive strategies, or historical factors leading to genetic isolation. The low Nm value indicates a relatively low level of gene migration between the populations, further reinforcing the conclusion of genetic isolation. Consequently, the limited gene exchange may hinder the transfer of genetic material, thereby reducing overall genetic diversity within these populations. The Ht (0.17) and Hs (0.08) indices further indicate that most gene diversity is concentrated within the populations, underscoring the presence of genetic structuring. Given these high levels of genetic differentiation and limited gene flow, it is crucial to consider the potential emergence of distinct adaptive races and their impact on agronomic characteristics. This situation necessitates periodic monitoring and the development of management strategies to preserve genetic diversity and ensure the long-term sustainability of these populations. Thus, the Moldavian populations exhibit significant

genetic differentiation and limited gene exchange, which are important factors for understanding their evolutionary dynamics and for designing effective agricultural management strategies.

The phylogenetic tree analysis of the genetic relationships among the three physiological races (E, G, and H) of *O. cumana* identified two separate groups (Figure 2B). One group includes races G and H, while race E forms its distinct branch. The analysis shows a close genetic connection (0.03) between races G and H, thus suggesting the possibility of a shared evolutionary background (Table 2). Notably, significant genetic divergence exists between these aggressive races and the ancestral race E, indicated by distances of 0.09 and 0.07, respectively. This divergence suggests that the accumulation of new mutations has facilitated their adaptation to changing climates and diverse host genotypes, promoting the rapid evolution of these races.

Population diversity analyses indicate higher coefficients of gene differentiation (Gst) for races H (0.62) and G (0.56) compared to race E (0.53) (Table 3). The genetic diversity indices (Ht: 0.22 for race E, 0.18 for race G, and 0.23 for race H; Hs: 0.10, 0.08, and 0.09, respectively) further confirm that most genetic variation occurs among populations (Table 3). The race tree analysis divides the populations into two main groups: races G and H, which are closely related, and race E, which forms a distinct branch. This separation indicates genetic divergence and suggests that various environmental conditions, combined with possible historical and ecological factors, have driven the differentiation of these races. The findings underscore the genetic complexity and diversity of *O. cumana's* population structure, which has significant implications for managing this parasitic plant in agronomic contexts. Understanding these genetic relationships can inform targeted management strategies that consider the unique adaptations and behaviors of different races. This knowledge is crucial for developing more effective control measures.

The phylogenetic tree analysis revealed distinct haplotypes within *O. cumana*. Populations from Serbia, Turkey, and China form one haplotype that is genetically distinct from those found in Moldova, Romania, and Bulgaria (Figure 2A). Analysis of the three physiological races revealed two haplotype groups: races G and H represented one group, whereas race E formed a separate group (Figure 2B). These phylogenetic results reflect evolutionary trends, showing that newer, more virulent races are increasingly dominating the older races. Significant gene differentiation among the populations of Moldova and Bulgaria (Gst values of 0.52 and 0.49) indicates potential seed interchange from different regions and genetic recombination between populations (Pineda-Martos et al., 2013). Gene exchange mechanisms within populations from Serbia (Nm = 0.88), Turkey (Nm = 0.90), and China (Nm = 0.85) can facilitate the emergence of new genetic variability and changes in virulence (Pineda-Martos et al., 2013).

This research highlights how environmental variations and evolutionary adaptations can affect genetic diversity within *O. cumana*. The findings underline the necessity of understanding broomrape's genetic structure to develop effective management strategies for controlling this parasitic plant in sunflower production. The elevated genetic potential suggests that more virulent races may arise, emphasizing the importance of ongoing monitoring and genetic assessments to inform control strategies. Knowledge of genetic relationships and diversity within local and regional populations of *O. cumana* will be essential for designing targeted and successful weed control programs.

Molecular studies have extensively examined the genetic diversity within and among *O. cumana* populations across diverse geographic regions, employing various markers to analyze intrapopulation and interpopulation genetic variation along with gene flow dynamics (Castejón-Muñoz et al., 1991; Gagne et al., 1998; Pineda-Martos et al., 2013; Martín-Sanz et al., 2016; Malek et al., 2017). Environmental factors, historical events, reproductive strategies, genetic mechanisms, and anthropogenic impacts influence the observed genetic variation. Understanding these factors is crucial for effective biodiversity management and conservation strategies for species like *O. cumana*. Prior research has identified molecular differences in *O. cumana*

populations from various countries (Gagne et al., 1998; Benharrat et al., 2002; Ciucă et al., 2004; Atanasova et al., 2005; Pineda-Martos et al., 2013; Molinero-Ruiz et al., 2013). Studies utilizing RAPD PCR technology have revealed low intrapopulation variability and limited gene exchange among Bulgarian, Spanish, Romanian, and Turkish populations (Gagne et al., 1998). Research highlighted weak polymorphism in populations from Spain, Yugoslavia, and Romania (Ciucă et al., 2004). It documented two distinct gene pools in Spain with minimal variation in the Cuenca province and Guadalquivir Valley (Pineda-Martos et al., 2013). Additionally, genetic homogeneity was noted within highly virulent populations, with no significant molecular differences found (Molinero-Ruiz et al., 2013).

Recent findings on European populations (Bulgaria, Romania, Turkey, and Spain) indicate low intrapopulation (Hs) and high interpopulation genetic variation (Hg), with minimal gene flow (Gagne et al., 1998). In contrast, Ivanović et al. (2021) reported higher genetic variation and lower intrapopulation variability in Serbian populations, potentially due to seed introductions and genetic recombination from diverse regions. A study in Bulgaria identified two contrasting gene pools, where central area weedy populations exhibited low intrapopulation diversity relative to more diverse wild populations along the eastern coast, suggesting genetic exchange through cross-fertilization or seed dispersal (Pineda-Martos, 2014a). Guchetl et al. (2014a) found poorly differentiated gene pools between Russian-Kazakh and Romanian populations, with the former displaying higher polymorphism and intrapopulation diversity. Employing dominant markers such as RAPDs and ISSRs has been common for estimating genetic diversity in *O. cumana* (Katzir et al., 1996; Gagne et al., 1998; 2000; Benharrat et al., 2002; Román et al., 2002; Ciucă et al., 2004; Atanasova et al., 2005). Recently, SSR markers have gained traction due to their co-dominant inheritance, multiple alleles, and high polymorphism (Pineda-Martos et al., 2013; 2014a; 2014b; Guchetl et al., 2014b; 2014c; Martín-Sanz et al., 2016). Pineda-Martos et al. (2013) noted significant genetic recombination effects driving race evolution in 50 *O. cumana* populations from Spain. Furthermore, bidirectional gene flow between Bulgarian and Spanish populations was documented through SSR analysis (Pineda-Martos et al., 2014a, 2014b). Jebri et al. (2017) utilized SSR and SNP approaches to assess genetic diversity among nine Tunisian populations. Furthermore, Bilgen (Bilgen et al., 2019) reported that in six *O. cumana* populations from Turkey's Thrace region, 66% of genetic variation was attributable to within-population variance, while the remaining 34% was due to among-population differences. In summary, significant advancements in understanding the genetic diversity of *O. cumana* have been achieved; however, continued research remains essential for developing effective management and conservation strategies regarding this economically significant parasitic plant.

The phylogenetic tree analysis revealed distinct haplotypes within *O. cumana*. Populations from Serbia, Turkey, and China form one haplotype that is genetically distinct from those found in Moldova, Romania, and Bulgaria (Figure 2A). The analysis of the three physiological races identified two haplotype groups: races G and H represented one group, while race E formed a separate group (Figure 2B). These phylogenetic results reflect evolutionary trends, showing that newer, more virulent races are increasingly dominating over older ones.

Comparative studies utilizing SSR markers on the same populations have corroborated these findings, revealing similar genetic differentiation patterns. For instance, SSR analyses have also indicated significant gene differentiation among the populations of Moldova and Bulgaria (Gst values of 0.52 and 0.49), suggesting potential seed interchange and genetic recombination within these regions (Pineda-Martos et al., 2013). Additionally, SSR studies have shown a high level of genetic variability within populations from Serbia, Turkey, and China, which aligns with the Nm values observed in our ISSR analysis (Nm = 0.88 for Serbia, Nm = 0.90 for Turkey, and Nm = 0.85 for China). This consistency across different molecular markers highlights the robustness of the findings and suggests that gene exchange mechanisms facilitate the emergence of new genetic variability and changes in virulence.

This research underscores how environmental variations and evolutionary adaptations can affect genetic diversity within *O. cumana*. The findings emphasize the necessity of understanding broomrape's genetic structure to develop effective management strategies for controlling this parasitic plant in sunflower production. The elevated genetic potential suggests that more virulent races may arise, reinforcing the importance of ongoing monitoring and genetic assessments to inform control strategies. Additionally, the consistency of findings from SSR studies indicates a comprehensive understanding of genetic relationships and diversity within local and regional populations of *O. cumana*, which will be essential for designing targeted and successful weed control programs.

CONCLUSIONS

Genetic variation analysis of O. *cumana* populations at both the country and race levels revealed high interpopulation diversity across all physiological races (E, G, H), with Moldavian populations exhibiting similar patterns. In contrast, for the Bulgarian, Serbian, Turkish, and Chinese populations, the primary genetic differentiation occurred within their respective populations.

Each phylogenetic clade represents genetically homogeneous groups that are closely related yet distinct from other populations and races. The classification and arrangement of clades identified through ISSR sequences provide critical insights into the ongoing evolutionary mechanisms associated with *O. cumana* and indicate potential strategies for its monitoring and control.

These phylogenetic trees underscore the complexity of *O. cumana* population structure and highlight the significance of genetic adaptation studies in managing agronomic risks linked to different physiological races across various regions. The results of this study are vital for clarifying the evolutionary dynamics and geographical distribution of *O. cumana*, thereby supporting the development of enhanced agricultural management practices designed to reduce the detrimental impacts of this parasitic weed.

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EXPLORING THE BACTERIOCINOGENIC PROPERTIES OF *Lactococcus lactis* R152 ISOLATED FROM A TRADITIONALLY MADE CHEESE

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Abstract

Lactococcus lactis R152 was isolated from a traditionally made cheese and identified through 16S rRNA gene sequencing. This strain exhibited antibacterial activity against five bacterial strains, including four potential pathogenic strains. In this study, we investigated the nature of the inhibition and characterized the active compound. The pH neutralization of the culture supernatant did not affect the inhibitory activity against Lactobacillus delbrueckii subsp. bulgaricus LMG6901^T, but this was lost after proteinase K treatment, indicating that the antibacterial compound is proteinaceous, likely a bacteriocin. Ammonium-sulfate precipitation of the culture supernatant increased the activity from 1,600 AU/ml to 12,800 AU/ml. Tricine-SDS-PAGE analysis indicated an estimated molecular mass of the presumptive bacteriocin of less than 6,500 Da. This compound was resistant to heat (preserving activity after autoclaving) and to pH variation in the pH 1 and 9 interval. These findings, combined with the identification of nisin A gene in the bacterial genome, suggest that Lactococus lactis R152 produces nisin A, a bacteriocin with potential applications in the food industry.

Key words*: antimicrobial activity, bacteriocin, food-borne pathogens, Lactococcus lactis.*

INTRODUCTION

The increasing prevalence of antibiotic-resistant bacteria poses a significant threat to global health, driving the search for novel antimicrobial agents (WHO, 2023). One promising avenue of research lies in the exploration of naturally occurring antimicrobials produced by microorganisms, particularly by lactic acid bacteria (LAB). Because food fermentation relies heavily on LAB, these bacteria have a well-established history of safe use in food production. Moreover, they can inhibit bacteria responsible for food spoilage or pathogenic bacteria derived from food. Several metabolites produced by LAB, such as organic acids, bacteriocins, hydrogen peroxide, reuterin, diacetyl, are involved in this activity (Ibrahim et al., 2021). Among the inhibitory compounds, bacteriocins stand out as a particularly promising group by enhancing the ability of LAB to limit the growth of undesirable bacteria, especially in food products (Dal Bello et al., 2012; Kondrotiene et al., 2018). Many LAB have been shown to produce bacteriocins, which usually exhibit antimicrobial activity against closely related bacterial species (Simons et al., 2020). Among LAB, *Lactococcus lactis* gained considerable reputation due to its widespread use in dairy fermentations and its ability to produce nisin, a well-characterized bacteriocin with broad-spectrum antimicrobial activity (Liu et al., 2021). Discovered in 1947 by Mattick and Hirsch (Mattick & Hirsch, 1947) from a strain of *Lactococus lactis*, nisin is a class I lantibiotic (Wayah & Philip, 2018) and it was approved by the EU, WHO, and FDA to be used in over 48 countries for the preservation of food, especially in cheese production (Favaro et al., 2015; Singh, 2018). Nisin has a bactericidal mode of action against many Gram-positive bacteria, such as *Bacillus cereus*, *Clostridium botulinum*, *Staphylococcus aureus*, and *Listeria monocytogenes*, among others. It works by disrupting the bacterial cell membrane and interfering with cell wall

synthesis in the sensitive strains (Tavares et al., 2024). In contrast, Gram-negative bacteria demonstrate significantly greater tolerance to nisin, primarily due to their less permeable cell walls (Gong et al., 2018). Along with its applications in food preservation, nisin is also being explored for its potential therapeutic uses, including its role in combating antibiotic-resistant bacteria (Field et al., 2016). Overall, nisin is a valuable tool in food safety and preservation, contributing to the reduction of foodborne illnesses and enhancing the quality of various food products (Dal Bello et al., 2012; Kondrotiene et al., 2018).

Traditional fermented foods (such as fermented vegetables, fermented dairy products or fermented cereals - *bors*), particularly those produced using artisanal methods, represent a rich reservoir of diverse microbial communities, potentially harboring novel LAB strains with unique functional properties (Grosu-Tudor et al., 2014; Grosu-Tudor S. & Zamfir M., 2014).

Lact. lactis R152 was isolated from a traditionally made cheese from Romania and was selected for further investigation due to its observed antimicrobial activity. This paper presents the results of a study investigating the antibacterial activity of *Lact. lactis* R152 and the characterization of the antibacterial compound, aiming to assess its potential for applications in food preservation and as a possible alternative to conventional antimicrobials.

MATERIALS AND METHODS

Bacterial strains and growth media

Lact. lactis R152 was isolated from an artisanal sheep cheese sourced from Romania and identified through 16S rRNA gene sequencing (results not published). The antibacterial activity of this strain was tested against another LAB strain, *Lactobacillus delbrueckii* subsp. *bulgaricus* LMG6901^T, and against six potential pathogenic bacteria: *Listeria monocytogenes* ATCC1911, *Escherichia coli* ATCC25922, *Salmonella enterica* ATCC14028, *Bacillus cereus* CBAB, *Bacillus subtilis* ATCC6633, and *Staphylococcus aureus* ATCC25923. All strains were preserved at -80°C in the presence of glycerol (25%). LAB strains were grown in MRS medium (De Man et al., 1960), while the others in BHI (Brain Heart Infusion) medium (Carl Roth GmbH, Germany). For solid media, 1.5% agar was added to the broth, while for the indicator top-layer medium, 0.7% agar was added.

Antibacterial activity of *Lact. lactis* R152

Evaluation of antimicrobial effect

The inhibitory activity against the seven bacterial strains mentioned above, was initially evaluated by using the spot-on-the-lawn method (Lewus & Montville, 1991). Briefly, 10 µl of an overnight culture of *Lact. lactis* R152 were spotted onto the surface of MRS agar and incubated at 28°C to allow bacterial growth in the spots. A thin top-layer containing 100 µl of overnight indicator culture inoculated in 5 ml of MRS/BHI supplemented with 0.7% agar was then added and further incubated for 24 h. The antibacterial activity was indicated by the presence of a clear halo around the spots of *Lact. lactis* R152.

Bacteriocinogenic activity of Lact. lactis R152

To investigate the nature of the inhibition, the supernatant obtained from an overnight culture of *Lact. lactis* R152 was further used. After removing the bacterial cells by centrifugation (13000 x g, 10 min, 4°C), the pH of the supernatant was adjusted to approximately 6.5-7.0, to eliminate any inhibition caused by organic acids. The antibacterial activity was then assessed using the agar spot method (De Vuyst et al., 1996). In this method, the indicator top-layer was poured onto the solid medium, allowed to solidify, and then 10 µl of the neutralized supernatant were spotted on top.

Additionally, the activity of the neutralized supernatant was determined after treatment with proteinase K (Carl Roth, Karlsruhe, Germany), for 1 h, at 65°C. This test would allow to establish if the inhibition was due to a proteinaceous compound.

Concentration and quantification of the putative bacteriocin

Ammonium sulphate was added to the neutralized cell-free supernatant to 40% saturation and allowed to precipitate overnight with a gentle agitation. The resulting precipitate, along with the floating pellicle, was then dissolved in

5 mM potassium phosphate buffer, pH 6.5, to yield the crude bacteriocin.
Antibacterial activity was quantitatively measured against *L. delbrueckii* subsp. *bulgaricus* using the agar spot method, as described elsewhere (De Vuyst et al., 1996). One unit of activity (AU/ml) was defined as the reciprocal of the highest dilution that shows inhibition.

Characterization of the putative bacteriocin

Inhibition of (potential) pathogenic bacteria

The inhibitory activity of the crude bacteriocin was evaluated against the indicator strains used in the initial screening. The well diffusion method (Valgas et al., 2007) was employed, as it allows the use of larger sample volumes. Briefly, the indicator strains were inoculated onto BHI agar medium using sterile cotton swabs. After inoculation, wells were cut in the medium and filled with 50 µl of the crude bacteriocin. Plates were left for about 30 min to allow the bacteriocin to diffuse into the medium, then incubated for 24 h at the optimal growth temperature of each indicator.

Influence of pH and temperature on the inhibitory activity of the crude bacteriocin

The pH stability of the inhibitory compound was assessed by adjusting the pH to various values ranging from 1 to 9, and testing after one hour of treatment.
To evaluate the heat stability, the crude bacteriocin sample was incubated at different temperatures, as shown in Table 3. The residual activity was quantitatively determined by the agar spot method.

Molecular mass

The molecular mass of the putative bacteriocin was estimated by Tricine-SDS-PAGE (Tricine sodium dodecylsulphate-polyacrylamide gel electrophoresis), according to Schägger and von Jagow (1987). Electrophoresis was performed in a Biometra Minigel Twin (Biometra, Germany). The gel was then thoroughly washed for 5 h with sterile ultrapure water and covered with a top-layer, containing *L. delbrueckii* subsp. *bulgaricus* LMG6901^T. Molecular mass was estimated in comparison with polypeptide SDS-PAGE molecular weight standards (BioRad Laboratories, Hercules CA, USA).

Genetic screening

Pure Link Genomic DNA kit (Invitrogen, MA, USA) was used for DNA extraction from *Lact. lactis* R152 cells. PCR reactions were conducted with the extracted DNA, using specific primers (Table 1) for the genes responsible for encoding nisin A (Li & O'Sullivan, 2002), lactococcin 972 (Alegría et al., 2010), and plantaricin A (Diep et al., 1996). The reaction mixture and PCR protocol were previously detailed (Zamfir et. al, 2016). Amplification was carried out in a Mastercycler pro S (Eppendorf, Germany). The amplified products were visualized by agarose gel electrophoresis.

Table 1. List of primers and annealing temperatures

Primer	Sequence (5'-3')	Annealing temperature (°C)
NisA-F	GGATAGTATCCATGTCTG	54
NisA-R	CAATGATTTCGTTCGAAG	
plnA-F	GTACAGTACTAATGGGAG	54
plnA-R	CTTACGCCAATCTATACG	
Lcn972-F	TTGTAGCTCCTGCAGAAG GAACATGG	50
Lcn972-R	GCCTTAGCTTTGAATTCTT ACCAAAAG	

RESULTS AND DISCUSSION

Antibacterial activity of *Lact. lactis* R152

When testing the bacterial culture, *Lact. lactis* R152 showed a clear inhibition against *L. delbrueckii* LMG6901^T and against four of the (potential) pathogenic strains used as indicators (Table 2).
The largest inhibition zone was observed against the *Listeria monocytogenes* strain (approximately 50 mm in diameter) and *L. delbrueckii* subsp. *bulgaricus* LMG6901^T (around 40 mm). Inhibition zones greater than 30 mm in diameter were also observed against indicator strains belonging to *E. coli*, *S. enterica,* and *B. cereus* species. However, when the neutralized cell-free supernatant was tested, the inhibitory activity was only detected against *L. delbrueckii* LMG6901^T (results not shown).

Table 2. Antibacterial activity of *Lact. lactis* R152 against the bacterial strains used as indicators

Indicator strain	Diameter of the inhibition zone (mm)*
L. delbrueckii subsp. *bulgaricus* LMG6901^T	40
Listeria monocytogenes ATCC1911	50
Escherichia coli ATCC 25922	35
Salmonella enterica ATCC 14028	32
Bacillus cereus CBAB	33
Bacillus subtilis ATCC 6633	ni
Staphylococcus aureus ATCC 25923	ni

*ni = no inhibition

Furthermore, no inhibition of the supernatant was shown after proteinase K treatment (results not shown). These findings suggest that the antibacterial activity of *Lact. lactis* R152 against (potential) pathogenic strains is primarily due to the production of organic acids, while the activity against *L. delbrueckii* LMG6901^T is due to other compounds, most probably a bacteriocin.

In general, bacteriocins have a narrow inhibitory spectrum, typically affecting bacterial species closely related to the producing bacteria (Putri et al., 2024), while organic acids exert a more broad-spectrum, and non-specific antimicrobial effects. Various bacteria, but also yeasts and molds that contaminate food, are sensitive to the organic acids produced by LAB (Ibrahim et al., 2021). For instance, the organic acids produced by *Lactococcus lactis* may prevent contamination of food with *E. coli* and many spoilage bacteria (Alizadeh Behbahani & Noshad, 2024; Maaty et al., 2025; Sanca et al., 2023).

Characterization of the putative bacteriocin

The inhibitory activity of the culture supernatant was determined to be 1,600 AU/ml. Ammonium sulphate precipitation resulted in an increase of activity to 12,800 AU/ml (Table 3). A mild inhibitory activity of the crude bacteriocin was detected against *B. cereus* CBAB, through the agar well method (Figure 1), suggesting a combined mechanism of action against this particular indicator strain.

Table 3. Influence of pH and heat treatment on the inhibitory activity of the bacteriocin produced by *Lact. lactis* R152

Treatment		Inhibitory activity (AU/ml)
Cell free supernatant		1,600
Crude bacteriocin		12,800
60°C	10 min	12,800
	30 min	6,400
	60 min	6,400
100°C	10 min	6,400
	30 min	6,400
	60 min	6,400
121°C	15 min	3,200
pH 1		12,800
pH 3		12,800
pH 5		12,800
pH 7		12,800
pH 9		3,200

Figure 1. Antibacterial activity of the crude bacteriocin produced by *Lact. lactis* R152 against *Bacillus cereus* CBAB

It has been shown that *Lactococcus lactis* is able to produce several bacteriocins, including nisin, lacticin, and lactococcin. These antibacterial peptides have a broad spectrum of activity, including various spoilage or pathogenic bacteria that may develop in food (Negash & Tsehai, 2020; Putri et al., 2024). However, this broad spectrum of activity was not confirmed in our study.

The crude bacteriocin was further incubated at different temperatures and pH values, and the results are summarized in Table 3. As shown, the activity remained high (6,400 AU/ml) after heating for 1 h at 60°C and 100°C.

Additionally, 25% of the initial activity was retained even after autoclaving at 121°C for 15 min, indicating a high heat stability of the inhibitory compound. The compound also demonstrated remarkable stability to pH changes. The activity remained constant (12,800 AU/ml) between pH 1 and pH 7, with a decrease to 3,200 AU/ml only observed at an alkaline pH of 9.

The molecular mass of the crude bacteriocin was estimated using Tricine-SDS-PAGE. After electrophoresis, when the gel was overlaid with a top-layer inoculated with the indicator strain, a clear inhibition zone was detected, corresponding to a protein band of less than 6.5 KDa (Figure 2).

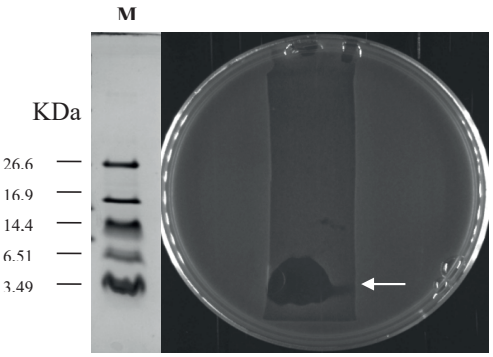

Figure 2. Estimation of the molecular mass of the bacteriocin produced by *Lact. lactis* R152. The protein band corresponding to the bacteriocin is indicated by the arrow

Given this very low molecular mass (< 6.5 KDa), and high stability at pH variations and at elevated temperatures, the bacteriocin produced by *Lact. lactis* R152 can be classified as a class I bacteriocin, which includes nisin, among others (Nes et al., 1996). This remarkable stability under different pH conditions and at high temperatures could offer a technological advantage for its potential use as a preservative in the food industry, (Grosu-Tudor et al., 2014).

Genetic screening

Using primers targeting genes encoding known bacteriocins, a specific amplification was observed for the nisin A gene, but no amplification occurred for lactococcin or plantaricin (Figure 3). The amplicon corresponded to the expected size of 298 bp. This confirms that *Lact. lactis* R152 possesses the genetic material required for nisin synthesis.

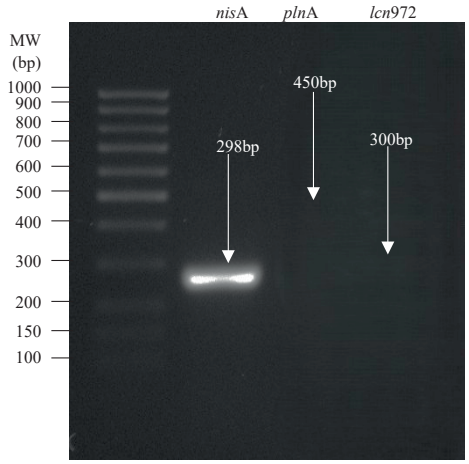

Figure 3. Amplification of genomic DNA extracted from *Lact. lactis* R152 using specific primers for *nis*A gene. First arrow indicates the amplified product of nisA, corresponding to about 298 bp; no specific amplicons were obtained for *pln*A and *lcn*972.

CONCLUSIONS

Based on our findings, we can conclude that *Lact. lactis* R152, isolated from artisanal sheep cheese, produces a small bacteriocin, most likely nisin A. This bacteriocin demonstrated excellent stability at high temperatures and at pH variations, making it promising for use in food preservation. Additionally, the antibacterial activity of *Lact. lactis* R152 against other bacteria may also be attributed to the production of organic acids.

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CHARACTERIZATION OF FUNCTIONAL GLUTEN-FREE BREADSTICKS WITH CUMIN IMPROVED BY ADDITION OF CRUCIFEROUS EXTRACTS

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Abstract

People suffering from celiac disease must exclude gluten products from their diet. The lack of gluten in bakery products causes both technological problems for processors and nutritional deficiencies for consumers. The purpose of this paper is to obtain functional gluten-free breadsticks with cumin improved by the addition of cruciferous extracts. The 3xBiotics commercial extracts was used for the experiments, which is a mixture of fermented cruciferous powders. In addition to the gluten-free breadsticks with cruciferous extracts, two control samples were made: gluten-free breadsticks with cumin whithout cruciferous extracts and breadsticks with gluten and cumin improved by the addition of cruciferous extracts. All the samples were evaluated from microbiological point of view to determine the shelf life, from physico-chemical point of view to determine the nutritional value and from sensory point of view, using a panel of consumers, to characterize the sensorial attributes. Following the comparison with the control samples, it was found that the energy value for functional gluten-free breadsticks with cumin and cruciferous extracts was higher compared to the control samples.

Key words*: bakery products, technological process, shelf life, nutritional values, sensory analysis.*

INTRODUCTION

Gluten is a protein made up of gliadin and glutenin that form disulfide bonds. It is found in wheat, barley, rye and oats (Abedi, 2021). Glutenin gives elastic properties while gliadin gives viscosity. Therefore, gluten is a viscous-elastic mass. In some predisposed individuals, gluten consumption triggers the activation of an immune-mediated enteropathy called celiac disease (CD).

Celiac disease is thought to affect 1% of consumers. The effects of gluten consumption are an inflammatory response that causes the destruction of the villi of the small intestine by reducing the absorption surface and the amount of secreted enzymes. These effects are caused by gliadin (Thompson, 2017).

Because the gluten matrix, which also contains starch granules and fiber fragments determines the properties of the dough such as extensibility, gas retention capacity, mixing tolerance and tensile strength, the elimination of gluten results in poor nutritional and sensory qualities for gluten-free products (Arendt & Dal, 2008).

It is considered that gluten-free products have a higher price compared to variants with gluten and also have lower nutritional values and sensory properties (Xhakollari et al., 2019), being caused problems during the technological process (Matos, 2014).

For people with celiac disease, a gluten-free diet is mandatory. It was also found that 20% of the population follow this gluten-free diet without a medical diagnosis, but as a lifestyle chosen from a civic, cultural, historical, ecological and ethnic point of view (Foschia, 2016).

However, the gluten-free diet also has the disadvantage of a deficiency in nutritional compounds. For this reason, it is very important to expand the market by introducing new products, fortified with food matrices rich in micronutrients that fulfill the needs of the consumer (Polo et al., 2020).

Thus, functional foods are developed by adding ingredients with antioxidant, anti-inflammatory, immunomodulatory or antitoxic effects. These foods are very important for the prevention of health problems, including chronic diseases (Vattem, 2016).

The leaven is very important in the technological process because it improves the aroma, texture, taste, shelf life and nutritional value by the presence of lactic bacteria (*Lactobacillus fermentum, Lactobacillus graminis, Lactobacillus plantarum, Lactobacillus sakei, Lactobacillus gallinarum and Pediococcus pentosaceus*) (Gobbetti, 2018).
The leaven is also important in the process of gluten-free breadsticks because the lack of gluten causes technological problems. Breadsticks are bakery products with a stick shape and are very well known for their taste and long shelf life.
The main purpose of this study is to obtain functional gluten-free breadsticks. In order to be improved from a nutritional point of view, a variant is the addition of 3 x Biotics cruciferous extracts (products manufactured by Pro Natura factory) which represent a mixture of cabbage, broccoli, cauliflower, Maca root and fermented polyfloral pollen in the form of powder. These powders were obtained by fermentation in symbiotic cultures of bacteria and yeast and dried on a bed of maltodextrin.

MATERIALS AND METHODS

The raw materials were microbiologically and physico-chemically analyzed, while the finished products were analyzed from both a microbiological and physico-chemical point of view, as well as sensory.

Microbiological analysis of raw materials

The raw materials that were analyzed from a microbiological point of view are wheat flour, rice flour and brown sugar.
Method that was used for determination of yeasts and molds number was according to SR ISO 21527-2:2009 - Microbiology - General guidelines for counting yeasts and molds. In order to have a greater specificity it was used the technique for counting colonies in products with water activity less than 0.95.
Enterobacteriaceae enumeration method was performed according to SR EN ISO 21528-2/2017- Food chain microbiology, respectively horizontal method for detecting and counting Enterobacteriaceae, namey part 2 - Colony counting method.
The determination of yeasts and molds number per gram of product (N) and Enterobacteriaceae number, after reading the colonies grown on selective media, was calculated by applying the following formula:

$$N = \frac{\sum C}{(n_1 + 0.1n_2) \times d},$$

where:
ΣC = the sum of the colonies counted in all retained plates;
n_1 = the number of plates retained at first dilution;
n_2 = the number of plates retained at the second dilution;
d = the dilution from which the first counts were made.
The results obtained are mentioned in Table 1.

Table 1. Microbiological analysis results for raw materials

Sample	Microbiological indicators	
	Yeasts and molds (cfu/g)	Enterobacteriaceae (cfu/g)
Wheat flour	1.4×10^2	-
Rice flour	< 10	-
Brown sugar	< 10	-

Physico-chemical analysis

The raw materials used to make breadsticks, respectively wheat flour, rice flour and brown sugar, were initially analyzed from a physico-chemical point of view. Moisture, ash, protein and fat content along with the acidity of the product were determined, according to the methods given below.
Sugar moisture determination was performed according to SR 110-3:1995 method.
Determination of moisture in cereal flours was performed according to SR 90:2007 point 11 with reference to SR EN 712:2010.
Determination of ash was performed according to SR ISO 2171:2009. The results calculation method was applied as follows:

$$wad = (m_2 - m_1) \times \frac{100}{m_0} \times \frac{100}{100 - Wm},$$

where:
m_0 = the work sample mass (g);
m_1 = the calcination crucible mass (g);
m_2 = the mass of the calcination crucible and calcined residue (g);
Wm = the moisture content of the sample as a mass percentage.

Determination of fat was carried out according to the Soxhlet method, where the results have been calculated as follows:

$$w = \frac{m2-m1}{m} \times 100,$$

where:
m_1 = the extraction flask mass;
m_2 = the mass of the extraction flask with fat;
m = the mass of the sample taken for drying.

Determination of protein content was performed according to the Kjeldahl method.
Raw materials acidity determination was done according to SR 90:2007 point 12 and the results calculation method is presented below:

$$\text{Acidity} = \frac{V \times 0.1}{m} \text{ (degrees)},$$

where:
V = the volume of 0.1 N NaOH solution (mL);
m = the work sample mass (g);
0.1 = the normality of the NaOH solution.
Determination of crude fiber (cellulose) content was achieved according to SR EN ISO 91:2007. In order to calculate the results, the following formula was used:

$$wf = \frac{m3-m1-m4-m5}{m2} \times 100,$$

where:
$m_5 = m_7 - m_6$;
W_f = the crude fiber content, %;
m_1 = the FiberBag mass, g;
m_2 = the initial sample mass, g;
m_3 = the calcination crucible mass with dried FiberBag, g;
m_4 = the mass of calcination crucible and residue obtained after calcination, g;
m_5 = the empty FiberBag blank mass, g;
m_6 = the calcination crucible mass, g;
m_7 = the mass of calcination crucible and ash content of empty FiberBag, g.
The results obtained from physico-chemical analysis of raw materials, are mentioned in Table 2.

Table 2. Physico-chemical analysis results for raw materials

Sample	Acidity (°)	Moisture (%)	Fat (%)	Ash (%)	Protein (%)	Raw fiber (%)
Wheat flour	3.5	13.58	1.08	0.65	10.83	1.17
Rice flour	1.6	13.42	0.39	0.39	8.58	0.51
Brown sugar	-	0.16	-	0.09	-	-

Formulation of products
3xBiotics (product manufactured by Pro Natura) cruciferous extracts consists of a mix of Cabbage powder (*Brassica oleracea* var. *capitata*), Cauliflower powder (*Brassica oleracea* var. *botrytis*), Maca root powder (*Lepidium meyenii*) and Broccoli sprout powder (*Brassica oleracea* var. *italica*), this ingredients being fermented in synbiotic cultures of bacteria and yeast and dried on a maltodextrin bed.
Three experimental breadsticks variants were obtained, as follows: functional gluten-free breadsticks with cumin enhanced by the addition of cruciferous extracts (Figure 2), gluten-free breadsticks with cumin and without cruciferous extracts (Figure 3) and breadsticks with gluten and cumin enhanced by the addition of cruciferous extracts (Figure 4).
The recipe for **functional gluten-free breadsticks with cumin enhanced by the addition of cruciferous extracts** with a net mass of 0.100 kg includes the following ingredients:
- Leaven which was obtained from: 0.0242 l water; 0.0157 kg rice flour; 0.0024 kg brown sugar; 0.0024 kg dry yeast.
- Dough which involved the mixing of the following ingredients: 0.058 kg rice flour; 0.012 kg eggs; 0.009 l olive oil; 0.007 l water; 0.007 kg cruciferous extracts; 0.0024 kg starch; 0.0014 kg cumin; 0.0012 kg chia; 0.0012 kg salt; 0.0007 kg carboxymethyl cellulose (CMC); 0.0002 kg Xanthan gum.
The recipe for **gluten-free breadsticks with cumin and without cruciferous extracts** with a net mass of 0.100 kg includes:
- Leaven obtained by mixing: 0.0286 l water; 0.0185 kg rice flour; 0.0028 kg brown sugar; 0.0028 kg dry yeast.
- Dough that was obtained from: 0.068 kg rice flour; 0.012 kg eggs; 0.011 l olive oil; 0.011 l water; 0.0028 kg starch; 0.0014 kg chia; 0.0014 kg salt; 0.0012 kg cumin; 0.0008 kg carboxymethyl cellulose (CMC); 0.0002 kg Xanthan gum.
The recipe for **breadsticks with gluten and cumin enhanced by the addition of cruciferous extracts** with a net weight of 0.100 kg includes:
- Leaven made of the ingredients such as: 0.0213 l water; 0.0138 kg white flour; 0.0021 kg brown sugar; 0.0021 kg dry yeast.

- Dough obtained by mixing the following ingredients: 0.051 kg white flour; 0.012 kg eggs; 0.0085 l olive oil; 0.0064 kg cruciferous extracts; 0.0053 l water; 0.0021 kg starch; 0.0012 kg cumin; 0.001 kg chia; 0.001 kg salt; 0.0006 kg carboxymethyl cellulose (CMC); 0.0002 kg Xanthan gum.

The technological process for obtaining the three experimental variants of breadsticks described above, involved the performance of a series of unitary operations, which are described below.

All raw materials are dosed according to the manufacturing recipe, using a scale.

The water is slightly heated using a stove or oven to facilitate the dissolution operation. Dissolution is the operation by which a homogeneous liquid mixture is formed and involves mixing water with salt in order to uniformly disperse the salt. At the same time, the yeast is dissolved in water to facilitate its activation.

The eggs are beaten with a mixer. Mixing is the technological operation that involves the uniform incorporation of raw materials. In the first phase, the ingredients for the leaven are mixed, followed by the incorporation of the remaining ingredients into the leaven.

The leaven is fermented in a bowl at room temperature for 60 minutes, meanwhile the bowl being covered.

The kneading is done at room temperature for 10 minutes in a one-arm mixer to form the dough.

The dough is shaped using a grinder with funnel, the shape of the by-products obtained being cylindrical and the cutting to the desired lengths is done with a knife.

The fermentation of the by-products takes place into the leavener for 20 minutes.

The baking process is performed at 190°C for 38 minutes, in a hearth oven with the by-products placed on a tray.

The cooling is realised with the trays placed on the work tables, for 2 hours, at ambient temperature.

The final products are packed in a normal atmosphere, in bags made of biaxially oriented polypropylene which is a highly valued material in the food industry, at different weights depending on consumer requirements, using the Impulse Bag Sealer equipment.

The products labeling shall be in accordance with Regulation (Eu) No 1169/2011 Of The European Parliament And Of The Council.

Breadsticks are stored at room temperature, in air-conditioned spaces, with controlled temperature, humidity and ventilation, for 7 weeks. Because the lack of gluten causes technological problems, Xanthan gum and carboxymethyl cellulose (CMC) were added in order to bind the dough components.

The technological operations are shown in Figure 1.

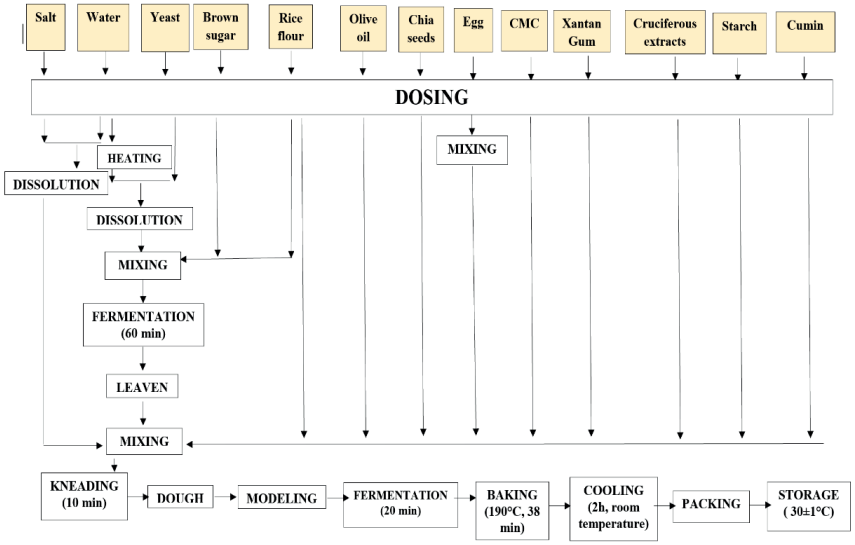

Figure 1. Technological diagram for functional gluten-free breadsticks with cumin enhanced by the addition of cruciferous extracts

Microbiological analysis of finished product use the same methods for yeasts and molds cfu/g and Enterobacteriaceae cfu/g, as previously described for the raw materials. The results are mentioned in Table 3.

Table 3. Microbiological analysis results for finished products

Sample	Microbiological and stability indicators	
	Yeasts and molds (cfu/g)	**Enterobacteriaceae** (cfu/g)
Initial analysis		
Functional gluten-free breadsticks with cumin and cruciferous extracts	< 10	< 10
Gluten-free breadsticks with cumin, without cruciferous extracts	< 10	< 10
Breadsticks with gluten, cumin and cruciferous extracts	< 10	< 10
Analysis at three weeks		
Functional gluten-free breadsticks with cumin and cruciferous extracts	< 10	< 10
Gluten-free breadsticks with cumin, without cruciferous extracts	< 10	< 10
Breadsticks with gluten, cumin and cruciferous extracts	< 10	< 10
Analysis at six weeks		
Functional gluten-free breadsticks with cumin and cruciferous extracts	< 10	< 10
Gluten-free breadsticks with cumin, without cruciferous extracts	< 10	< 10
Breadsticks with gluten, cumin and cruciferous extracts	< 10	< 10
Analysis at seven weeks		
Functional gluten-free breadsticks with cumin and cruciferous extracts	< 10	< 10
Gluten-free breadsticks with cumin, without cruciferous extracts	< 10	< 10
Breadsticks with gluten, cumin and cruciferous extracts	< 10	< 10

Determination of water activity is based on the formula: aw = relative humidity (%)/100.
The results are mentioned in Table 4.

Table 4. Results for water activity

Sample	Water activity
Initial analysis	
Functional gluten-free breadsticks with cumin and cruciferous extracts	0.750
Gluten-free breadsticks with cumin, without cruciferous extracts	0.752
Breadsticks with gluten, cumin and cruciferous extracts	0.751
Analysis at three weeks	
Functional gluten-free breadsticks with cumin and cruciferous extracts	0.751
Gluten-free breadsticks with cumin, without cruciferous extracts	0.753
Breadsticks with gluten, cumin and cruciferous extracts	0.755
Analysis at six weeks	
Functional gluten-free breadsticks with cumin and cruciferous extracts	0.755
Gluten-free breadsticks with cumin, without cruciferous extracts	0.756
Breadsticks with gluten, cumin and cruciferous extracts	0.758
Analysis at seven weeks	
Functional gluten-free breadsticks with cumin and cruciferous extracts	0.755
Gluten-free breadsticks with cumin, without cruciferous extracts	0.756
Breadsticks with gluten, cumin and cruciferous extracts	0.758

Physico-chemical analysis of finished products use the same methods for moisture, ash and fat, as previously described for the raw materials. Determination of proteins is based on the Kjeldahl method.
Determination of total carbohydrates is based on the formula:
Total carbohydrates = 100 – (Moisture + Protein + Fat + Ash)
The energy value measured in kilocalories is based on the formula:
E.V. (kcal)/sample = (4 x protein/sample) + (4 x total carbohydrates/sample) + (9 x fat/sample)
The energy value measured in kilojoules is based on the formula:
E.V. (kj) / sample = (17 x protein/sample) + (17 x total carbohydrates/sample) + (37 x fat/sample).
The results are mentioned in Table 5.

Table 5. Physico-chemical analysis results for finished products

Sample	Moisture (%)	Ash (%)	Protein (%)	Fats (%)	Total carbohydrates (%)	Energy value (kcal/ 100 g)
Functional gluten-free breadsticks with cumin and cruciferous extracts / 100 g	7.93	1.42	9.84	9.85	70.96	411.85
Gluten-free breadsticks with cumin and without cruciferous extracts / 92.74 g	7.24	1.50	9.68	10.24	64.08	387.20
Breadsticks with gluten, cumin and cruciferous extracts / 100 g	9.9	1.78	12.18	9.95	66.19	403.03

Sensory analysis of finished products

A questionnaire, which includes the following attributes, was completed by a panel of consumers: appearance (shape), surface color, inside color, smell, texture / chewing sensation.

Following the tasting, the panel of consumers completed a questionnaire and chose the answer by giving notes according to the individual assessment from: I dislike it extremely (1)/I dislike it a lot (2)/I dislike it moderately (3)/I dislike it lightly (4)/I don't care (5)/I like it lightly (6)/I like it moderately (7)/I like it very much (8)/I like it extremely (9).

The results were interpreted as a percentage and mentioned in Table 6. Sensory analysis results for functional gluten-free breadsticks with cumin and cruciferous extracts and Table 7. Sensory analysis results for gluten-free breadsticks with cumin, without cruciferous extracts.

Table 6. Sensory analysis results for functional gluten-free breadsticks with cumin and cruciferous extracts

Appearance (form)	I dislike it moderately (3)	I don't care (5)	I like it lightly (6)	**I like it moderately (7)**	I like it very much (8)
	16.67 %	8.33 %	16.67 %	**33.33 %**	16.67 %
Surface colour	I dislike it moderately (3)	I don't care (5)	I like it lightly (6)	**I like it moderately (7)**	I like it very much (8)
	16.67 %	8.33 %	8.33 %	**41.67 %**	25.00 %
Inside colour	I dislike it moderately (3)	I don't care (5)	I like it lightly (6)	**I like it moderately (7)**	I like it very much (8)
	-	16.67 %	8.33 %	**41.67 %**	33.33 %
Smell	I dislike it moderately (3)	I dislike it lightly (4)	I like it lightly (6)	**I like it moderately (7)**	I like it very much (8)
	8.33 %	8.33 %	25.00 %	**41.67 %**	16.67 %
Texture / chewing sensation	I dislike it moderately (3)	I don't care (5)	I like it lightly (6)	**I like it moderately (7)**	I like it very much (8)
	8.33 %	16.67 %	25.00 %	**41.67 %**	8.33 %
Aroma / taste	I dislike it a lot (2)	I like it lightly (6)	**I like it moderately (7)**	I like it very much (8)	I like it extremely (9)
	8.33 %	8.33 %	**41.67 %**	25.00 %	8.33 %
Sweetness	**I don't care (5)**	**I like it lightly (6)**	I like it moderately (7)	I like it very much (8)	I like it extremely (9)
	25.00 %	**25.00 %**	16.67 %	8.33 %	8.33 %
Acidity	**I don't care (5)**	I like it lightly (6)	I like it moderately (7)	I like it very much (8)	I like it extremely (9)
	41.67 %	8.33 %	25.00 %	8.33 %	8.33 %
Remaining taste	I dislike it extremely (1)	I dislike it moderately (3)	**I like it lightly (6)**	I like it moderately (7)	**I like it very much (8)**
	16.67 %	16.67 %	**25.00 %**	16.67 %	**25.00 %**
Total acceptability	I dislike it a lot (2)	I dislike it moderately (3)	I like it lightly (6)	**I like it moderately (7)**	I like it very much (8)
	8.33 %	8.33 %	16.67 %	**50.00 %**	16.67 %

Table 7. Sensory analysis results for gluten-free breadsticks with cumin, without cruciferous extracts

Appearance (form)	**I don't care (5)**	I like it lightly (6)	**I like it moderately (7)**	I like it very much (8)	-
	30 %	20 %	**30 %**	20 %	-
Surface colour	I don't care (5)	**I like it moderately (7)**	I like it very much (8)	-	-
	30 %	**40 %**	30 %	-	-
Inside colour	I don't care (5)	**I like it moderately (7)**	I like it very much (8)	-	-
	30 %	**50 %**	20 %	-	-
Ssmell	I dislike it lightly (4)	I don't care (5)	**I like it moderately (7)**	I like it very much (8)	-
	10 %	20 %	**50 %**	20 %	-
Texture / chewing sensation	I dislike it lightly (4)	I don't care (5)	I like it lightly (6)	I like it moderately (7)	**I like it very much (8)**
	10 %	20 %	20 %	20 %	**30 %**
Aroma / taste	I dislike it extremely (1)	I don't care (5)	I like it lightly (6)	**I like it moderately (7)**	-
	10 %	20 %	20 %	**50 %**	-
Sweetness	I dislike it a lot (2)	**I don't care (5)**	I like it lightly (6)	I like it moderately (7)	I like it very much (8)
	20 %	**30 %**	10 %	20 %	20 %
Acidity	I dislike it lightly (4)	**I don't care (5)**	I like it lightly (6)	I like it moderately (7)	I like it very much (8)
	10 %	**30 %**	20 %	10 %	20 %
Remaining taste	I dislike it a lot (2)	I dislike it moderately (3)	I like it lightly (6)	**I like it moderately (7)**	I like it very much (8)
	10 %	20 %	20 %	**30 %**	10 %
Total acceptability	I dislike it moderately (3)	I dislike it lightly (4)	I like it lightly (6)	**I like it moderately (7)**	I like it very much (8)
	10 %	20 %	20 %	**30 %**	10 %

RESULTS AND DISCUSSIONS

Among the raw materials analyzed, only wheat flour exhibited contamination with yeasts and molds at a level of 10^3 cfu/g. According to Order no. 27/2011, issued by ANSVSA, for the product category "flours for bakery" the maximum allowed limit is 10^3 cfu/g, but the analyzed sample shows contamination with yeasts and molds below this limit. The results obtained from the microbiological analyses fall within the accepted limits.

Comparing the physico-chemical analysis results for raw materials by analyzing acidity, moisture, fat, ash, protein and raw fiber, it can be seen that rice flour has lower values than white flour.

From a technological point of view, three experimental variants were obtained, as follows: Variant 1 - Functional gluten-free breadsticks with cumin enhanced by the addition of cruciferous extracts, whose appearance can be seen in Figure 2.

Figure 2: Functional gluten-free breadsticks with cumin enhanced by the addition of cruciferous extracts

Variant 2 - Gluten-free breadsticks with cumin, without cruciferous extracts whose appearance can be seen in Figure 3.

Figure 3. Gluten-free breadsticks with cumin, without cruciferous extracts

Variant 3 - Breadsticks with gluten, cumin and cruciferous extracts, whose appearance can be seen in Figure 4.

Figure 4. Breadsticks with gluten, cumin and cruciferous extracts

Under normal storage conditions, breadstick samples remained stable for seven weeks, showing no microbiological contamination by molds or Enterobacteriaceae during this period.
Both aerobic mesophilic bacteria and molds are common spoilage microorganisms that contaminate the technological flow under inadequate hygiene conditions. To prevent contamination of finished products by microorganisms, it is essential to rigorously monitor raw materials, equipment, personnel hygiene, the production environment, and processing methods. It is well known that these are potentially contaminating elements of the technological flow. The results obtained from the microbiological analyses fall within the accepted limits.
Based on the results presented in Table 4, the following conclusions can be drawn:
- after the initial analysis, gluten-free breadsticks with cumin, without cruciferous extracts had the highest water activity and functional gluten-free breadsticks with cumin while cruciferous extracts had the lowest water activity;
- the analysis made after three weeks showed that breadsticks with gluten, cumin and cruciferous extracts had the highest water activity while the functional gluten-free breadsticks with cumin and cruciferous extracts had the lowest water activity;
- at six-week evaluation, breadsticks with gluten, cumin and cruciferous extracts had the highest water activity and the functional gluten-free breadsticks with cumin and cruciferous extracts had the lowest water activity;
- after seven weeks, the analysis revealed that breadsticks with gluten, cumin and cruciferous extracts had the highest water activity while the functional gluten-free breadsticks with cumin and cruciferous extracts had the lowest water activity.
During the shelf-life monitoring period, the water activity index showed a slight increase but the final values are not significantly different from those obtained during initial testing.
Functional gluten-free breadsticks with cumin and cruciferous extracts, gluten-free breadsticks with cumin and without cruciferous extracts and breadsticks with gluten, cumin and cruciferous extracts were analyzed from a physico-chemical point of view. Comparing the results, we can see that moisture, ash and protein have the highest values for breadsticks with gluten, cumin and cruciferous extracts. Total carbohydrates and energy value have the highest values for functional gluten-free breadsticks with cumin and cruciferous extracts.
For functional gluten-free breadsticks with cumin and cruciferous extracts, the appearance was evaluated as „ I like it moderately" by 33% from the panel consumers, the surface colour, inside colour, smell, texture/chewing sensation and aroma/ taste were evaluated as "I like it moderately" by 41.67% from the panel consumers, the sweetness was evaluated as "I don't care " and "I like it lightly" by 25% from the panel consumers, the acidity was evaluated as "I don't care" by 41.67% from the panel consumers, the remaining taste was evaluated as "I like it lightly" and "I like it very much" by 25% from the panel consumers, the total acceptability was evaluated as "I like it moderately" by 50% from the panel consumers.
For gluten-free breadsticks with cumin, without cruciferous extracts, the appearance was evaluated as "I don't care" and "I like it moderately" by 30% from the panel consumers, the surface colour was evaluated as "I like it moderately" by 40% from the panel consumers, the inside colour was evaluated as "I like it moderately" by 50% from the panel consumers, the smell was evaluated as "I like it moderately" by 50% from the panel consumers, the texture/chewing sensation was evaluated as "I like it very much" by 30% from the panel consumers, the aroma/taste was evaluated as "I like it moderately" by 50% from the panel consumers, the sweetness was evaluated as "I don't care" by 30% from the panel consumers,

the acidity was evaluated as “I don't care” by 30% from the panel consumers, the remaining taste was evaluated as “I like it moderately” by 30% from the panel consumers, the total acceptability was evaluated as “I like it moderately” by 30% from the panel consumers. Functional gluten-free breadsticks with cumin and cruciferous extracts was preferred by consumers.

CONCLUSIONS

The purpose of this paper was to obtain functional gluten-free breadsticks with cumin improved by the addition of cruciferous extracts. The 3xBiotics commercial extracts was used for the experiments, which is a mixture of fermented cruciferous powders. The sulfur compounds naturally present in cruciferous vegetables are known to support liver detoxification at the cellular level. These vegetables strengthen the immune system and absorb free radicals. At the same time, sulfur compounds improve heart health and digestion.
From microbiological point of view, the shelf life of the functional gluten-free breadsticks with cumin and cruciferous extracts and the shelf life of the control samples were of seven weeks, all the samples being free of microbiological contamination with mold or Enterobacteriaceae. From physico-chemical point of view, the highest energy value was for functional gluten-free breadsticks with cumin and cruciferous extracts, compared to the control samples.
Following the results of sensory analysis performed, it can be noticed that total acceptability for breadsticks with cumin and cruciferous extracts is appreciated as “I like it moderately” by 50% of consumers and “I like it very much” by 16.67 % of consumers.

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VARIATION OF FLAVOPROTEIN CONCENTRATION AFTER SWEETENING IN A GREEN-TEA BASED FOOD SUPPLEMENT

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Abstract

Flavoproteins are proteins that contain a nucleic acid derivative of riboflavin. Flavoproteins removes the free radicals that contributes to oxidative stress and have an important role in DNA repair. The raw material used, is free from plant growth hormones and pesticides, helping to maintain high levels of nutrients in the finished product. The objective of this paper work is to identify the variant with the highest concentration of flavoproteins following the green tea sweetening process, in order to produce the most effective product for an innovative food supplement. The results of this phase of research have identified the variant with the highest concentration of flavoproteins following the green tea sweetening process. The best variant obtained at this stage of research is characterized by sensory improvements and minimal changes in chemical composition compared to the unsweetened variant.

Key words*: enzyme, flavoprotein, green tea, sweeteners.*

INTRODUCTION

Tea is one of the most consumed beverages. Beneficial effects on the circulatory system, improvement of heart function, prevention of atherosclerosis and obesity, anticancer, anti-inflammatory, and antibacterial properties are some of the properties of tea. Most of them result from the presence of biologically active substances in tea leaves, mainly polyphenolic compounds, but also alkaloids (theobromine, theophylline, and caffeine) and theanine (Savescu, 2016a; Savescu, 2021; Kowalska et al., 2021).

Abbas & Sibirny write in their paper that riboflavin [7,8-dimethyl-10-(1′-d-ribityl) isoalloxazine, vitamin B_2] is an obligatory component of human and animal diets, as it serves as a precursor of the flavin coenzymes flavin mononucleotide (FMN) and flavin adenine dinucleotide (FAD). Flavin mononucleotide (FMN) and flavin adenine dinucleotide (FAD) are involved in oxidative metabolism and other processes (Abbas & Sibirny, 2011; Tița et al., 2021).

Succinate dehydrogenase is an example of a flavoenzyme; it catalyzes the removal of two hydrogen atoms from the succinic acid molecule and their transfer to the prosthetic FAD group. Many of these flavoenzymes additionally contain a metal, which is essential for the function of metal-flavoprotein enzymes (Savescu et al., 2019; Savescu et al., 2020b).

Nobel laureate Szent-Györgyi showed that flavoproteins act as electron carriers between dehydrogenases and iron-containing cytochromes. The segments of the electron transport chain have been reconstituted in vitro using purified components (Isenberg & Szent-Györgyi, 1958).

NAD-dependent oxidoreductases are enzymes from the class of anaerobic dehydrogenases and have as coenzymes, Nicotinamide Adenine Dinucleotide (NAD^+) or reduced ($NADH+H^+$) and Nicotinamide Adenine Dinucleotide Phosphate Oxidate ($NADP^+$) or reduced ($NADPH+H^+$). These coenzymes consist of a derivative of vitamin PP, nicotinamide and an adenine-derived nucleus (Savescu, 2021).

NAD^+ and $NADP^+$ are anaerobic, because the transferred hydrogen acceptor is not oxygen, but another element. They catalyze redox reactions by the generally reversible transfer of protons. The transfer of hydrogen in the redox reactions catalyzed by NAD^+ and $NADP^+$ is carried out at the level of the nicotinamide component in the structure of these coenzymes (Savescu, 2021).

In certain liquid foods, these mechanisms can be disturbed by certain food additives

(sweeteners, preservatives, colorings). Sweeteners can have an oxidative effect on these redox systems, and this effect can significantly reduce the antioxidant capacity of the food. In green tea, sweeteners can reduce the concentration of reduced active forms of some oxidoreductases (in particular metal-flavoprotein enzymes) (Savescu, 2019; Savescu, 2021).

$NADH+H^+$ is the form in which electrons are collected from various substrates by NAD^+-dependent dehydrogenases. These electrons are passed up the respiratory chain via flavoproteins (NADH dehydrogenases) (Savescu, 2021).

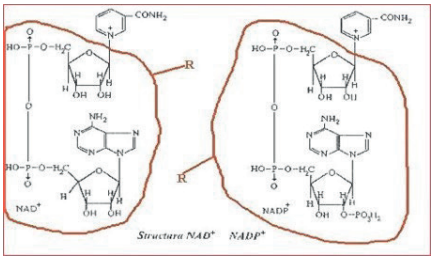

Figure 1. Structure of NAD^+ and $NADP^+$ - according Savescu, 2021

There are two types of flavoproteins with dehydrogenase activity. L-amino acid oxidase is localized in the endoplasmic reticulum and D-amino acid oxidase is localized in the microsomes of liver cells (Savescu, 2016b; Savescu, 2017b).

The flavin nucleotides of these oxidases ($FADH_2$ and $FMNH_2$) react directly with molecular oxygen, generating H_2O_2 which is in turn broken down into water and oxygen under the influence of catalase. This enzymatic process occurs in the peroxisomes of liver cells. FMN (Flavin mononucleotide) is a prosthetic (protease) group of flavoprotein. It is similar in structure to FAD (Flavin adenine dinucleotide), but lacks adenine as a nucleotide. FMN (like FAD) can accept 2 e- + 2 H+ to yield $FMNH_2$. When bound to the active site of some enzymes, FMN can accept e-, converting to a semiquinone radical (Savescu, 2017a).

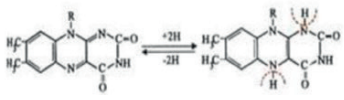

Figure 2. FMN Mechanism of redox processes (Flavin Mononucleotide) (Savescu, 2017)

FAD-dependent oxidoreductases are enzymes of a heteroproteinic nature from the group of aerobic dehydrogenases having as active groups derivatives of vitamin B2 (riboflavin or 7,8-dimethyl-10-ribithyl-isoaloxazine), namely: flavin adenine mononucleotide (FMN) and flavin dinucleotide (FAD) (Savescu, 2021). Flavin enzymes (FMN, FAD) are involved in electron and proton transfer reactions mediated by the isoalloxazine nucleus. They accept either an electron or a pair of electrons (unlike NAD^+ and $NADP^+$ which only accept electron pairs) (Savescu, 2021).

Flavinenzymes have the standard redox potential Eo between +0.19V (oxidants stronger than NAD^+) and –0.49V (reducing agent stronger than NADH), which shows a wide range of variation of redox properties depending on environmental conditions and the nature of the substrate (Savescu, 2021).

Theophylline is a dimethylxanthine having the two methyl groups located at positions 1 and 3. It is structurally similar to caffeine and is found in green and black tea. It has a role as a vasodilator agent, a bronchodilator agent, a muscle relaxant, an EC 3.1.4.* (phosphoric diester hydrolase) inhibitor, an anti-asthmatic drug, an anti-inflammatory agent, an immunomodulator, an adenosine receptor antagonist, a drug metabolite, a fungal metabolite and a human blood serum metabolite (National Center for Biotechnology Information, 2025).

The main objective was to determine which of the sweeteners could induce changes in the concentrations of the active forms of flavoproteins in green tea.

Using a statistical program such as origin pro 2020, positive correlations were found between the concentrations of reduced forms of some oxidoreductases and certain sweeteners (brown sugar) and negative correlations (sucrose, fructose) between other sweeteners and these reduced forms. Most previous studies have concerned an analysis of the positive/negative correlations between certain NAD- and NADP-dependent oxidoreductases and certain sweeteners (Savescu, 2020b).

This paper aimed to analyze the variation of flavoprotein concentrations in tea upon additivities with certain natural and synthetic sweeteners using analytical methods. These

data will be further statistically analyzed using an updated statistical software.

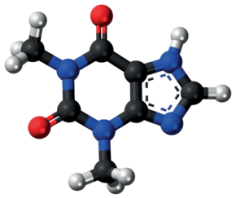

Figure 3. Chemical structure of theophylline
https://en.wikipedia.org/wiki/Theophylline#/media/File:Theophylline_3D_ball.png

MATERIALS AND METHODS

A range of natural and synthetic sweeteners were used in the laboratory to study the effects of these sweeteners on the chemical composition of green tea. The raw material utilized (green tea) was subjected to testing using A.A.S. (Atomic Absorption Spectroscopy), with the purpose of determining the presence of any contamination by residues or heavy metals. The influence of sweeteners on the flavoprotein activity was ascertained through the implementation of UV-VIS optical spectrometry and mathematical statistics. Approximately 10 grams of green tea plant per 1000 ml of water was added to a bowl to obtain the control version of unsweetened green tea. The tea was heated, cooled and filtered to produce the experimental V0. The present experiment involved the creation of ten sweetened tea variants, which were derived from the experimental variant (Figure 4). The resulting variants are listed below:

V0 - an unsweetened green tea variety;
V1 - green tea variety sweetened with white sugar;
V2 - variant of green tea sweetened with brown sugar;
V3 - green tea variant sweetened with honey;
V4 - green tea variant sweetened with saccharin;
V5 - green tea sweetened with Sucrazit variant;
V6 - variant of green tea sweetened with Diamond;
V7 - variant of green tea sweetened with fructose;
V8 - variant of green tea sweetened with xylitol;
V9 - green tea sweetened with sorbitol;
V10 - green tea sweetened with stevia.

The experimental version V5, Sucrazit, is the trade name of the synthetic sweetener containing 20% saccharin, citric acid, whose acidity has been buffered with sodium bicarbonate.

The experimental variant V6, Diamond, is the trade name for a synthetic sweetener containing a combination of sodium cyclamate and sodium saccharine.

Molecular absorption spectra of flavoproteins from experimental variants were obtained using certified reference substances and a T92 Plus UV-VIS spectrophotometer manufactured by PG Instruments U.K.

Certified reference substances (pure analytical substances) were used to make calibration scales of different concentrations, which helped to find the wavelength at which the maximum molecular absorption spectra for flavoproteins and riboflavin are recorded. Using the single addition method and interpolation on both axes, specific wavelengths were determined.

As we pointed out in a previous paper, the spectrophotometer was configured to function within a wavelength bandwidth of 1 cm, thereby enabling the measurement of nanometre-to-nanometre molecular absorption values across the UV (190-400 nm) and visible (400-700 nm) ranges. This configuration permitted the acquisition of data in both the UV (190-400 nm) and visible (400-700 nm) ranges (Ionescu et al., 2023).

The equipment automatically records spectral curves, changing the deuterium and tungsten lamps at 361 nm by automatic programming. To double-check the values obtained, at each measurement the T92 Plus spectrophotometer was set to develop an automatic retracking (Ionescu et al., 2023).

Absorbance measurements were made in special quartz UV cuvettes.

The first stage of sample preparation for atomic absorption spectrometry is mineralization (microwave digestion) (Savescu, 2017c). Microwave digestion is a method of sample preparation for elemental analysis by ICP, ICP-MS or AA. It is applicable to a wide range of samples, including those from plant, soil, food and pharmaceutical sources. The principle behind this method is to dissolve the sample before introducing it into the analyzer. In contrast, acid digestion is used to break down

the sample matrix, leaving the elements of interest in solution and ready for analysis (Savescu, 2017c). CEM microwave digestion systems rapidly break down a wide variety of sample matrices, leaving behind a clear solution containing the analytes of interest. (Savescu, 2017c).

The experiment employed a 1200 W MARS mineralized microwave CEM system, a multimode platform equipped with a magnetic stir plate and a rotor that facilitates parallel processing of multiple vessels per batch. The MARS CEM system is notable for its versatility, offering a range of modes that include a Teflon insert (TFA) (HP-500) (80 ml vessel volume, max pressure 350 psi, max temperature 210ºC) and Greenchem (glass insert (borosilicate)) (80 ml vessel volume, max pressure 200 psi, max temperature 200ºC) vessel types, both based on a 14-position rotor (Savescu, 2017b). The system provides a constant power output ranging from 0 to 1200 watts. The temperature is regulated internally by a fiber optic probe situated within a control reference vessel (Savescu, 2017b).

Method: In summary, 10 g of tea solids were measured with analytical precision. For the mineralization step, 10.00 g of product (green tea), 6 ml of concentrated nitric acid and 3 ml of 30% hydrogen peroxide were added to each digestion cartridge. A digestion cartridge containing no product but only reagents was used as a control (L'vov, 2005; Belitz, 2009; Savescu, 2017b).

Figure 4. Experimental variants

RESULTS AND DISCUSSIONS

During the stages of obtaining innovative dietary supplements from green tea, it is recommended to monitor the active forms of flavoproteins and riboflavin in green tea additivities to maintain the antioxidant capacity of the final product. When monitoring and controlling subsequent products, only those experimental variants that show the smallest changes in flavoprotein and riboflavin concentrations compared to the control variants after sweetening should be emphasised. These will also be the variants recommended to consumers, especially those with diabetes or nutritional disorders.

The use of the AAS technique revealed high concentrations of potassium, magnesium and calcium in the parts of the tea plant used, thus increasing the nutritional density of the final product - green tea dietary supplement.

Table 1. Atomic Absorption Spectroscopy baseline for green tea

Indicator/ Constituent	GREEN TEA	
	Dry matter ppm (mg/kg)*	Watery extract 1:10 ppm (mg/L) Average value
Na^{+}	872±1.74	77.86
K^{+}	1484±1.86	72.43
Ca^{2+}	376±0.96	2.04
Mg^{2+}	212±0.88	2.10
Zn^{2+}	24.6±0.02	0.62
Mn^{2+}	288±0.94	0.95
Fe^{2+}	8.74±0.03	0.08
Al^{3+}	6.86±0.03	0.31
Cu^{2+}	4.267±0.03	0.18
Pb^{2+}	0.018±0.002	lipsa

V3 (honey) and V4 (saccharin) gave the highest concentrations of NAD^{+} and $NADH+H^{+}$ and FMN and $FMNH_2$.

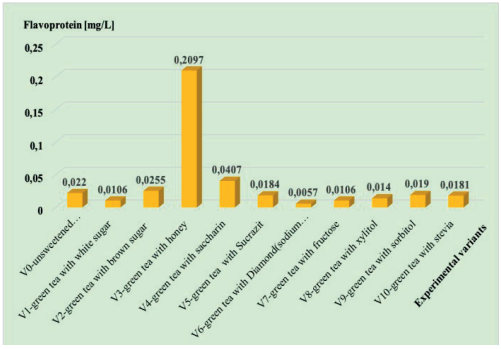

Figure 5. Green tea flavoprotein concentration

The flavoprotein concentrations reached the highest value in the V3 variant (honey) and the

lowest one in the V6 variant (sodium cyclamate and saccharin).
Honey (used in the experimental variant V3) exerts a strong effect on flavoproteins in green tea. According to the graphs, increased concentrations of the oxidized and reduced forms of riboflavin in green tea are present in this variant (V3 - Figures 5, 6, 7). It is thus proven to increase the flavoprotein activity of green tea when honey is used as a sweetener.

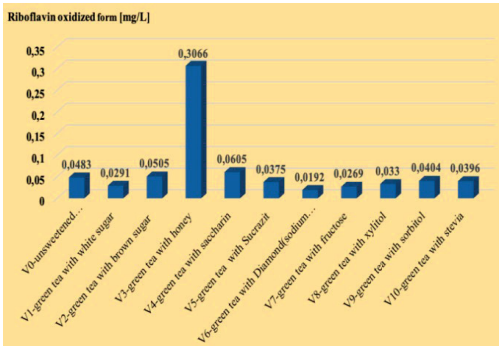

Figure 6. Concentration of the oxidized form of riboflavin in green tea

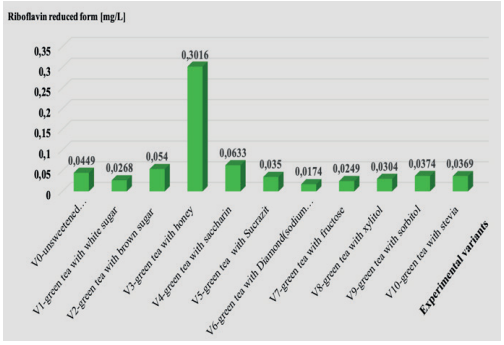

Figure 7. Concentration of the reduced form of riboflavin in green tea

Saccharin induces strong oxidative effects in the chemical composition of green tea and alters both the hue and intensity of the colour, causing visible disturbances. When Sucrazit is added to the medium, the oxidation effects are much less as the coloured pigments are better protected. The additional activation of the isoalloxazine nucleus of riboflavin was proved when saccharin was acidified with an organic acid and buffering of this nucleus with bicarbonate (Sucrazit sweetened variant). Evidence for this phenomenon has been demonstrated in the case of oxidoreductase activity within green tea (Ionescu, 2023).
A decrease in the concentration of active forms of riboflavin can be observed when fructose (V7) is used (Figures 6, 7).
The experimental variants V8 and V9 with sorbitol and xylitol showed similar variations of the molecular absorption spectra curves, inducing almost the same effects as the unsweetened variant (V0).

CONCLUSIONS

From analyzing the results of the laboratory experiments and interpreting the values, a number of clear conclusions emerge.
Brown sugar (V2) produce the smallest alterations in the concentration of reduced forms and induces a reduced oxidizable state.
Honey (V3) denatures the sweetened variant the most compared to the control variant (V0).
The use of honey (V3) and saccharin (V4) increases the concentrations of some NAD- and FMN-dependent coenzymes.
The same trends are seen for flavoprotein concentrations (the highest concentrations were found in the V3 variant - honey and the lowest in the V6 variant – Diamond).
The use of fructose (V7) induces a decrease in the concentrations of the active forms of riboflavin.
Sorbitol (V8) and xylitol (V9) produce minimal changes, very close to the control variant.
Although sweetened products benefit from improved sensory qualities, sweetening must be done carefully so that valuable biocompounds are not altered. Consequently, the optimal sweetener is that which generates minimal alterations in the chemical composition, whilst preserving all the advantageous characteristics of green tea. This sweetener is brown sugar (V2).
It is inadvisable to use sweeteners containing saccharin and sodium cyclamate to sweeten green tea. This is because these sweeteners (Diamond, trade name) have been shown to produce very high changes in the activity of flavoprotein.
The development of innovative methods and technologies for the management of food additives facilitates the production of high-quality and safe food end products.

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RESEARCH ON THE VALORISATION OF PLANT-BASED BY-PRODUCTS TO PRODUCE FOOD LINKED TO THE CIRCULAR ECONOMY CONCEPT

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Abstract

Circular economy is a sustainable approach that can help reduce waste, by valorising by-products and other materials for obtaining new products or for obtaining value added compounds. The aim of this study is to present a potential valorisation of various plant by-products through the brewing of Kombucha teas. This approach not only contributes to reducing food waste but also encourages the creation of more sustainable food systems by promoting resource efficiency. The plant-based Kombuchas were prepared separately using grape, lemon, broccoli, green tea and apple by-products. After the required aerobic fermentation process, the obtained samples showed regular pH values (3.3-3.5), Brix values (5.6-10.9), ethanol levels (<3 g/100 mL) and acidity levels (< 0.8% acetic acid). All of the samples, which were sterilised at 75 and 98°C respectively, showed no signs of any bacteria (Enterobacteriaceae and Listeria monocytogenes colonies) or fungi (yeasts and molds colonies). The results showed that discarded plant by-products have the potential to be used to obtain refreshing and natural Kombucha drinks.

Key words*: circular economy, valorisation, food residues, Kombucha tea, plant by-products.*

INTRODUCTION

Food waste is an increasingly serious problem leading to many future negative consequences such as severe environmental degradation, significant economic losses and social inequalities (Peng et al., 2024).

A 2011 report by the Food and Agriculture Organization (FAO) states that approximately one-third of the food produced globally is thrown away or wasted (Gustavsson et al., 2011).

In 2021, members of the United Nations Environment Programme (UNEP) estimate that 931 million tonnes of food, or 17% of the total food available to consumers in 2019, went into the waste bins of households, retailers, restaurants and other food services. According to new UN, research has been conducted to support global efforts to halve food waste by 2030 through the Food Waste Index report (UNEP, 2021).

In this context, the objective of this paper is to highlight the valorisation of agro-food by-products in the context of food preservation especially in the case of fruits and vegetables. This study strongly relates to the premises of circular economy by aiming to show beneficial and healthy alternatives to wasting precious resources.

MATERIALS AND METHODS

Materials

The materials utilized for the research were obtained from various suppliers in the Murcia region. The by-products that were intended to be discarded were: whole grapes, Verna lemon peels, broccoli stems, commercial green tea bags and Jonagold apple peels (a cross between Jonathan and Golden varieties).

To prepare the Kombucha tea, it has been used a liquid which was obtained by homogenizing the waste materials in an industrial mixer and removing the solids and all the polyphenols through an osmotic nanofiltration process. Afterwards, this liquid was boiled at 98°C for 2-3 minutes to sterilize it and let it cool down at a temperature of around 6°C for 2 hours. It is very important that it is left to cool off as to not kill the microorganisms which will be added later on.

Methods and equipment

In order to use the liquid in making tea, it must have a certain amount of sugar in its composition. By using a refractometer (Figure 1), the evolution of the Brix values of the beverages has been determined. These represent the number of solid compounds dissolved in a liquid and are commonly used to measure the dissolved **sugar content** of an aqueous solution.

Figure 1. The Brix Refractometer

The value of one Brix degree is the equivalent of one gram of sucrose in 100 grams of solution and represents the strength of the solution as part of a percentage by weight (% w/w). If the solution contains dissolved solids other than pure sucrose, then the °Bx only approximates the dissolved solid content. (Ryan, 2013)

By adding sugar to enhance the concentration level of the liquid, the Brix value was adjusted between 9 and 12°Bx (Table 1).

Table 1. The added sugar that was needed for each Kombucha tea

Sample	Added sugar (g)	Initial Brix value (°Bx)	Final Brix value (°Bx)
Grape	100	9	11
Lemon	0	12.2	12.2
Broccoli	640	3	10.6
Green Tea	140	0	10.9
Apple	250	3	10.8

During two weeks of incubation, 100 mL of each sample was collected every day under aseptic conditions using a micropipette and plastic sample containers. The analyses resumed two weeks after bottling and consisted in determinations such as: pH value, Brix degrees, total acidity, the amount of ethanol produced, the total *Enterobacteriaceae* colonies, the presence of *Listeria monocytogenes*, yeast and mold colonies, and the total number of lactic acid bacteria.

The **pH values** of all samples were measured every day using a pH meter, after which it was calibrated accordingly with sodium hydroxide (NaOH) representing an alkaline solution, and with hydrochloric acid (HCl) representing an acidic solution respectively. The electrode was kept immersed in the sample until the final result was displayed on the screen of the measuring device.

The **total acidity** values were determined by titrating the distillate sample with a 0.1 N sodium hydroxide (NaOH) solution in the presence of the indicator "phenolphthalein" under continuous stirring. The final values resulted from applying the mathematical formulas (Aveiga, 2024).

The **amount of ethanol** in the samples has been determined using a 785nm excitation laser with a Raman spectroscopy (Lucindo et al., 2023).

The method for the determination of the **total *Enterobacteriaceae* colonies** involves homogenization and serial decimal dilutions to ensure uniform distribution of microorganisms. In a Petri dish, 1 mL + 2% of the sample is inoculated into the culture media using the "lawn" technique with a Drigalski loop. 10-15 mL of pre-tempered Rapid *Enterobacteriaceae* agar is added and mixed thoroughly to allow to solidify. The mix is overlayed with an additional 5-10 mL of the same medium to create semi-anaerobic conditions. After solidification, the plates are inverted and incubated at 37 ± 1°C for 24 ± 2 hours. The colonies are subsequently counted using a magnifying glass on plates with 10 to 300 colonies. The number of total *Enterobacteriaceae* is calculated using the formula:

$$Total\ Enterobacteriaceae = \frac{(No.\ of\ colonies * Dilution\ factor)}{Volume\ of\ liquid\ on\ the\ plate}$$

The final result is measured in CFU ("colony forming units")/mL (Silbernagel, 2002).

The **presence of *Listeria monocytogenes*** determination requires prior homogenization to

ensure uniform distribution of bacteria in the samples. 25 ml of each sample is introduced into a nutrient broth medium enriched with BLEB ("Buffed Listeria Enrichment Broth") to promote the growth and development of *Listeria monocytogenes*. The samples are incubated for 24-48 hours at 30°C. After incubation, a portion of each sample is transferred to another Fraser-type nutrient broth medium. A second incubation is carried out for 24-48 hours at 35-37°C. At the end of the second incubation, the samples are inoculated onto Oxford media containing lithium chloride and antibiotics to inhibit other bacteria, except *Listeria*, whose colonies will appear black coloured. The third and final incubation is carried out at 35-37°C for 24-48 hours. Finally, they are observed under a microscope according to the colour acquired and counted. The measurements are made in numbers of colonies/25 ml (Singh, 2025).

The method for determining **colonies of yeast and molds** requires preliminary homogenization and decimal dilutions for a uniform distribution of microorganisms in the samples. In a Petri dish 1 mL + 2% of the sample is inoculated into the culture media using the "lawn" technique with a Drigalski loop. 10–15 mL of pre-tempered Symphony Agar is added and mixed thoroughly to allow to solidify. After solidification, the plates are inverted and incubated upright at 25°C for 54-72 hours. The colonies are subsequently counted using a magnifying glass on plates with 10 to 150 colonies. The total number of yeast and mold colonies is calculated using the formula:

$$Total\ yeasts\ and\ molds = \frac{(No.\ of\ colonies * Dilution\ factor)}{Volume\ of\ liquid\ on\ the\ plate}$$

The final result is measured in CFU ("colony forming units")/ml (Silbernagel, 2002).

The determination of the **total number of lactic acid bacteria** involves homogenization and serial decimal dilutions to ensure uniform distribution of microorganisms. In a Petri dish, 1 mL + 2% of the sample is inoculated into the culture media using the "lawn" technique with a Drigalski loop. 10-15 mL of selective MRS Agar (de Man, Rogosa and Sharpe) medium. The samples are incubated for 24-72 hours at 30-37°C. Is added and mixed thoroughly to allow to solidify. The mix is overlayed with an additional 5-10 mL of the same medium to create semi-anaerobic conditions. After solidification, the plates are inverted and incubated at 30 ± 1°C for 72 ± 3 hours. The colonies are subsequently counted using a magnifying glass on plates with 10 to 300 colonies. The number of total lactic acid bacteria is calculated using the formula:

$$Total\ lactic\ acid\ bacteria = \frac{(No.\ of\ colonies * Dilution\ factor)}{Volume\ of\ liquid\ on\ the\ plate}$$

The final result is measured in CFU ("colony forming units")/mL (Silbernagel, 2002).

Product preparation

In a glass container it was added the liquid, a starter which is composed of nutrients and over 1 billion live microorganisms, promoting the development of the culture medium but also a SCOBY disc ("symbiotic culture of bacteria and yeast") (Figure 2), where the SCOBY community creates a thick cellulose biofilm and a sparkling acidic broth with the infusion (El-Shall et al., 2023).

Figure 2. The SCOBY disc

The mixture is covered with a filter which allows both maintaining the sterility of the medium and its access to oxygen which is needed for the aerobic fermentation. The containers were stored in a dry and dark place at an ambient temperature.

After the 2 designated weeks for fermentation, the teas were divided into 3 distinct categories, each with the same amount of liquid. The first category was the blank sample to which no modification was applied. The second category was subjected to sterilisation by boiling for 1 minute at a temperature of 75°C, and the third at a temperature of 98°C (Figure 3).

Figure 3. Grape Kombucha tea bottles at the three boiling temperatures

Comparison of the samples with commercial Kombucha tea

To make a proper comparison with the commercial products, a bottle of lemon Kombucha tea from a supermarket has been subjected to the same set of analyses as the samples prepared in the laboratory. Being lemon tea, the results were compared to those of the one sterilised at 98°C in the laboratory.

After the release of the microbiological analysis results, a sensorial examination was carried out on the samples sterilized at the 98°C temperature, and compared with the commercial lemon sample. The examination followed 3 sensory parameters, namely the taste, colour, and smell of the samples, as well as the global value represented by the average of the 3 parameters. The analysis was carried out on a group of approximately 50 untrained consumers, part of Murcia's Technological Center staff. The subjects appreciated each category with grades on a 5-point hedonic scale.

RESULTS AND DISCUSSIONS

After the first two weeks, due to the added microorganisms through the starter solution but also with the help of the optimal growth and development conditions created, through the aerobic fermentation process, the SCOBY disc grew on the surface of the liquids forming a gelatinous film around it with the role of ensuring a much more stable connection with the oxygen they need to survive (Figures 4 and 5).

Figure 4. The Grape Kombucha tea freshly brewed on day 0

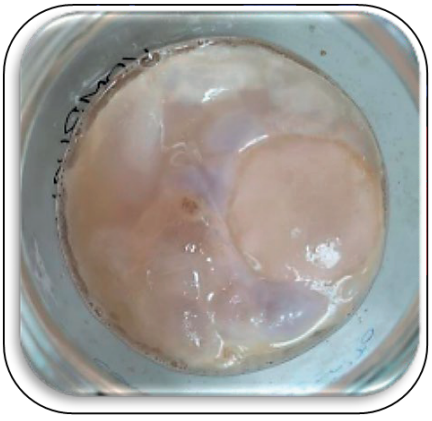

Figure 5. The Grape Kombucha tea on the 15th day of fermentation

During the fermentation process, the acidity level of the samples increased, achieving a lower pH, which can also be observed by the formation of gas bubbles in the sample jars, with the lids slightly bulging from the accumulated pressure (Figure 6), due to the increasing level of carbon dioxide (CO_2) which also holds the SCOBY disc at the surface of the liquid throughout the entire process.

Figure 6. Lemon Kombucha sample on the 10th day of fermentation

After the initial fermentation timeframe of 15 days, both the SCOBY disc and other solid particles were removed and the liquid was poured into the bottles.

The following data has been obtained from the course of various determination methods during a total period of 5 weeks starting at the very beginning of the fermentation process (day 0) and towards the end of the 3 weeks of bottling (day 35).

Over time, the pH values of most samples had a favourable evolution. Due to the fermentation process, the acidity level in the media increased, resulting in a significant decrease in pH (Figure 7).

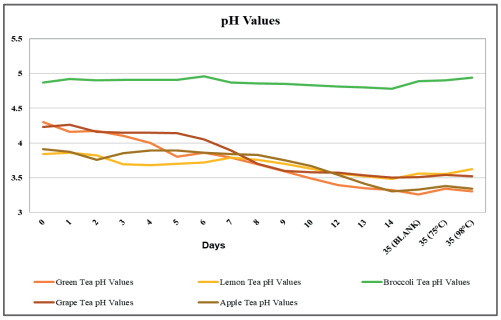

Figure 7. Evolution of pH levels throughout the fermentation period

Green, grape and apple teas showed the largest variations in 2 weeks, with values from 4.3 to 3.32, 4.23 to 3.5 and 3.91 to 3.3, respectively. These values are similar to other studies (Barbosa et al., 2021; Morales et al., 2023; Zubaidah et al., 2018). In their research, after 2 weeks of fermentation the pH values were: 3.8 for green tea (Barbosa et al., 2021), 2.8 for grape tea (Morales et al., 2023) and 3.00 for "Red Delicious" apple tea (Zubaidah et al., 2018). However, the evolution of the Broccoli Kombucha Tea proved to be problematic, because the fermentation process didn't occur. The explanation could be that, compared to the other samples, broccoli being a vegetable instead of a fruit, meant that its sugar content was too low to start a fermentation, requiring additional amounts to kick-start the process. As such, for future experiments, sugar must be added in order to have a proper fermentation.

The Brix values of the samples decreased during the fermentation, subsequently increasing back to their initial values for some of them (Figure 8).

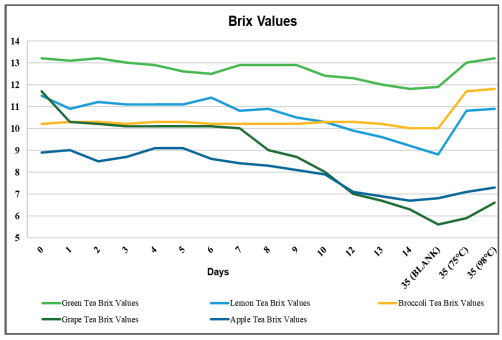

Figure 8. Evolution of Brix values throughout the fermentation period

For the lemon tea with values from 11.5° on day 0 to 10.9° on day 35, and for the green tea from 13.2° on day 0 to 13.2° on day 35. The largest decrease was observed in the grape tea, which from the initial value of 11.7° reached 5.6° for the blank sample after bottling. In regards to the apple teas evolution from 8.9° initially to 6.7° after 2 weeks, the results correlate to Zubaidah et al. (2018) once more, having achieved 6.00° for "Red Delicious" apple tea during the 14 days of fermentation.

The total acidity levels had a relatively slow initial increase during the first week of preparation (Figure 9).

Green tea, apple tea, and lemon tea had significantly different evolutions from each other during the fermentation period but reached similar values after bottling. The green tea had a relatively fast peace, increasing from 0.07% acetic acid to 0.16% acetic acid in seven

fermentation days. The result is similar to that of Tu (2008) where the green tea had 0.12% acetic acid in a single week.

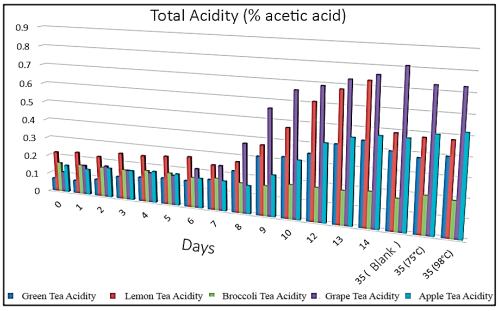

Figure 9. Total Acidity of the samples

Ethanol production was measured after a preliminary fermentation period of approximately 4-5 days. These values increased significantly up to 3 g/100 mL initially but decreased slightly with a maximum level of 1.5 g/mL lost after bottling (Figure 10).

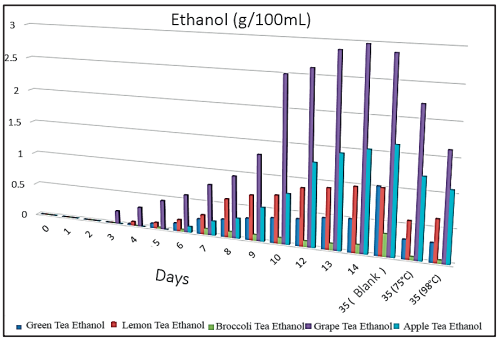

Figure 10. Ethanol production chart

The quick increase of the green tea from 0.02 g/100 mL on day 4 to 0.5 g/100 mL on day 14 is similar to the green tea of Barbosa et al. (2021) which had a total of 0.7 g/100 mL during the two weeks period.

As for the microbiological determinations, both for the presence of *Listeria monocytogenes* and the formation of *Enterobacteriaceae* colonies, the results indicated no signs of either of them in any of the analysed samples.

The formation of yeast and mold colonies was analysed after bottling the 5 types of teas. For the thermally treated samples, insignificant values of viable yeast and mold were detected, namely < 1 cfu/mL. As for the blank samples, the highest quantity was detected in the lemon tea, reaching a value of 780,000 cfu/mL (Figure 11).

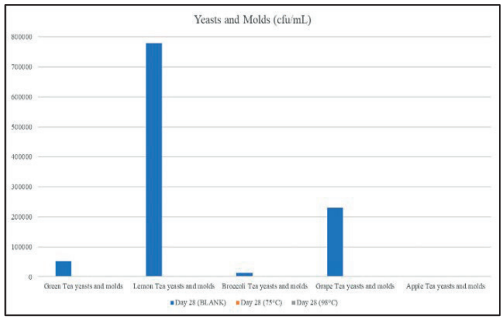

Figure 11. Total yeast and molds CFU before and after sterilization

Similar to the analyses performed to detect the formation of yeast and molds, for determining the total lactic acid bacteria, the samples subjected to boiling had insignificant values of < 1 cfu/mL. The most significant value was found in the grape tea, producing quantities of up to 2,400,000 cfu/mL of total lactic acid bacteria (Figure 12).

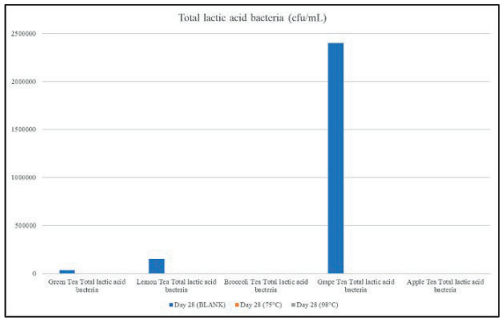

Figure 12. Total Lactic Acid Bacteria CFU before and after sterilization

The commercial lemon Kombucha tea was analysed similar to the sample teas obtained in the laboratory and the results were compared with the laboratory lemon tea sterilised at 98°C (Table 2).

The pH and Brix values were relatively similar, but the total acidity and ethanol values differ significantly indicating the presence of a possible acidity enhancer or different sweeteners that give the drink a more refreshing taste and a longer shelf life than in the case of the laboratory teas subjected to traditional and more natural preparation methods.

Table 2. Comparison between commercial lemon Kombucha tea and laboratory lemon Kombucha tea sterilised at 98°C

	Commercial lemon Kombucha tea	Laboratory lemon Kombucha tea
pH	3.8	3.62
Brix Values (°)	9.7	10.9
Total Acidity (% acetic acid)	0.7	0.48
Ethanol (g/100 mL)	1.82	0.64
Total *Enterobacteriaceae* (cfu/mL)	<1	<1
Listeria monocytogenes (/25 mL)	0	0
Yeast and Molds (cfu/mL)	<1	<1
Total Lactic Acid Bacteria (cfu/mL)	<1	<1

In addition, all the sample teas sterilized at 98°C and the commercial lemon Kombucha tea were subjected to a sensory analysis carried out with a panel of 50 untrained consumers. The examination followed 3 sensory parameters, namely taste, colour, and smell of samples, as well as the global value represented by the average of the 3 parameters. The subjects appreciated each category with grades on a 5-point hedonic scale. The results indicated that apple tea was the most overall appreciated having all 3 of the parameters with the most points compared to the others with a score of 4.23 points and the grape tea obtaining the second position with a score of 3.47 points (Table 3).

Table 3. Organoleptic examination results

Sample	Parameter	Result
Grape Kombucha	Smell	2.23
	Appearance	3.98
	Taste	4.22
	Global value	**3.47**
Lemon Kombucha	Smell	3.23
	Appearance	3.77
	Taste	2.92
	Global value	**3.30**
Broccoli Kombucha	Smell	1.88
	Appearance	3.13
	Taste	2.09
	Global value	**2.36**
Green Tea Kombucha	Smell	2.29
	Appearance	3.46
	Taste	4.08
	Global value	**3.27**
Apple Kombucha	Smell	4.01
	Appearance	4.12
	Taste	4.56
	Global value	**4.23**
Lemon Kombucha (commercial)	Smell	3.31
	Appearance	3.69
	Taste	2.92
	Global value	**3.30**

Regarding the other teas, except for Broccoli tea, they all achieved similar results with the commercial lemon Kombucha tea.

CONCLUSIONS

The results obtained indicate that the Kombucha samples represent an ideal example for highlighting the potential of a circular economy approach in the food and beverages sector. By providing them with the optimal growth and development conditions specific to an aerobic fermentation process, refreshing and natural drinks from food waste can be successfully obtained. The best results were registered for grape and apple teas, which were highlighted by their chemical and microbiological descriptors, as well as the overall organoleptic parameters (from the slight acidity to the sweet aroma specific for the fruit sample).

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GLOBAL TRENDS AND RESEARCH EVOLUTION IN FLOUR AND BAKERY PRODUCT FORTIFICATION: A BIBLIOMETRIC ANALYSIS

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Abstract

This Scopus-based bibliometric study evaluates global trends and the impact of wheat and bakery product fortification on public health. The search query (flour AND fortification) OR (bakery AND products AND fortification*) yielded 1,917 articles, from which 1,239 English-language publications were selected for in-depth analysis in the domains of Agricultural and Biological Sciences and Medicine. Publications date back to 1942, although the volume of papers surged markedly post-2000. The most pertinent sources were Food and Nutrition Bulletin, Food, and Food Chemistry. Most authors were associated with institutions in the USA, India, and Brazil, whereas the most frequently cited publications were produced in the USA, Canada, and India. Frequently identified key words in abstracts included "flour", "female", "article", "human", and "folic acid". Trend analysis since 2002 revealed a rapid diversification of research topics, reflecting the dynamic evolution of fortification research. Although articles span eight decades, the and most impactful research directions emerged after 2014. In conclusion, this bibliometric analysis evaluates global trends, key research areas, and the impact of flour and bakery product fortification.*

Key words*: flour, female, article, human, folic acid.*

INTRODUCTION

Biotechnologies represent an interdisciplinary field at the intersection of life sciences, engineering, and technology, with significant applications in agriculture, the food industry, and medicine. In the food sector, biotechnologies contribute significantly to improving the nutritional value and functional properties of products. One of the essential techniques for enhancing the nutritional quality of foods is fortification (Chadare et al., 2019; Echegaray et al., 2020).

Food fortification is a fundamental biotechnological method that helps combat nutritional deficiencies by adding vitamins, minerals, or bioactive compounds to staple foods (e.g., bakery products). This approach plays an important role in vulnerable populations and public health policies (Crider et al., 2022; Olson et al., 2021; Keats et al., 2021).

Scientific literature in the field of biotechnologies applied to fortification is continuously evolving, with diverse studies covering both fundamental aspects and practical applications (Klassen, 2020). Although the first published data in this field date back to the year 1951 (Rosenberg & Rohdenburg, 1951), the number of articles addressing fortification strategies for bakery products and their impact on human health has increased significantly since 2014. Initially, fortification focused on essential vitamins and minerals, such as iron and folic acid, to combat nutritional deficiencies (Kaur et al., 2022). Later, the beneficial effects of fiber- and protein-enriched products began to be explored. The large number of studies in the field of food product fortification has led to the development of review papers (Vieth, 2020; Das et al., 2019), systematic reviews, meta-analyses (Antonic et al., 2020; Tam et al., 2020), and bibliometric analyses.

Bibliometric analysis is an essential tool for exploring and synthesizing information to gain an overview of the scientific impact and evolution of food fortification biotechnology. The use of quantitative methods and advanced data visualization allows the identification of the most influential works in the field, key associated concepts, the most prolific authors, and the journals that publish the most articles on the topic.

Objective:

The aim of this study is to conduct a bibliometric analysis of the scientific literature on the fortification of bakery products.

MATERIALS AND METHODS

For this bibliometric analysis, the Scopus database was queried, and studies on bakery product fortification published in the last 10 years were selected. The syntax from which the study started was: [(TITLE-ABS-KEY (flour AND fortification*) OR TITLE-ABS-KEY (bakery AND products AND fortification*) AND (LIMIT-TO (SRCTYPE, "j")) AND (LIMIT-TO (PUBSTAGE, "final")) AND (LIMIT-TO (SUBJAREA,"AGRI")) OR LIMIT-TO (SUBJAREA, "MEDI")) AND (LIMIT-TO (DOCTYPE, "ar")) AND (LIMIT-TO (LANGUAGE, "English")). After a more in-depth analysis, the syntax was adapted and modified to obtain a database more suitable for the research topic.

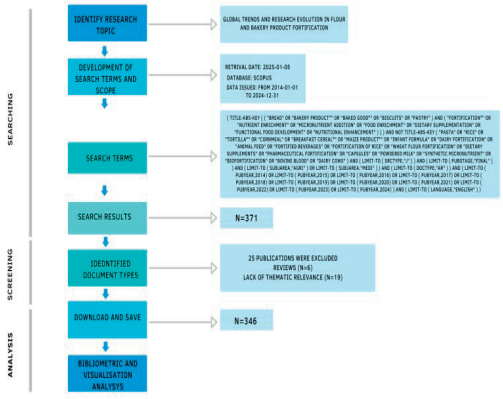

Figure 1. Research Methodology for the Bibliometric Study

The search syntax used in the database was designed to be as precise as possible for the field of interest.

(TITLE-ABS-KEY ((("bread" OR "bakery product*" OR "baked good*" OR "biscuits" OR "pastry") AND ("fortification*" OR "nutrient enrichment" OR "micronutrient addition" OR "food enrichment" OR "dietary supplementation" OR "functional food development" OR "nutritional enhancement"))) AND NOT TITLE-ABS-KEY ("pasta" OR "rice" OR "tortilla*" OR "cornmeal" OR "breakfast cereal*" OR "maize product*" OR "infant formula" OR "dairy fortification" OR "animal feed" OR "fortified beverages" OR "fortification of rice" OR "wheat flour fortification" OR "dietary supplements" OR "pharmaceutical fortification" OR "capsules" OR "powdered milk" OR "synthetic micronutrient" OR "biofortification" OR "bovine blood" OR "dairy cows") AND (LIMIT-TO(SRCTYPE,"j")) AND (LIMIT-TO (PUBSTAGE, "final")) AND (LIMIT-TO (SUBJAREA, "AGRI")) OR LIMIT-TO (SUBJAREA, "MEDI")) AND (LIMIT-TO (DOCTYPE, "ar")) AND (LIMIT-TO (PUBYEAR, 2014)) OR LIMIT-TO (PUBYEAR, 2015)) OR LIMIT-TO (PUBYEAR, 2016)) OR LIMIT-TO (PUBYEAR, 2017)) OR LIMIT-TO (PUBYEAR, 2018)) OR LIMIT-TO (PUBYEAR, 2019)) OR LIMIT-TO (PUBYEAR, 2020)) OR LIMIT-TO (PUBYEAR, 2021)) OR LIMIT-TO (PUBYEAR, 2022)) OR LIMIT-TO (PUBYEAR, 2023)) OR LIMIT-TO (PUBYEAR, 2024)) AND (LIMIT-TO (LANGUAGE, "English")).

The fields of agriculture, environmental sciences, medicine, and nutrition were included (Figure 1).

To analyze and visually represent keyword networks, relationships between terms, authors, sources, and clusters in the field of bakery product fortification, the VOSviewer software (version 1.6.20, Van & Waltman, 2011) was used. After uploading the database and the thesaurus text file, the image was exported from VOSviewer.

For the quantitative analysis of the scientific literature (e.g., publication trends, high-impact journals, and the most influential authors), the Bibliometrix package in R (version 4.3.2) was used.

RESULTS AND DISCUSSIONS

This bibliometric study analyzes research trends, influential authors, relevant publications, and emerging directions in the field of bakery product fortification, highlighting existing gaps and future research opportunities. In this bibliometric analysis, data was collected from Scopus, one of the most extensive scientific databases, ensuring a relevant and up-to-date source for evaluating research trends in bakery product fortification. Given the significant increase in research interest over the last decade, the search focused on scientific articles written between 2014 and 2024. To obtain the most relevant studies on bakery product fortification and to exclude topics unrelated to the study's objective, multiple selection criteria were applied. Specific key words relevant to bakery products and nutritional fortification were used through the "AND" and "OR" operators, while irrelevant terms were excluded using the "AND NOT" filter.

The VOSviewer platform was used for analyzing and visualizing bibliometric data. After uploading the database into the platform, Figure 2 was generated, displaying a density visualization of key words in the scientific literature related to bakery product fortification. Red and yellow areas indicate highly mentioned topics, while other regions contain less frequent but still relevant concepts.

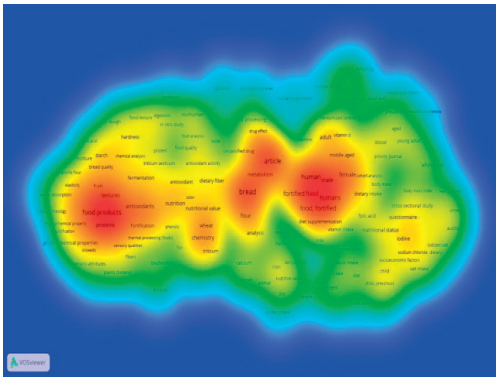

Figure 2. Key word Density Map in VOSviewer

The presented image includes 206 terms, each mentioned at least five times in the analyzed literature. These terms were grouped into four distinct clusters. Cluster 1 reflects research on the impact of fortification on the biochemical composition, physicochemical properties, and sensory quality of bakery products. Studies in this category focus on technological processes to obtain fortified products with improved nutritional value. Cluster 2 highlights the effects of food fortification on human health. Cluster 3 emphasizes the role of micronutrients in the diet of different population groups. Cluster 4 focuses on the impact of fortification through clinical studies, nutrient absorption, and optimization of fortification strategies for bakery products.

Repeating the analysis in VOSviewer, with the thesaurus dictionary, there were identified 161 terms that were mentioned at least five times in the analyzed literature (Figure 3).

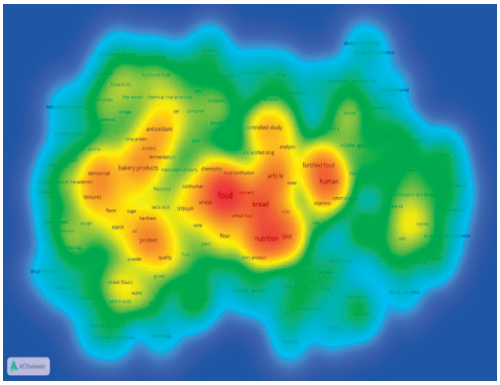

Figure 3. Optimized Key word Density Map after Thesaurus application

These terms were grouped into three major clusters, containing 89, 47, and 25 terms. Cluster 1 reflects research on the impact of fortification on the nutritional composition, physicochemical properties, and acceptability of bakery products. Studies in this cluster focus on optimizing technological ingredients to obtain fortified products with high nutritional value. Cluster 2 highlights the importance of fortification in preventing nutritional deficiencies and its impact on human health; the terms in this cluster reflect findings from clinical studies. Cluster 3 emphasizes the relationship between micronutrients used in bakery product fortification and their impact on public health.

The main items in this cluster focus on mineral and vitamin absorption and their biological effects.

The analysis of the two images revealed that there are common clusters of ideas/terms, as well as distinct clusters. In both cases, the first cluster includes details about the impact of fortification on biochemical composition. The second cluster is also similar, it is focused on the importance of fortification and its impact on human health. The third cluster shares similarities in highlighting the role of micronutrients. The major difference appears in the fourth cluster, which is present only in one of the images and focuses on clinical studies and the effectiveness of fortification strategies.

For a more detailed perspective on the bibliometric analysis, the Bibliometrix platform was used.

After uploading the database into the platform, a series of relevant images were exported for this analysis. The WordCloud obtained illustrates the most frequently used terms in the scientific literature analyzed on bakery product fortification (Figure 4). The largest and most visible words (bread, fortified, food, female, human, article, flour) indicate the terms with the highest frequency in the analyzed database. Terms such as "fortified food" and "iodine" highlight the importance of micronutrient fortification in research. Additionally, the presence of "female" and "male" suggests differences in how fortification impacts different genders.

Figure 4. Word Cloud Representation of Key Terms in Bibliometrix

The analysis of the Co-occurrence Network image in Bibliometrix (Figure 5) reveals the relationships between the central concepts in the analyzed literature.

The two major clusters, colored blue and red, indicate different but interconnected research areas. The blue cluster focuses on the impact of bakery product fortification on human health, emphasizing gender differences and clinical studies. The red cluster is oriented towards biotechnological and compositional aspects of fortification, analyzing the physicochemical and nutritional properties of fortified bakery products.

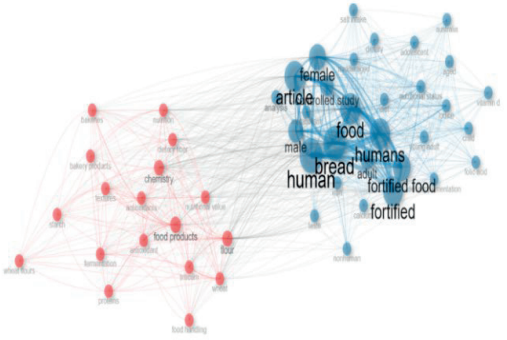

Figure 5. Co-occurrence Network of Key words in Bibliometrix

Figure 6 represents the thematic evolution of research in the field of bakery product fortification over the years 2014-2024.

The lines connecting the terms indicate the continuity and transformation of topics over the years. From 2014 to 2016, the research focused on bakeries, bakery products, fatty acids, food products, and controlled study, which shows an interest in the nutritional characteristics of bakery products and their technological properties. During 2017-2018, studies placed greater emphasis on terms such as bread, human, article, and yeast, which marks a transition towards human health. Between 2019-2020, interest appeared in terms such as female, textures, proteins, and wheat flours, suggesting the emergence of the importance of sensory and nutritional aspects, as well as gender differences in the consumption of fortified foods.

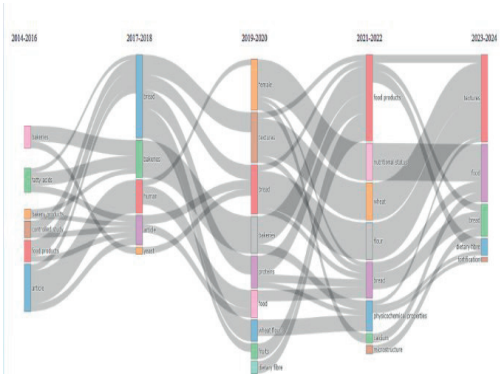

Figure 6. Thematic Evolution of Research on Bakery Production Fortification

Over 2021-2022, the increased interest in nutritional status, physicochemical properties, wheat, and calcium highlights a focus on the impact of fortification on nutritional status and product properties. During 2023-2024, the highlighted terms textures, food, bread, dietary fiber, and fortification continue to emphasize the improvement of structure, nutritional value, and ingredient optimization.

Figure 7 presents the most relevant authors in the field of bakery product fortification, based on the number of documents published in the analyzed literature. Swieca M. is the author with the highest number of publications (8 documents), followed by Dziki D. and Gawlik-Dziki U., each with 7 publications. These researchers are important for the field of bakery product fortification.

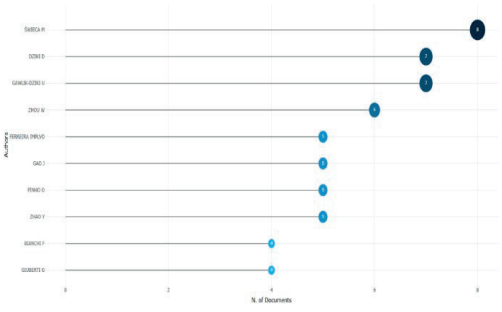

Figure 7. Most Relevant Authors in Bakery Product Fortification Research

The authors in this image have between 4 and 8 publications, indicating that the field has active researchers. This analysis helps in identifying influential researchers, which is useful for analyzing research trends and reviewing the literature.

CONCLUSIONS

This bibliometric analysis provided an insight into the evolution of research in the field of bakery product fortification.

The study highlighted the most important scientific trends, influential authors, and collaboration networks identified in the specialized literature.

The results showed that, starting from 2019, there has been an increase in the diversity and development of the fortification field. Through visual analysis, between three and four major clusters were identified, focusing on the impact of fortification on biochemical composition and sensory quality, the effects of fortification on human health, and the role of micronutrients in nutrition.

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THE LATEST DEVELOPMENT OF RADIO-FREQUENCY APPLICATIONS IN THE FOOD INDUSTRY: ADVANTAGES AND DRAWBACKS

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Abstract

The quest for innovative processing solutions that meet both food safety and quality standards and sustainability goals (in terms of processing, preservation, and consumption) has led to a growing interest in technologies like radiofrequency (RF) technology. Although the mechanisms behind this technology have been thoroughly explained and are well-documented in the literature, and its advantages for industrial applications are recognized in several studies, the widespread adoption of RF technology for various agri-food products thermic treatment, remains limited due to unsolved issues. One of the main challenges in applying RF technology on a large scale is the need to adapt equipment and technology according to the specific characteristics of the product (e.g. raw material, finished product, liquid, semi-liquid, solid, packaged, unpackaged) and the intended purpose (e.g., processing, preservation, sanitization). This paper aims to review recent findings in this area, highlighting both the benefits and challenges of RF technology application for different food products thermal processing. Addressing these challenges requires continued scientific research in various directions within this field.

Key words*: benefits and challenges, food processing, RF technology.*

INTRODUCTION

Radiofrequency (RF) technology is an emerging and effective approach for food processing. It operates on the principle of capacitive dielectric heating, enabling rapid and uniform heating, even in solid food products (Lu et al., 2007; Piyasena et al., 2003; Y. Zhao et al., 2000).

Radio frequency (RF) technology utilizes electromagnetic waves within the 3 kHz to 300 MHz range to generate heat in food products (Altemimi et al., 2019; Radhakrishnan, 2013; Sun et al., 2023; 2025; Zeng et al., 2022). It is extensively used in the food industry for applications such as drying, thawing, and pest control (Alfaifi et al., 2014; Farag et al., 2011; Gao et al., 2010; Jumah, 2005; Kocadağlı et al., 2012; Wang et al., 2014). Studies indicate that RF technology has potential for pasteurizing and sterilizing packaged foods (Cui et al., 2023; Lara et al., 2022). However, a major challenge with RF heating is the tendency for overheating at the edges and corners of food samples (Jiao et al., 2015). RF technology is also applied to the control of microorganisms, especially fungi that contaminate food products (Berni and Brutti, 2023).

In RF heating, a high-voltage alternating current (AC) is applied to parallel electrodes configured as a capacitor, generating electromagnetic waves at a frequency of 27.12 MHz - significantly higher than those used in ohmic and pulsed electric field (PEF) treatments (Costa & Marra, 2024; Dong et al., 2021; Li et al., 2024; Ozturk et al., 2020). Unlike microwave heating, where waves are dispersed by a magnetron within a cavity, RF heating directly immerses food between the electrodes. This capacitive nature, combined with the longer and more penetrating wavelengths of RF waves, ensures a more uniform heating process compared to microwave technology (Zhao et al., 2000).

Thermal processing is widely used for pasteurization and decontamination to improve food safety and extend shelf life. However, this method affects the physical and technological properties of food, primarily due to alterations in gluten structure and starch configuration. (Gelinas & McKinnon, 2004; Neill et al., 2012; Sun et al., 2014).

Researchers have extensively investigated RF heating for processing flours and powders. For instance, studies have examined RF heating's effects on wheat (Kim et al., 2004), corn (Ozturk et al., 2017), and potato flours (Zhu et al., 2021), as well as its use in pasteurizing wheat flour (Boreddy et al., 2019; Liu et al., 2018b). Other work has explored RF treatment of spice powders like red pepper (Choi et al., 2018; Jiao et al., 2019; Zhang et al., 2020), paprika and cumin (Ozturk et al., 2017), alongside applications in infant formula (Zhang Zhu et al., 2020) and egg white powder (Kar et al., 2020). More recently, the effects of superheated steam on wheat flour properties have also been studied (Ma et al., 2021).

RF heating offers several additional benefits, including improved energy efficiency through volumetric heating, which minimizes energy waste. Its compact design reduces the required production floor space, making it ideal for facilities with limited room. Furthermore, RF technology is highly compatible with automated production systems, supporting both batch and continuous flow processing, thereby enhancing operational efficiency and scalability (Manzocco et al., 2008).

RF HEATING APPLICATIONS IN BAKERY INDUSTRY

Zhang et al. (2025) demonstrated that strategically positioning RF wave transmission holes along the metal susceptor's long sides, particularly near colder areas, significantly improved heating uniformity, speed, and energy efficiency. The researchers found that the energy transmission area was crucial for overall efficiency. Simulations confirmed that the ideal hole diameter increased with the sample's length-width ratio and height, but exceeding a certain size reduced electric field concentration. Optimized susceptor designs boosted energy efficiency by approximately 10%, with specific configurations reaching 15% and 18% improvements for different susceptor sizes. Furthermore, the study established a precise relationship between sample dimensions and optimal hole diameter, with minimal error. These results provide vital guidance for enhancing RF heating efficiency in metallized food packaging for industrial applications. Radio frequency (RF) heating presents a promising approach for food preservation and extending the shelf life of food products.

In their study, Mitelut et al. (2015) inoculated rye bread with three food spoilage fungi - *Aspergillus flavus*, *Penicillium expansum*, and *Fusarium graminearum* - before undergoing RF technology at varying temperatures (60°C, 80°C, and 100°C) to evaluate its effectiveness in inhibiting fungal growth. The results indicated that RF technology successfully inhibited fungal development on the surface of rye bread, demonstrating its potential as an effective method for controlling bread spoilage fungi.

RF drying is widely used after baking in the production of cookies, crackers, cereals, and snacks (McHugh, 2016). Traditional drying methods often create moisture gradients within these foods, leading to surface cracks. RF drying addresses this issue by delivering energy uniformly, reducing moisture differences and preventing cracks. Additionally, this method prevents flavour degradation and discoloration caused by excessive heat buildup.

RF drying also enhances production efficiency by increasing conveyor speed, stabilizing moisture levels, lowering drying temperatures, and minimizing surface marks from processing equipment (Palamthodi et al., 2021). Research by Anese et al. (2008) examined the impact of RF heating on acrylamide formation in baked goods. Their findings indicated that incorporating RF heating in the final baking stages, particularly when residual moisture levels were still high (around 10%), helped maintain low acrylamide levels. This suggests that combining conventional hot air baking with RF heating can be an effective approach to improving both product quality and food safety (Costa & Marra, 2024).

RF HEATING APPLICATIONS IN MEAT AND FISH INDUSTRY

To minimize quality degradation and flavour loss associated with autoclaving, a pickering emulsion (ZS) was formulated using zinc oxide nanoparticles (ZnO NPs) loaded with star anise essential oil (SAEO) to treat cooked tiger skin chicken feet. This emulsion was then combined with radiofrequency (RF) technology for pasteurization, ensuring enhanced preservation of quality and sensory attributes. The experimental results conducted by indicate that ZSRF pasteurization effectively keeps microbial levels in chicken foot samples low, outperforming the combined treatment of ZnO and RF. Compared to conventional high-pressure sterilization (HPS), this combined pasteurization approach minimizes flavour loss and the development of undesirable flavours while also regulating colour changes and preventing lipid and protein oxidation (Wei et al., 2025).

The dielectric properties of lean beef were analysed across different frequencies and temperatures, ranging from frozen to unfrozen states, to better understand its behaviour during RF heating (Bedane et al., 2017). Researchers conducted experiments to thaw lean beef using radio frequency (RF) energy, testing both stationary and continuous processes. In stationary setups, the distance between electrodes affected how evenly the meat heated. In continuous thawing using a conveyor belt, both electrode spacing and belt speed were critical factors. Notably, the moving conveyor system resulted in slightly more uniform heating. The data from these experiments can be used to develop models and simulations that optimize RF thawing parameters (Bedane et al., 2017).

Beef sausage heated using superheated water (SW)-assisted RF technology exhibited enhanced quality attributes, such as improved texture, colour, and microstructure, compared to those heated with SW alone. These enhancements were due to the shorter heating duration and more uniform temperature distribution achieved with RF technology. Consequently, this research suggests that SW-assisted RF technology could be a rapid and efficient method for safely and effectively heating various food products (Wang et al., 2024).

Cao et al. (2021) investigated using a combination of hot air and radio frequency heating (RF-HA) to dry tilapia fillets. Their aim was to achieve faster, more even drying and improve the fillet's quality. They tested different fillet thicknesses and examined the impact of wrapping the fillets with skin or gauze.

The results showed that RF-HA drying significantly speed up the drying process compared to just hot air, with drying rates increasing by 1.1 to 1.4 times. Fillets dried with RF-HA rehydrated better and shrank less, especially the thicker ones.

RF HEATING APPLICATIONS IN MILK PROCESSING INDUSTRY

The superior thermal efficiency and enhanced heating uniformity of radio frequency (RF) and microwave processes have been shown to effectively achieve comparable or improved bacterial and enzymatic inactivation in liquid and semi-solid foods. Additionally, these methods help preserve the sensory and nutritional quality of fresh products more effectively than conventional pasteurization (Abea et al., 2023).

In their study, Tonti et al. (2024) explored the influence of two intrinsic food factors namely fat content and matrix structure, on the RF inactivation of *S. typhimurium* and *L. monocytogenes* in dairy model systems. Regarding fat content, the effect on microbial inactivation varied depending on the system type and microorganisms. However, there was a general trend where increased fat content (5.9%) led to higher inactivation (greater “k” values, where “k” is defined as inactivation rate constant according to Arrhenius relation) at elevated temperatures (above 70°C). This phenomenon was attributed to the faster dielectric heating of fat compared to water, potentially exposing microorganisms to higher temperatures at the microscale when fat content is higher. For matrix structure, the k value was typically higher in gel matrices than in liquid matrices, which is contrary to the behaviour observed with conventional heating. This effect was believed to be linked to the direct heating

of microorganisms by RF and the slower dissipation of heat in gel matrices (Zhang H. et al., 2025).

RF HEATING APPLICATIONS IN THE FRUIT AND VEGETABLE PROCESSING INDUSTRY

Pang et al. (2024) studied the impact of RF heating on polyphenol oxidase (PPO) activity by applying four kinetic models, with the Logistic and Bi-phasic models demonstrating the best fit. The study revealed that RF heating modified the secondary and tertiary structure of mushroom PPO, decreasing α-helix content while increasing surface hydrophobicity. Spectral analysis across different RF heating temperatures and durations showed that both factors influenced PPO inactivation. A redshift in maximum fluorescence intensity was observed as heating time and temperature increased, indicating structural disruption, exposure of hydrophobic groups, and enhanced surface hydrophobicity. These structural alterations were directly correlated with changes in PPO activity. Additionally, colour test results demonstrated that RF heating effectively inhibited mushroom browning. By correlating enzyme activity with structural modifications and establishing a kinetic model for PPO inactivation, this study provides a theoretical foundation for future applications in food processing.

The research conducted by Ranjan et al. (2024) highlight the effectiveness of radiofrequency cold plasma (RF-CP) treatment in significantly reducing microbiological contamination in cashew nuts while preserving their sensory and nutritional properties. The study revealed that a plasma mixture of N_2 (70%) and O_2 (30%) was more effective in microbial decontamination than argon (Ar) used in percent by 70% and mixed with O_2 (30%), requiring lower power and shorter processing times to achieve the desired results. These findings suggest that cold plasma technology is a promising and innovative approach for microbial reduction in cashew nuts without compromising their quality.

Novel-assisted RF technology enhances the drying rate of ready-to-use food products while preserving heat-sensitive compounds. For instance, applying a hot air assisted radio frequency (HA-RF) for carrot slices drying, it can reduce drying time by 30% compared to hot air drying alone, with the final samples exhibiting acceptable colour, rehydration properties, and the highest total carotenoid levels (Gong et al., 2020). Du et al. (2024) applied a conjugated nisin-assisted RF technology to pre-treated carrots, resulting in significant retention of carotenoids and ascorbic acid, effective inactivation of *Staphylococcus aureus*, *Bacillus subtilis*, *E. coli*, and an extension of shelf life to 42 days. Additionally, when compared to hot water blanching, HA-RF-treated broccoli preserved better texture, bioactive compounds, and microstructure. The ascorbic acid, sulforaphane, and total glucosinolate levels in HA-RF samples were 251.1%, 131.9%, and 36.7% higher, respectively, than those in hot water blanched samples (Qing et al., 2023).

In a study, Cui et al. (2021) applied RF heating during the reheating of soymilk for packed tofu production. Computer simulations revealed that the electric field became distorted and deviated in sharp areas, leading to overheating at the edges and corners of the material. Consequently, a cylindrical container (50 mm in diameter, 100 mm in height) with minimal sharp areas was found to be the most suitable for RF heating of soymilk, as confirmed by experimental results. Additionally, packed tofu heated using RF technology at 80°C for over 20 minutes demonstrated improved texture properties, enhanced odour and taste values, and a denser network structure compared to commercially available packed tofu. These findings suggest that RF-heated packed tofu has strong consumer acceptance potential. RF heating could serve as an alternative to conventional heat treatment in packed tofu production, offering a promising approach for improved quality and efficiency.

As an alternative method, in-package RF processing was assessed for its effectiveness in inactivating *Salmonella* on black peppercorns and dried basil leaves, as well as preventing cross-contamination during storage after processing. In-package steaming involves heating the samples inside a steam vent package, which generates and retains steam throughout the treatment. This approach

ensured uniform heating, likely due to steam circulation within the package. A one-way steam vent allowed excess steam to escape once a certain pressure threshold was reached and then returned to its original position to reseal the package once RF energy was removed. In-package RF steaming of black peppercorns and dried basil leaves for 135 seconds and 40 seconds, respectively, resulted in more than a 5-log reduction of Salmonella. After treatment, the steam vent remained intact and effectively sealed the package, protecting the product from external contamination (Wason et al., 2024).

Ballom et al. (2021) developed a thermal process using a pilot-scale radio frequency (RF) unit to inactivate *Salmonella* and *Listeria monocytogenes* in cocoa powder. To ensure the effectiveness of radio frequency (RF) heating for sanitizing cocoa powder, researchers tested it against two harmless bacteria, *Enterococcus faecium* and *Listeria innocua*. Heating cocoa powder to 90°C reduced *E. faecium* by approximately 1.8 log. Holding the heated powder in an insulated container for 48 minutes further decreased bacteria levels by 3.65 log. *L. innocua* proved more susceptible, showing a 5.64 log reduction after heating to 75°C followed by a shorter insulated holding period. The study concluded that *E. faecium* is a reliable indicator for assessing the process's ability to eliminate harmful pathogens like *Salmonella* and *L. monocytogenes*. Insulating the cocoa powder after RF heating is highly recommended for better microbial reduction and energy savings.

In Table 1 is presented an overview of the significant results obtained by RF heating applications in different food products treatments. These results demonstrate the effectiveness of the RF heating for food products decontamination and shelf-life extension.

Table 1. Effects of RF technology applied for thermic treatment of food products

Field	Product	Effects	Author, Year
Bakery Industry	Metallized food packaging	RF wave transmission holes placement, heating uniformity, energy efficiency optimization	(Zhang et al., 2025; Zhao et al., 2020)
	Rye bread	RF technology at 60°C, 80°C, and 100°C for fungal inhibition	(Mitelut et al., 2015)
	RF drying in cookie, cracker, cereal, and snack production	RF drying reduced moisture gradients, prevented surface cracks, improved conveyor speed, and stabilized moisture levels	(McHugh, 2016; Palamthodi et al., 2021)
	RF heating and acrylamide reduction	Incorporating RF in final baking stages with ~10% residual moisture minimized acrylamide levels	(Anese et al., 2008)
	Wheat flour	Reduction of *Enterococcus faecium*- 2.5-3.7 log a_w – 0.45 ± 0.02	(Zhang et al., 2021)
	Wheat flour	*Salmonella enteritidis* and *Enterococcus faecium* - D85°C was calculated to be 18 min to achieve 1 log reductions; 8.34 ± 0.12% (w.b.) a_w – 0.45 ± 0.02	(Liu et al., 2018a)
	Wheat germ	RF treatment reduced lipase activity by 18.2% at 100°C (15 min) and 22.5% at 110°C (5 min), enhancing wheat germ color and absorption capacity.	(Ling et al., 2019)
Meat and Fish Industry	Cooked tiger skin chicken feet	RF with ZnO NPs Pickering emulsion for microbial reduction and quality retention	(Wei et al., 2025)
	Lean beef meat	RF thawing in batch and continuous conditions, dielectric properties analysis	(Bedane et al., 2017)
	Beef sausage	Superheated water-assisted RF heating for texture, color, and microstructure improvement	(Wang et al., 2024)
	Tilapia fillets	RF-HA drying at 5, 8 and 10 mm thickness with wrapping materials	(Cao et al., 2021)

Field	Product	Effects	Author, Year
Milk Processing Industry	Dairy model systems	RF inactivation of *S. typhimurium* and *L. monocytogenes*, effect of fat content and matrix structure	(Tonti et al., 2024)
	Raw cow's milk	*Staphylococcus aureus* - 5.65 log reduction	(Soto-Reyes et al., 2022)
	Yoghurt	Destruction of yeasts and molds. LAB partially survive RF heating at 58°C and 65°C while inactivated by conventional treatment	(Altemimi et al., 2019)
	Milk	RF processing results in minimal alterations to the organoleptic properties of milk, ensuring that flavor and appearance remain largely intact	(Di Rosa et al., 2018)
Fruit and Vegetable Processing Industry	Mushrooms	RF heating effects on polyphenol oxidase (PPO) inactivation, kinetic modeling	(Pang et al., 2024; Zhang et al., 2018)
	Cashew nuts	RF cold plasma treatment for microbial decontamination and quality retention	(Ranjan et al., 2024)
	Carrot slices	HA-RF drying for reduced drying time and carotenoid preservation	(Gong et al., 2019; 2020)
	Pre-made carrots	Nisin-assisted RF technology for microbial inactivation and extended shelf life	(Du et al., 2024; Wang C. et al., 2021; Zhang R., Li, et al., 2020b)
	Broccoli	HA-RF technology for better texture, bioactive compound preservation	(Dekker et al., 2014; Qing et al., 2023)
	Soymilk for tofu production	RF heating for improved texture and sensory properties	(Cui et al., 2021)
	Black peppercorns and dried basil leaves	In-package RF steaming for *Salmonella* inactivation	(Wason et al., 2024)
	Cocoa powder	RF heating at 90°C with insulated holding for pathogen inactivation	(Ballom et al., 2021)
	Kiwi puree	The RF-treated sample retained more nutrients, had higher soluble solids, lower pH, and higher acidity than the control.	(Liu et al., 2019)
	Apple juice	RF-treated samples had similar soluble solids but higher pH than the control. RF heating preserved phenolic content and color better, with fewer objectionable components than conventional treatment	(Tian et al., 2018)
	Sweet potato	RF with hot water bleaching improved color, texture, and heating uniformity with a 90 mm gap and 60 mm thickness.	(Jiang et al., 2020)
	Soybean	RF-treated soy protein isolate had better functionality than conventional heating. RF also reduced hexanal and 1-hexanol levels, improving soy milk's sensory properties.	(Jiang et al., 2018)
	Green peas (*Pisum sativum* L.)	Increasing the temperature from 60 to 85°C significantly affected the weight, color, electrolyte leakage, and texture of peas, causing more pronounced changes.	(Zhang C. et al., 2021)
	Sweet corn (*Zea mays* L.)	RF bleaching improved texture, color, and nutrients over boiling, reducing enzymatic activity to 4.68% at 80°C.	(Sun et al., 2022)
	Pearl millet	Hot air-assisted RF treatment inactivated 97.3% of lipase in 15% hydrated millet (15 min), improving bonding and flour quality.	(Yarrakula et al., 2022)

CONCLUSIONS

Radio Frequency (RF) processing technology offers several advantages over conventional heat exchange systems in the food industry, including time savings, higher profitability, and improved efficiency in food safety under identical operating conditions. RF heating has been demonstrated to effectively eliminate pathogenic and spoilage microorganisms while preserving the physicochemical, sensory, and nutritional properties of food due to its shorter processing time.

Additionally, RF heating enables in-package processing, minimizing the risk of cross-contamination and aligning with HACCP principles (Dragoev et al., 2024).

RF processing stands as a promising alternative to conventional heating methods, offering multiple benefits, though continued advancements are necessary to overcome current challenges and optimize its use.

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A CROSS-COUNTRY CONSUMERS' STUDY ON THE ACCEPTANCE OF GRISSINI ENRICHED WITH WHEY

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Abstract

The study aimed to valorize the whey resulting from dairy technology by its incorporation in grissini food products. It aimed to evaluate the consumers' acceptability of the new product and the market potential through a cross-country trial and survey conducted in parallel in Romania and Italy. Whey ultrafiltration concentrate used in the grissini recipe had high protein, fat, and sugar content. Grissini obtained by replacing water with whey were characterized by a higher protein content, total phenolic content, and antioxidant activity than the control sample made with water. Acceptance of the new product obtained was assessed with 100 panelists in Italy and 102 in Romania, respectively. Respondents answered a questionnaire and tested the grissinis. When it came to the acceptability of the samples made with whey, in both countries, the consumers showed a higher appreciation than for the control sample. A similar percentage of respondents (50% in Italy and 48% in Romania) were willing to pay 1.5 euro (8 lei)/100 g product. In general, participants in the study are keen to pay for innovative products at the same price as for conventional products.

Key words*: grissini, protein content, total phenolic content, antioxidant activity, consumer acceptability.*

INTRODUCTION

In recent years, the consumers' demand for functional food products and the awareness of natural bioresources depletion has led to the necessity to come up with new products, nutritionally enriched. Among the high quantity of by-products resulting from the industry, there are resources that are edible and have the potential to be reintroduced into the food system.

In the dairy industry, whey, the liquid remaining after the cheese making and casein manufacture, is produced in high amounts annually, but around 50% of the whey globally obtained is not reintroduced in the food chains (Lavelli and Beccalli, 2022). This by-product from the dairy industry is an important source of bioactive compounds (Herrera-Ponce et al., 2022), being a rich source of nutritional, health, and functional compounds (Lavelli and Beccalli, 2022).

Whey contains 55% of milk's nutrients and is obtained by skimming and precipitation of proteins (casein), it is also known as the serum phase of milk. It comprises a water-soluble fraction that includes lactose, soluble minerals and whey proteins (Chandrapala et al., 2016; Guimarães et al., 2010). Whey, although it still has many nutritive compounds, is disposed of as wastewater and raises serious environmental problems (Zhao et al., 2023).

About 50% whey is treated for further processing into valuable products, and the rest is only disposed of as waste (Baldasso et al., 2011; Zandona et al., 2021). Estimates indicate that global whey production is around 200

million tons per year with a continuing upward trend as reported by Ostertag et al. (2023).

Whey is an important source of proteins and it can undergo the processes of microfiltration, ultrafiltration, and reverse osmosis with the purpose of obtaining a whey protein concentrate (Bacenetti et al., 2017). As a consequence of the fact that bakery products have a low content of proteins, studies were conducted in order to incorporate whey obtained in different forms in cereal-based products such as bread (Guiné et al., 2018; Tsanasidou et al., 2021; Ferreyra et al., 2021), grissini (Suhodol et al., 2022), biscuits (Ahmed et al., 2019), pasta (Boudalia et al., 2020). Additionally, by supplementing cereal-based products with whey, nutritionally enhanced products are obtained, which present improved technological, chemical, and sensory characteristics.

Grissini are dry oven-baked cereal-based products which have a long shelf-life due to the low water content, its packaging being important in order to prevent water absorption (Hui, 2007). Grissini are made usually with the addition of fat which can improve the palatability and sensory properties.

The objective of this study was to reuse a by-product from the cheese technology by incorporation in grissini samples and to obtain information regarding the acceptance and willingness to pay for these samples in selected countries, namely, Italy and Romania.

MATERIALS AND METHODS

Raw materials

To prepare grissini, the ingredients used were: whole-wheat flour (moisture: 14%, protein content: 13.3%, fats: 2.3%, carbohydrates: 56.2%, dietary fibers: 13.8%, from Velpitar 7 Spice SA Romania), fresh compressed yeast, salt, sesame and flax seeds, olive oil (from the market), and water/liquid whey concentrate (pH 4.7). Two grissini products were manufactured: control-using water as ingredient and sample-using whey obtained by concentration through ultrafiltration by ENEA, Italy (composition: moisture 89.60 ± 0.06%, protein: 5.50 ± 0.05%, lipid: 0.21 ± 0.01%, ash: 0.29 ± 0.08%, salt: 0.2%, sugar, lactose: 3.97 ± 0.09%, acidity: 53.50 ± 0.71%, pH value 4.17 ± 0.01).

Manufacturing recipe of grissini samples

Grissinis with whey were prepared in the pilot plant experiments of IBA Bucharest, Romania. Initially, fresh compressed yeast (50 g) and salt (100 g) were dissolved in water (1.8 L) for the control or in whey (1.7 L) for the sample and added to the solid mixture of ingredients (whole-wheat flour: 3 kg, sesame seeds: 208 g, flax seeds: 358 g, and olive oil: 350 ml). Thus, all ingredients were mixed slowly for 3min at a lower speed in the mixer (Diosna Original, Dierks & Söhne, Maschinenfabrik, Osnabrück, Germany), followed by a faster step for 5 min (step II) for dough development. Then, the dough was divided into pieces which were shaped automatically in the form of grissinis using grissini-forming equipment. The shaped grissini were placed on baking pans for proofing: at 36°C, 80% humidity, for 40 min in the M.C.E Meccanica fermenter, model DAT TECH 1, Buttapietra, Italy. Then, the products were baked at 240°C for 30 min in the Mondial Forni oven. After baking, the grissinis were allowed to cool for 1-2 hours before performing the analysis. For consumer tests in Italy, the grissini were shipped to Italy.

Grissini proximate composition

The physico-chemical composition of the samples was investigated using the following methods: (1) moisture content by the drying method (AOAC Method 925.23, AOAC 935.36, AOAC 926.12 and AOAC 927.05); (2) protein content by the Kjeldahl method with a conversion factor of nitrogen to protein of 6.25 (AOAC Method 991.20 and AOAC 950.36); (3) fat content by extraction with petroleum ether under reflux conditions in a Soxhlet (AOAC Method 922.06 and AOAC 963.15); (4) ash content by gravimetric method by burning at 550°C in a furnace (AOAC Method 923.03 and AOAC 945.46); (5) acidity by titrimetric method (AOAC Method 947.05); (6) the total carbohydrate contents were assessed by subtracting the values of the moisture, protein, fat, and ash content from 100; (7) calorie contents were calculated using the following conversion factors: 9 for fat, 4 for carbohydrates, 4 for protein and 2 for fiber following the Commission Regulation no. 1169/2011. All analyses were performed in

duplicate and results are expressed as mean ± standard deviation (SD).

Phenolic compounds and antioxidant activity determination

The extraction procedure for total phenolic content and antioxidant activity determination is described below.

To assess polyphenolic content and antioxidant activity, 2 g of the sample were weighed and mixed with 80% methanol. The extracts were obtained through an extraction process employing vortex equipment (Vortex Heidolph Instruments Multi Reax) for 4 h at room temperature. Following this, the resulting extracts were centrifuged for 30 min at 4°C and 10,000 rpm to remove any secondary materials (Multescu et al., 2022).

Determination of total phenolic content (TPC)

The determination of TPC was conducted using the Folin-Ciocalteu method with minor adjustments (Wojdyło et al., 2007). Briefly, a volume of 500 µl of the extract was mixed with 5 ml Folin-Ciocalteu reagent 1: 15, and 500 µl of 20% sodium carbonate. Following a 20 min incubation at room temperature, the absorbance was measured at 752 nm using a Specord 210 UV-VIS spectrophotometer (Analytic Jena, Germany). A standard curve was prepared by using various concentrations (0.005- 0.175 mg/ml) of gallic acid under the same condition with samples (R^2 = 0.9999). Total phenolic content was expressed as mg gallic acid equivalent/100g of sample (mg GAE/100 g).

Determination of antioxidant activity through the DPPH method

DPPH radical scavenging activity was assessed based on the reduction in DPPH radical, following the method described by Culeţu et al. (2016). The reaction mixture consisted of 400 µl of sample and 6ml of DPPH radical solution, which was incubated for 20 min in the absence of light. Subsequently, the absorbance was recorded at 517 nm using a Specord 210 UV-VIS spectrophotometer (Analytic Jena, Germany). Antioxidant activity was calculated using a calibration curve (0.05-0.60 mmol/l) obtained with Trolox (R^2 = 0.9995). The results were expressed in mg Trolox/100 g of sample.

Determination of colour

The color of the grissini (ground samples) was measured with Konica Minolta Colorimeter (Spectrophotometer CM-5, Osaka, Japan). Three parameters were determined: parameter L*-the brightness of the sample on a scale from 0 to 100 (0 is black and 100 is white); parameter a*- the color of the sample on the scale from pure green to pure red (−a = green and +a = red), and parameter b*- the color of the sample on a scale from pure blue to pure yellow (−b = blue and +b = yellow). Before starting the analysis, the equipment was calibrated. The preparation of the samples consisted of filling the special glass cylinder of the device (diameter of 45 mm and height of 17 mm) with a sample without leaving air gaps. The sample thus prepared was placed on the measuring area and the glass was rotated in different positions to obtain 10 values for each color parameter. Means and standard deviation were calculated for each color parameter.

Consumers study

202 participants from two countries, namely Italy (100 consumers) and Romania (102 consumers), were recruited based on some screening criteria: to be part of the general population; the number of males and females to be balanced; to ensure a good spread of age, higher than 18 and younger than 60-year-old. Each participant completed a written consent form where they mentioned that they were available for participation, that they have no medication, have no food allergies or food intolerances.

Acceptance of the new product was measured based on product trials with tasting and on a survey. Both the trial and the survey were conducted in Italy and Romania.

The control sample and the grissini with whey were presented to the panelists at room temperature, in plastic plates, coded with three distinct digits. For testing, 20 g (2 grissini/person) of the control sample (grissini with water) and sample grissini with whey were provided to panelists. The samples were analyzed individually, randomly, and water was used as a palate cleanser.

Participants of the trial were informed regarding the composition of the products. In order to evaluate the acceptability of the

product, the hedonic test was used, using a scale from 1 to 9, where 1 means "I dislike it extremely", and 9 "I like it extremely".

Survey questions

A questionnaire was designed for this study to evaluate the consumers' acceptance of the new product compared to the control sample and had 10 questions with open or closed answers. In both countries, the study was run through face-to-face interviews with easily understandable questions. All the consumers received detailed information about the study.

The respondents had to answer questions regarding their gender, age, allergies, knowledge about the benefits of whey, the overall acceptability of the two samples, the improvement that they consider should be made to the whey grissini, and the advantages or drawbacks of this kind of product. Additionally, they had to rate the samples after information regarding the nutritional composition and benefits of whey were provided. After rating the products based on the aspect and the data presented, grissini were tasted by panelists in a randomized order. Aspects related to willingness to pay for a new product obtained with whey and the price that they will pay for it were also measured.

Statistical analysis

All physical-chemical analyses were performed in duplicate and results were expressed as mean ± sd. For the statistical analysis Minitab statistical software version 20 was used and one-way analysis of variance (ANOVA) was performed to study the differences among the samples, followed by Tukey's test, at a confidence level of $p < 0.05$.

RESULTS AND DISCUSSIONS

Proximate composition, hardness and color parameters were determined for grissini samples. Moreover, we determined the total phenolic content and antioxidant activity using the DPPH method.

Proximate composition and nutritional properties of whey and grissini samples

Whey ultrafiltration concentrate was physicochemical and nutritional characterized and then was incorporated into grissini samples. The comparison between grissini made with water and grissini enriched with whey was investigated.

Images with grissini samples are presented in Figure 1.

Figure 1. Grissini samples: a - control (grissini with water) and b - grissini with whey

The proximate composition of the whey and grissini samples are presented in Table 1.

Table 1. Proximate composition of the whey and grissini samples

Parameters	Grissini – control	Grissini with whey
Acidity, °	4.5 ± 0.14b	5.9 ± 0.14a
Sugar, saccharose %	2.22 ± 0.08b	3.15 ± 0.07a
Salt, %	3.11 ± 0.01a	2.90 ± 0.01b
Lipid, %	15.43 ± 0.18a	15.35 ± 0.08a
Moisture content, %	4.40 ± 0.08b	5.82 ± 0.13a
Protein, %	16.07 ± 0.00b	17.22 ± 0.04a
Ash, %	3.58 ± 0.09a	3.37 ± 0.14a
Fiber, %	4.33 ± 0.08a	3.72 ± 0.08b
Starch, %	53.06 ± 0.11a	49.75 ± 0.18b
Carbohydrates	56.19	54.55
Energetical value, kcal	437	433
Energetical value, kJ	1834	1817

Values followed by different letters (a–b) for grissini samples are statistically different at $p < 0.05$%.

Based on the acidity, whey can be classified as sweet whey (pH = 6.2-6.4) and acidic whey (pH = 4.6-5.0) (Ganju and Gogate, 2017). The whey used in our experiments was an acidic whey (pH = 4.17), which presented a moisture content of 89.60%, comparable with the one reported by Dushkova and Dinkov (2009) of 89.11% for whey ultrafiltrate.

The protein content of whey was 5.50%, higher than the one reported by Dushkova and Dinkov (2009) of 3.65%. The lipid, sugar, and ash content of whey was 0.21%, 3.97%, and 0.29%, respectively.

When compared with the control sample, grissini with whey have significantly higher values for acidity, sugar, moisture content and for protein content.

When adding whey to the grissini, the acidity increased from 4.5°T for control to 5.9°T for whey grissini. This could be the result of the natural acids present in whey. Similar results were reported by Suhodol et al. (2022) who replaced the water from grissini with whey and

obtained grissini with a higher acidity than the control sample.
The sugar content of the grissini with whey was significantly higher (3.15%) than the sugar content of the control sample (2.22%). The addition of whey improves water retention in the dough (Paul et al., 2022), and the moisture content of grissini with whey (5.82%) is significantly higher compared to the one determined for the control sample (4.40%).
By replacing water with whey in the recipe, the protein content of the grissini increased significantly.
No significant differences were noticed between the lipid and ash content of both control and grissini enriched with whey. When it comes to fiber content, the control sample had a higher fiber content (4.33%) than grissini made with whey (3.72%). Also, when it comes to salt content, the control had a higher content than grissini with whey.
The starch and carbohydrate content were higher for the control sample compared to the ones obtained for grissini with whey.
The difference in the energetic value between the two samples is negligible.

Total phenolic compounds estimation (TPC)
Table 2 presents the TPC of whey ultrafiltration concentrate, grissini control and grissini with whey. The polyphenolic content of whey ultrafiltration concentrate was 5.31 mg GAE/100 g. Grissini control and grissini with whey showed higher TPCs, recording close values, 95.42 mg GAE/100 g and 96.05 mg GAE/100 g, respectively.

Table 2. Phenolic content in analyzed grissini samples

Sample	TPC (mg GAE/100 g)
Whey ultrafiltration concentrate	5.31 ± 0.12
Grissini - control	95.42 ± 0.21a
Grissini with whey	96.05 ± 0.08a

Values followed by different letters (a–b) for grissini samples are statistically different at $p < 0.05\%$.

Antioxidant Activity of analyzed samples
After whey addition, the antioxidant activity of grissini increased significantly from 48.53 ± 0.11 mg TE/100 g for control to 63.29 ± 0.09 mg TE/100 g for grissini with whey.

Color analysis of samples
The addition of whey brings an advantage regarding the lightness of the bread crumbs samples. Both samples have positive values for the two-color parameters (a and b), the control sample has a slightly reddish coloration (Table 3) while the sample with added control whey has a slightly yellowish coloration.

Table 3. The results of the color analysis of the bread samples

Sample	L*(D65)	a*(D65)	b*(D65)
Grissini - control	58.56 ± 0.3b	9.84 ± 0.23a	27.41 ± 0.44b
Grissini with whey	59.45 ± 0.32a	9.61 ± 0.24b	27.94 ± 0.29a

Values followed by different letters (a-b) for grissini samples are statistically different at $p < 0.05\%$.

The results are mean_standard deviation (n=10 determinations for color parameters). Values followed by different superscript letters in the same column are significantly different ($p < 0.05$).

Consumers' acceptance study
The tests of the new food product with whey were organized to evaluate the acceptance of the product prototype in two participating countries, Romania and Italy. After the consumers received detailed information about the study, the subjects were recruited from the general population based on the screening criteria mentioned before answering the questionnaire. One of these criteria was related to the allergy/intolerance to lactose (whey), sesame seeds, and gluten. In Italy, 100 consumers were recruited (56 women and 44 men, aged between 18-55+ years and overall age of 41) and completed the questionnaire. In Romania, among the 102 participants (40 men and 62 women aged between 18-55+ years and overall age of 40) to the study, 7 mentioned to have an allergy to one of the ingredients added to grissini samples or an intolerance to gluten, therefore the questionnaire ended for them without testing the grissini. 54% of Romanian participants and 62% of Italian participants declared they knew about the benefits of whey.
The overall acceptability (Figure 2) of the samples was scored using a 9-point Hedonic Scale from 9-Like Extremely to 1-Dislike Extremely.
In both countries, the whey grissini had a higher acceptability than the control.
The consumers' willingness to buy food from whey by-product was evaluated (providing a scale from: 1 euro/100 g, 1.5 euro/100 g or 2 euro/100 g). The results showed that the willingness to pay for products enriched with

by-products is a general attitude of the consumer. In Italy and Romania, a similar percentage of consumers (50% and 48%, respectively) have chosen to pay 1.5 euro/100 g product. In general, participants in the study would prefer to pay for the innovative products at the same price as for conventional products.

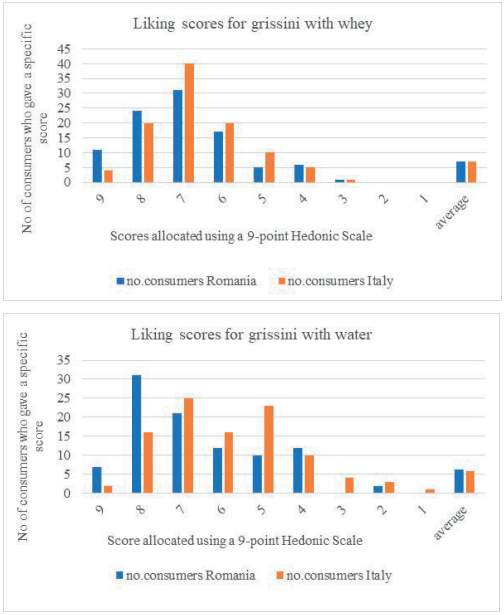

Figure 2. Overall acceptability of samples in Romania and Italy

CONCLUSIONS

The interest in reformulation of food products through incorporation of by-products started to increase. It is important to find possibilities to valorise whey in a most convenient way for reducing food waste. Incorporating whey into grissini production is a practical solution for dairy factories to valorize by-products, reduce waste, and offer healthier, sustainable food options. This approach aligns with both economic and environmental goals, contributing to a circular food system.

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LIPOSOMES IN REGENERATIVE COSMETICS: REVOLUTIONIZING SKIN CARE THROUGH ADVANCED NANOTECHNOLOGY

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Abstract

Liposomes play a vital role in the advancement of regenerative cosmetics due to their capacity to encapsulate, protect, and effectively deliver active ingredients deep within the skin. These versatile carriers mimic biological membranes, enhancing the stability and bioavailability of sensitive compounds such as peptides, growth factors, vitamins, and plant-derived bioactives. Regenerative cosmetics leverage liposomal systems to promote skin repair, boost collagen synthesis, and combat signs of aging by targeting cellular pathways with precision. This review explores the state-of-the-art advancements in liposome technology for cosmetic applications, emphasizing their role in addressing challenges such as poor solubility, rapid degradation, and limited penetration of active agents. Additionally, we examine the economic viability, scalability, and regulatory considerations associated with integrating liposome-based products into the commercial cosmetic market. Despite their transformative potential, challenges remain in ensuring formulation stability, achieving cost-effective production, and verifying long-term safety. By providing a comprehensive overview, this review underscores the potential of liposome technology to revolutionize regenerative cosmetics, paving the way for next-generation skincare innovations that restore, rejuvenate, and protect the skin.

Key words*: liposomes, regenerative cosmetics, natural ingredients, skin carre.*

INTRODUCTION

The field of regenerative cosmetics has witnessed significant advancements in recent years, with liposome technology emerging as a key innovation in the delivery of active ingredients for enhanced skin health (Dubey et al., 2022; Huang et al., 2024). Liposomes, vesicles composed of phospholipid bilayers, serve as highly efficient carriers that encapsulate and transport bioactive compounds deep into the skin. Their structural similarity to biological membranes allows for improved biocompatibility and enhanced absorption of otherwise poorly soluble or unstable molecules (Aziz et al., 2019; Dymek & Sikora, 2022; Giordani et al., 2023; Lee, 2020; Khater et al., 2021; Nsairat et al., 2022; Obeid et al., 2017).

One of the primary challenges in skincare formulations is ensuring the effective penetration of active ingredients, such as peptides, growth factors, vitamins, and botanical extracts, into the deeper layers of the skin. Traditional topical applications often suffer from limitations, including rapid degradation, insufficient bioavailability, and inefficient targeting of cellular pathways (Antimisiaris et al., 2021; Ashfaq et al., 2023; Aqil et al., 2013; Li et al., 2019; Musielak & Krajka-Kuźniak, 2024). Liposomal carriers overcome these challenges by providing controlled and sustained release, protecting sensitive compounds from environmental degradation, and optimizing their bioavailability.

Regenerative cosmetics, which focus on skin repair, rejuvenation, and protection, have greatly benefited from liposomal advancements. By facilitating targeted delivery of essential biomolecules, liposomes contribute to enhanced collagen synthesis, reduction of fine lines and wrinkles, and overall skin renewal. This cutting-edge approach is particularly relevant for anti-aging treatments, hydration therapies, and formulations designed to combat oxidative stress and inflammation (Huang et al., 2024; Lee et al., 2024; Pleguezuelos-Beltrán et al., 2024). Additionally, the ability of

liposomes to encapsulate both hydrophilic and lipophilic molecules has broadened their application scope, enabling the development of multifunctional cosmetic formulations that address a wide range of dermatological concerns.

Despite the promising benefits of liposomal technology in cosmetics, several hurdles remain in its widespread adoption. The high cost of production, challenges in ensuring long-term formulation stability, and the need for rigorous regulatory approvals pose significant barriers to commercial scalability (Agrawal et al., 2025; Liu et al., 2022; Sameer Khan et al., 2024). Moreover, the physicochemical properties of liposomes, including their size, charge, and composition, must be carefully optimized to ensure consistent performance in skincare applications (Gatto et al., 2024; Tran et al., 2022). Advanced techniques such as nanoliposomes, ethosomes, and deformable liposomes have emerged to further refine delivery efficiency, but their full potential is yet to be realized in mainstream cosmetic formulations (Ricci et al., 2024; Sapkota & Dash, 2021). This review explores the current state of liposomal technology in regenerative cosmetics, highlighting its transformative potential in overcoming traditional skincare limitations.

Additionally, we examine the economic viability, scalability, and regulatory considerations associated with integrating liposome-based products into the commercial cosmetic market. Despite their transformative potential, challenges remain in ensuring formulation stability, achieving cost-effective production, and verifying long-term safety. By providing a comprehensive analysis, this review underscores the role of liposomes in revolutionizing modern skincare, paving the way for next-generation cosmetic innovations that restore, rejuvenate, and protect the skin.

MATERIALS AND METHODS

This mini-review was conducted through an extensive literature search and analysis of various types of papers like reviews, books, research articles, etc. related to liposomal technology in regenerative cosmetics. A systematic search was performed in March 2025 using databases such as PubMed, Scopus, Web of Science, ScienceDirect, etc. to collect peer-reviewed journal articles, using keywords, such as ‘liposomes’, ‘regenerative cosmetics’, ‘nanocarriers in skincare’, ‘liposomal drug delivery’, ‘cosmetic formulations’ and ‘biocompatible nanoparticles’. Articles were selected based on their relevance, credibility, and impact on the field, prioritizing studies discussing the physicochemical properties, stability, efficacy, and safety of liposomal formulations in cosmetic applications.

Studies were included if they focused on the development, characterization, stability, and effectiveness of liposomal formulations in cosmetic applications, along with regulatory and economic considerations, while articles lacking experimental or clinical data, those centered exclusively on pharmaceutical applications, or reports with insufficient methodological details were excluded. Also, Regulatory guidelines for cosmetic liposomal formulations were reviewed, focusing on the European Union (Regulation EC No. 1223/2009) and FDA regulations.

RESULTS AND DISCUSSIONS

Liposomes in regenerative cosmetics

Liposomes are nanoscale vesicles composed of one or more concentric phospholipid bilayers enclosing an aqueous core, making them highly effective carriers for both hydrophilic and lipophilic molecules (Gregoriadis, 2017). Their amphipathic nature allows them to self-assemble in aqueous environments, forming stable bilayer structures known as lamellae, which can be classified as unilamellar (single bilayer) or multilamellar (multiple bilayers) vesicles (Aman Mohammadi et al., 2023). This unique architecture not only protects encapsulated compounds from environmental degradation but also enhances their stability and bioavailability in cosmetic formulations (Giordani et al., 2023; Musielak & Krajka-Kuźniak, 2024).

The composition of liposomes plays a crucial role in their structural integrity and performance. Typically, they are formed using natural or synthetic phospholipids, such as phosphatidylcholine, phosphatidylethanolamine, and cholesterol, which influence their stability,

charge, and permeability (Santonocito & Puglia, 2025). Cholesterol, in particular, is often incorporated to enhance membrane rigidity and reduce permeability, thereby increasing the shelf life of liposomal formulations (Ohishi et al., 2024).

The size and surface charge of liposomes also impact their ability to penetrate the stratum corneum, the outermost layer of the skin. Liposomes ranging from 50 to 200 nm in diameter have been shown to exhibit improved skin permeability, allowing for deeper penetration of active ingredients (Nayak et al., 2024). Additionally, surface modifications, such as PEGylation or functionalization with ligands, can enhance the targeting efficiency and stability of liposomes in cosmetic applications (Gatto et al., 2024).

Beyond their structural advantages, liposomes offer significant benefits in skincare by improving the bioavailability and controlled release of active compounds. They have been used to encapsulate a wide range of bioactive ingredients, including vitamins (e.g., vitamin C and E), antioxidants (e.g., resveratrol and coenzyme Q10), peptides, and botanical extracts (Tran et al., 2022). The encapsulation process protects these sensitive compounds from oxidation, UV degradation, and enzymatic breakdown, ensuring prolonged efficacy upon application (Agrawal et al., 2025; Sameer Khan et al., 2024; Pires et al., 2019).

Various techniques, including sonication, extrusion, and freeze-thaw cycles, are employed to reduce liposome size and improve their ability to penetrate the skin barrier (Lombardo & Kiselev, 2022; Santonocito & Puglia, 2025; Taouzinet et al., 2023). Sonication utilizes ultrasonic waves to break down liposomes into smaller vesicles, whereas extrusion forces liposomal suspensions through membranes of defined pore sizes, yielding uniform nano-sized vesicles (Martel-Estrada et al., 2022). The freeze-thaw method, which involves repeated cycles of freezing and thawing, enhances liposomal stability and encapsulation efficiency (Huang et al., 2005).

Liposomes offer multiple advantages as delivery systems for cosmetics. They protect bioactive compounds from degradation caused by light, oxidation, and environmental factors, thereby extending product shelf life and ensuring sustained release (Huang et al., 2024; Khorasani et al., 2018). Furthermore, liposomes improve ingredient bioavailability, enhance skin absorption, and allow for targeted delivery of both hydrophilic and lipophilic active agents (Musielak & Krajka-Kuźniak, 2024; Ricci et al., 2021).

Several encapsulation techniques, including emulsion, spray drying, extrusion, electrospinning, and coacervation, can be utilized to achieve efficient liposomal formulations (Dejeu et al., 2024; Filipczak et al., 2020). Among these, extrusion is regarded as the most efficient nanosizing method due to its ability to produce homogeneous liposomal structures with controlled particle sizes (Kaul et al., 2018; Mozafari et al., 2020).

To ensure successful encapsulation, several critical factors must be considered: (i) forming a stable liposomal shell around the active ingredient, (ii) preventing leakage of encapsulated compounds, and (iii) maintaining the exclusion of undesired external materials (Giordani et al., 2023; Huang et al., 2024). These characteristics contribute to improved product stability, extended efficacy, and enhanced bioavailability (Huang et al., 2024). Additionally, liposomes' physicochemical properties, such as size, lamellarity, and membrane rigidity, can be tailored to optimize solubility, pharmacokinetics, and biodistribution of active ingredients (Kaul et al., 2018; Allen & Cullis, 2013; Santonocito & Puglia, 2025).

Despite their numerous advantages, several challenges hinder the widespread adoption of liposomes in cosmetics. High production costs, potential leakage of encapsulated compounds, phospholipid oxidation or hydrolysis, fusion of active ingredients, low solubility, and short half-life present significant obstacles (Huang et al., 2024; Khorasani et al., 2018). The inherent instability of liposomal formulations can lead to aggregation, fusion, or precipitation, reducing their effectiveness in delivering active ingredients (Giordani et al., 2023). Furthermore, the interaction of liposomes with skin enzymes and external environmental factors, such as temperature and humidity, can degrade phospholipids, diminishing the overall bioavailability of encapsulated agents (Puri et al., 2020).

Another major concern is the structural integrity of liposomes upon skin application. The skin's stratum corneum acts as a formidable barrier, limiting the penetration of larger or unstable liposomal structures (Gupta et al., 2012; Musielak & Krajka-Kuźniak, 2024). While nanosized vesicles improve absorption, uncontrolled liposome fusion or premature release of active ingredients can reduce their targeted effectiveness (Nayak et al., 2024).

Additionally, the physicochemical properties of the liposomal membrane, including its charge, rigidity, and lamellarity, significantly influence its interaction with the skin surface and subsequent permeability (Cheng et al., 2022; Martel-Estrada et al., 2022).

Overcoming these limitations requires advancements in formulation techniques, cost-effective manufacturing strategies, and improved stabilization methods. Strategies such as polymer coating, cholesterol incorporation, and hybrid nanosystem development have shown promise in enhancing liposomal stability and prolonging their shelf life (Carita et al., 2018; Ferraris et al., 2021; Patra et al., 2018). Modified liposomal systems, such as ethosomes, transfersomes, and niosomes, have emerged as superior alternatives due to their enhanced flexibility, deeper skin penetration, and higher encapsulation efficiency (Lane, 2013; Santonocito & Puglia, 2025). With ongoing research and development, liposomes are poised to become a cornerstone in regenerative cosmetics, offering consumers high-performance skincare solutions with improved efficacy and safety (Kaul et al., 2018). Advances in nanotechnology continue to drive the evolution of liposomal formulations, incorporating stimuli-responsive vesicles that release active compounds based on pH, temperature, or enzymatic activity (Gupta et al., 2012). These innovations not only extend product longevity but also provide personalized skincare solutions tailored to individual skin needs. As the field progresses, liposome-based formulations will likely dominate the next generation of cosmeceuticals, setting new benchmarks in cosmetic science and dermatological care (Dejeu et al., 2024; Ferraris et al., 2021; Mozafari et al., 2020).

Economic viability, scalability, and regulatory considerations of cosmetic liposome-based products

Economic viability

The economic viability of liposome-based cosmetic products hinges on several key factors, including production costs, consumer demand, market potential, and scalability. The formulation and manufacturing of liposome-based products are inherently more complex than standard cosmetic products, as they require advanced techniques, specialized equipment, and precise control over physicochemical properties to maintain liposomal stability and encapsulation efficiency (Lombardo & Kiselev, 2022; Taouzinet et al., 2023).

One of the major cost drivers is the selection of high-purity phospholipids and stabilizers essential for preventing degradation, oxidation, and leakage of active compounds. Additionally, processing methods such as high-pressure homogenization, microfluidization, and freeze-drying further increase production costs (Cheng et al., 2022; Izadiyan et al., 2025). Despite these challenges, innovations in large-scale liposome production, such as continuous flow microfluidics and spray-drying techniques, have the potential to reduce costs while improving batch-to-batch consistency and scalability (Shah et al., 2020).

Consumer demand is another crucial economic aspect. With increasing awareness of advanced skincare technologies, there is growing interest in products that offer superior efficacy, such as liposome-based formulations (Assali & Zaid et al., 2022; Kaul et al., 2018). The global shift toward science-driven beauty solutions, particularly in the anti-aging, hydration, and dermatological skincare sectors, is expected to drive demand for liposomal skincare products. Additionally, the rise of personalized skincare – where formulations are tailored to individual needs – has further positioned liposome-based products as a preferred choice among consumers willing to invest in innovative solutions (Tran et al., 2022). Liposome-based cosmetics predominantly target the high-end skincare market, offering luxury formulations with clinically backed benefits.

The willingness of consumers to pay a premium for advanced skincare, particularly those that provide visible and long-term results, is a strong indicator of economic viability (Ferraris et al., 2021; Giordani et al., 2023). Furthermore, the clean beauty movement and demand for natural, bioavailable ingredients have created a niche for liposome-based products that utilize plant-derived phospholipids and encapsulated botanical extracts (Cheng et al., 2022). Although high production costs remain a concern, companies can mitigate these expenses through cost-effective formulation strategies, outsourcing specialized liposome manufacturing, and leveraging economies of scale. Strategic partnerships with biotechnology firms and research institutions can also facilitate access to novel liposomal encapsulation methods that enhance cost efficiency (Raszewska-Famielec & Flieger, 2022). Moreover, as production technologies continue to evolve, the cost gap between conventional and liposome-based cosmetic formulations is expected to narrow, making these products more accessible to a broader consumer base (Izadiyan et al., 2025).

Scalability

Scalability is a critical consideration when introducing liposome-based products into the market, as manufacturers must balance quality, cost, and volume.

The production of liposomal products requires specialized techniques, such as thin-film hydration, reverse-phase evaporation, or the hydration method (Dhawan et al., 2020). These methods, while effective, are more labor- and resource-intensive than conventional production processes.

Scaling up without compromising the quality and integrity of the liposomes can be challenging. Sourcing raw materials for liposome production, such as phospholipids and other bioactive ingredients, must be consistent and reliable. While these materials are available globally, fluctuations in their prices or availability could impact production scalability. Developing long-term supplier relationships and ensuring stability in the supply chain is essential for long-term success (Dhawan et al., 2020; Giordani et al., 2023; Patel et al., 2023).

Regulatory considerations

In the European Union, cosmetic products, including liposomal skincare products, are regulated under Regulation (EC) No. 1223/2009, which ensures that products are safe for consumers. Any new cosmetic product must be thoroughly tested for safety and efficacy before being marketed. Liposome formulations, due to their complex structure and the inclusion of active compounds, may require additional clinical testing to demonstrate their safety and efficacy. In addition to cosmetic product safety, individual ingredients must be compliant with EU regulations. Any novel ingredients encapsulated within liposomes must be assessed for safety, and certain ingredients may require approval or safety assessments by the European Medicines Agency (EMA) or other relevant bodies if they are used in therapeutic skin products (Ferraris et al., 2021).

The market for liposome-based regenerative cosmetics

Liposome-based formulations have been widely adopted in the cosmetic industry, enhancing the performance of various products. These formulations can be categorized into key areas of skin care: i) anti-aging and wrinkle reduction; ii) skin hydration and moisturization; iii) skin protection and regeneration; and iv) specialized treatments. Anti-aging and wrinkle reduction formulations assist in smoothing wrinkles, improving skin tone, and enhancing hydration, leading to a youthful appearance. Liposome-based formulations play a crucial role in reducing signs of aging by enhancing the delivery of bioactive compounds. Encapsulated ingredients such as antioxidants, peptides, retinol, and botanical extracts help combat oxidative stress, improve skin elasticity, and promote collagen synthesis. Skin hydration and moisturization focused formulations incorporate essential lipids, ceramides, hyaluronic acid, and botanical extracts to restore the skin's moisture barrier. Liposomal encapsulation improves the penetration of these ingredients, ensuring deeper and longer-lasting hydration. These formulations aid in revitalizing fatigued skin, reversing dullness, and protecting against environmental stressors that contribute to dryness and dehydration.

Liposomes enhance the efficacy of sunscreens, anti-pigmentation, and skin-repair treatments by facilitating the delivery of UV-blocking agents, antioxidants, and regenerative compounds such as vitamins C and E (Huang et al., 2024; Shaw et al., 2022). These formulations help shield the skin from environmental aggressors, reduce the appearance of dark spots, and accelerate skin renewal (Raza et al., 2013). Encapsulated polyphenols, ferulic acid, and plant-derived anthocyanins contribute to strengthening the skin's defense mechanisms while promoting a smooth, even complexion. Advanced liposomal systems are used in targeted treatments such as acne control, oil regulation, and skin nourishment. Active compounds like salicylic acid, tea tree oil, vitamins A and E, and plant-derived saponins are encapsulated for improved efficacy and controlled release (Lohani & Verma, 2017). These formulations help soothe inflammation, and enhance the absorption of nutrients, leading to healthier skin. In Table 1 are summarized the main cosmetic liposomal products available on market. Romania's skin care market is expanding, driven by a growing demand for anti-aging and dermatological products. Liposomal technology, being associated with high-quality, efficient skincare, fits well into the premium segment, which has seen growth in the past few years (De Leeuw et al., 2009; Huang et al., 2024; Shaw et al., 2022). In Romania, liposomal skin care products are likely to be available in high-end cosmetic stores, pharmacies, and online platforms. E-commerce is particularly growing, with more Romanian consumers purchasing beauty products online, often seeking products with a high scientific basis like liposome-based formulations. As Romania follows EU regulations for cosmetics, manufacturers will need to ensure compliance with all necessary safety tests and certification for liposomal skin care products before entering the market.

Table 1. Main cosmetic liposomal products

Cosmetic application	Product name	Manufacturer	Key ingredients
Anti-aging	Capture Totale	Dior	Longoza flower extract, niacinamide, lactiflora Root Extract, *Lilium candidum* Bulb Extract, Jasmine flower extract etc.
Anti-aging	Ageless Facelift Cream	I-Wen Naturals	Coenzyme Q10, tetrahexyldecyl ascorbate, glycan booster peptide, matrix peptide, hydrolyzed oat proteins, cranberry seed oil etc.
Anti-aging	Royal Jelly Lift Concentrate	Jafra Cosmetics	Extracts of winter cherry, lotus flower, sunflower etc.
Anti-aging	Derma Stemness Reviving Serum	Kaya Skin Clinic	Argan plant stem cell extract, hyaluronic acid etc.
Anti-aging	Isocell MAP	Lucas Meyer	Hydrogenated lecithin, magnesium, ascorbyl phosphate etc.
Anti-aging	Daeses Lifting Cream	Sesderma	Silicone, jojoba oil, vitamin e, dimethyl mae, liposomal organic silicon, immediate tensile complex (*Caesalpinia spinosa* fruit extract, *Kappaphycus alvarezii* extract) etc.
Anti-aging	Liposome Concentrate	Russell Organics	Vegetable oils & organic floral water, superoxide dismutase, beta glucan from oats etc.
Anti-aging and skin repair	Liposome Face and Neck Lotion	Clinicians Complex	Sunflower oil, squalane, sodium hylauronate, super oxide dismutase, Vit E etc.
Anti-aging and skin repair	Rovisome ACE Plus	Evonik	Ascorbyl palmitate, lecithin, retinol, tocopherol etc.
	Bio Performance Liposome	Dead Sea Premier	Dead Sea minerals,Vit A & E, Dunaliella seaweed extracts and Aloe vera
Anti-aging and skin repair	Acglicolic Classic Crema Hidratante SPF 15	Sesderma	8% liposomal glycolic acid, Vit C+E, Aloe vera, *Eryngium maritimum* stem cells, hyaluronic acid, milk proteins, bisabolol, ergothioneine, ceramides etc.
Anti-wrinkle and anti-	Rovisome® Q10 NG	Evonik	Coenzyme Q10, Vit A, C and E and *Camellia*

Cosmetic application	Product name	Manufacturer	Key ingredients
oxidant			*sinensis* extract
Anti-aging skin brightening	C-Vit	Sesderma	Vitamin C, extracts of mulberry and hyaluronic acid
Anti-aging, hydration, anti-wrinkle	Ferulac Liposomal Serum	*Sesderma*	Ferulic acid from Apple polyphenol extract
Anti-aging, anti-wrinkle	Resveraderm Antiox Serum	*Sesderma*	Resveratrol, quercetin and EGCG from Red grape extract
Anti-wrinkle	Lumessence EyeCream	AubreyOrganics	*Rosa Mosqueta* seed oil, *Camellia sinensis* leaf oil, evening primrose oil, *Laminaria digitata* extract, rye seed extract etc.
Anti-pigmentation	Azelac Ru Serum	Sesderma	*Bellis perennis* flower extract, pummelos fruit extract, lecithin etc.
Moisturizer	Rehydrating Liposome Day Creme	Kerstin Florian	Vitamin E, shea butter & horse chestnut
Moisturizer	Moisture Liposome: Eye Cream/Face Cream	Decorte	Ginkgo biloba leaf extract, *Lagerstroemia speciosa* leaf extract, oyster extract, Panax ginseng root extract, polyglutamic acid etc.
Skin repair	Advanced Night Repair Protective Recovery Complex	Estee Lauder	Tripeptide-32, hyaluronic acid
Skin repair	Skin rejuvenate cream	*Mythos*	*Punica granatum* (pomegranate) extract
Skin repair	Holistic Age Defense Eye Cream	*Apivita*	Greek royal jelly extract liposomes
Anti-acne and oil control	Clearly It!® Complexion Mist	Kara Vita	Vit A & E, Lecithin, Complex Origanum, Lyphazomes.
Anti-acne	Acnel Lotion N	Dermaviduals	Natural oils and Vit F

CONCLUSIONS

Liposomes have emerged as a transformative technology in regenerative cosmetics, offering enhanced stability, bioavailability, and targeted delivery of active ingredients. Their unique phospholipid bilayer structure mimics biological membranes, enabling deeper penetration of bioactive compounds such as peptides, antioxidants, and vitamins. This capability translates into improved skin repair, collagen synthesis, hydration, and protection against environmental stressors, making liposomes a powerful tool in modern skincare formulations. Despite their numerous benefits, challenges persist in the widespread adoption of liposomal formulations, including high production costs, formulation stability, and regulatory requirements. Advances in nanotechnology, such as ethosomes and deformable liposomes, continue to refine their efficiency, while scalable production methods and strategic collaborations may help mitigate economic barriers. Furthermore, compliance with regulatory frameworks remains essential to ensuring product safety and consumer confidence. As scientific advancements drive the evolution of liposomal formulations, their integration into regenerative cosmetics is expected to expand, setting new benchmarks in skincare innovation. With ongoing research and development, liposomes have the potential to revolutionize the cosmetics industry, offering more effective, long-lasting, and scientifically driven skincare solutions that cater to the growing demand for high-performance beauty products.

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ANTIRHEUMATIC CREAM BASED ON NATURAL INGREDIENTS

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Abstract

Choosing organic and natural products should be a top priority for a healthy skin. The aim of this research was to formulate and test a cream based on natural ingredients, used for skincare and also to relief rheumatic diseases. The main active ingredients used to obtain the cream were the following: burdock oil, macadamia nut oil, shea butter, ylang-ylang oil, harpagophytum oil, capsicum oil, and vitamin E. The properties of each ingredient have been taken into account in the preparation of the product, in accordance with the general methods for making creams. Organoleptic and physicochemical analyses confirmed the quality of the product, pleasant appearance and odor and a creamy consistency.

Key words*: active ingredients, burdock, cream, skin.*

INTRODUCTION

Rheumatic diseases, also known as musculoskeletal inflammatory diseases, affect everyone from children, adolescents, young people to the elderly (Hersh et al., 2011). Chronic inflammatory diseases that affect bones, joints, muscles, ligaments and tendons are grouped together under the term "rheumatism" and can have consequences for the health of the skin, heart, kidneys and lungs. They have a significant impact on quality of life (Benenson, 2010).

Cosmetic products are mainly used to protect the skin against various exogenous and endogenous harmful agents. Herbal cosmetics are formulated using certain base-forming substances, in which one or more herbal ingredients are incorporated to provide certain benefits (Liu, 2022).

Currently, plants are intensively used for the development of new types of cosmetic products called cosmeceuticals (Mahesh et al., 2019).

Cosmeceuticals are products that combine the benefits of cosmetics and pharmaceuticals (Radd, 2002) (Figure 1). They are designed to provide skincare benefits that go beyond basic beauty enhancement by addressing specific skin concerns (Choi et al., 2024). The use of cosmeceuticals is multi-purpose because they offer a range of skin benefits that bridge the gap between cosmetic beauty products and medical treatments (Morganti & Coltelli, 2019). People use cosmeceuticals for ***targeted skin treatment*** (acne, aging, pigmentation, rosacea) (Callender et al., 2017; Diguță et al., 2014), ***anti-aging benefits*** (wrinkles, fine lines, and sagging) (Shanbhag et al., 2019**),** ***improved skin health*** (hyaluronic acid help to hydrate and plump the skin, while antioxidants - vitamin C - help protect the skin from environmental damage and premature aging) (Boo, 2022; Bukhari, et al., 2018; Pirvu et al., 2011), ***enhanced skin function*** (improving skin's natural barrier function and/or hydration levels and protection against environmental stressors) (Selwyn & Govindaraj, 2023), ***non-invasive solutions*** (alternative to aggressive dermatological treatments, such as laser procedures or injections) (Tanha et al., 2023).

By containing active ingredients with therapeutic effects, cosmeceuticals are used to enhance cosmetic beauty while also addressing skin health. They are often recommended by dermatologists and are commonly found in professional skincare lines (Millikan, 2001; Callender et al., 2017).

The purpose of using cosmeceuticals is to achieve more than just superficial beauty enhancement (Draelos, 2009). They are meant to provide deeper, science-based benefits that improve skin function, treat specific skin

issues, and contribute to long-term skin health (Zhang & Falla, 2009).

Natural ingredients in cosmeceuticals for **rheumatic pain relief** help reduce inflammation, improve circulation, and soothe joint and muscle discomfort (Alamgir, 2017). These ingredients are often used in **creams, gels, balms, and oils** for conditions like arthritis, osteoarthritis, and fibromyalgia (Mahajan et al., 2023).

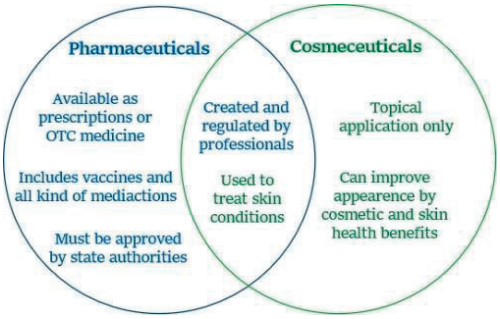

Figure 1. Similarities and differences between pharmaceuticals and cosmeceuticals

The main pain relief natural ingredients in cosmeceutical formulations for rheumatic disease include:

- Natural analgesics as pain-relieving agents (capsaicin from chili pepper extract, menthol from peppermint oil, methyl salicylate from wintergreen oil, eucalyptus oil, harpagophytum oil) (Kopustinskiene et al., 2022);
- Anti-inflammatory agents to reduce swelling and stiffness (turmeric, *Boswellia serrata* resin, ginger extract, *Arnica montana* extract, burdock oil, harpagophytum oil, willow bark extract, green tea, *Uncaria tomentosa extract*) (Sharma et al., 2023);
- Circulation-boosting agents to enhance blood flow and reduce pain **(**capsaicin from cayenne pepper, rosemary extract, cinnamon extract) (Kopustinskiene et al., 2022);
- Skin barrier and hydration support to prevent irritation from chronic inflammation (*Aloe vera*, shea butter, coconut oil, macadamia nut oil, jojoba oil, olive oil) (Kopustinskiene et al., 2022).

The aim of this research was to formulate a cream based on natural ingredients used both for the relief of rheumatic conditions and for skin care.

The main objectives consisted in formulating and obtaining a stable cosmeceutical cream (1) and highlighting its quality by organoleptic and physio-chemical tests (2).

MATERIALS AND METHODS

The formulated cream has been prepared under laboratory conditions, from natural ingredients, in compliance with the European regulations on ingredients and their admissible concentrations. The properties of each ingredient have been taken into account in the preparation of the product, in accordance with the general methods for making creams.

The ingredients used to formulate the cream are listed in Table 1.

Table 1. Ingredients used and their role in the cream formulation

Ingredient	Role in the formulation
Distilled water	Solvent
Cetyl alcohol	Emulsifier
Euxyl PE 9010	Conservant
Vegetable glycerin	Moisturizing agent
Lanolin	Emolient
Sodium lauryl sulfate	Surfactant
Paraffin oil	Emulsifier
Burdock oil	Active substance
Macadamia nut oil	Active substance
Shea oil	Active substance
Ylang-ylang oil	Odorizing agent
Harpagophytum oil	Active substance
Capsicum oil	Active substance
Vitamin E	Active substance

The cream was formulated under laboratory conditions, according to a particular recipe, in compliance with European pharmacological standards.

The main steps for making the cream are described below:

I. Mix ingredients of phase A: cetyl alcohol, lanolin, paraffin oil, glycerine;
II. Mix ingredients of phase B: sodium lauryl sulfate and euxyl;
III. Bring the two phases to 70°C in a water bath;
IV. Add phase B over phase A;
V. Add water over the A + B mixture;
VI. Add the thermosensitive ingredients (phase C): burdock oil, macadamia nut oil, shea

oil, ylang-ylang oil, harpagophytum oil, capsicum oil, vitamin E.

The cream was obtained using two Berzelius beakers. In the first one, the ingredients of phase A are introduced, in the second one, the ingredients of phase B, according to Table 2. Both beakers were heated to 70ºC using a water bath, to dissolve the solid ingredients. Then, the beakers were removed from the water bath and phase B is added over phase A. After homogenization with a mixer, the mixture is left to cool until the temperature reaches 40°C, after which the thermolabile ingredients of phase C are added, stirring continuously.

Table 2. Cream formulation

	Ingredient	Quantity for 100 g product
1.	Distilled water	46 mL
2.	Cetyl alcohol	18 mL
3.	Euxyl PE 9010	0.6 mL
4.	Vegetable glycerin	6 mL
5.	Lanolin	9 g
6.	Sodium lauryl sulfate	1.4 g
7.	Paraffin oil	5 mL
8.	Burdock oil	6 mL
9.	Macadamia nut oil	1 mL
10	Shea oil	1 mL
11.	Ylang-ylang oil	1 mL
12	Harpagophyum oil	3 mL
13.	Capsicum oil	2 mL
14.	Vitamin E	2 mL

After the preparation, the quality of the cream was determined by organoleptic and physico-chemical analysis, according to the regulations specified in the Romanian Pharmacopoeia X (R.P. X, 1998).

The organoleptic analyses focused on appearance, smell and color.

To determine the **appearance**, 1 g of sample was pressed between two 10 x 25 cm glass plates until a uniform layer of about 0.5 mm was obtained. The product was then examined in natural light. No drops of oil, water or grease were allowed and the cream layer must be free from crystalline particles or solid agglomerations.

The **smell** was determined by spreading a thin layer of sample on a glass plate or paper filter strip and then examined closely. The smell should be characteristic of the substances present in the cream, with no musty or rancid smell (R.P. X, 1998).

Color was determined by spreading the sample in a thin layer on a white paper and visual examination in natural light (R.P. X, 1998).

The physico-chemical analyses focused on pH, solubility, type of emulsion, spreadability, peroxide value and stability under accelerated conditions.

The **pH value** was determined by usual colorimetric or potentiometric methods in the aqueous solution obtained by shaking 5 g sample with 20 ml distilled water. The mixture was filtered and the pH was determined from the filtrate.

The determination of **spreadability** was performed using the Del Pozo Ojeda-Suñé Arbussá extensometer. 1 g of sample was placed between two 11 cm wide glass plates, over which weights between 100 and 500 g were added at 1 min intervals. The diameter of the cream layer was measured after the addition of each weight (Garg et al., 2002).

Several solvents were used for **solubility** determination of the cream: methanol, carbon tetrachloride, benzene, petroleum ether, hot distilled water. The sample is considered completely dissolved when the solution examined with the naked eye is free of suspended particles.

The **type of emulsion** was determined by the conductivity method. Two electrodes were inserted in the cream, if the cream allows current to pass through it means that the mass of the current forms water, so the type of emulsion is O/W. If the cream does not allow electric current to pass between the two electrodes, the emulsion has continuous oil phase type W/O.

The **peroxide value** indicates the degree of oxidative rancidity of fats. It represents the content of peroxide and other oxidizing substances in the sample that oxidize potassium iodide releasing iodine. The sample was dissolved in a mixture of acetic acid and chloroform, potassium iodide was added, then the free iodine was titrated with sodium thiosulphate (R.P. X, 1998).

The cream was also tested for **stability** under accelerated degradation in different temperature values. The sample was placed in closed vials which were kept for 8 hours, first at 4°C, then at 40°C. The cream is considered stable if the two phases, aqueous and oily, do

not separate and if, after testing, no significant differences are reported compared with the quality tests carried out on freshly prepared cream.

RESULTS AND DISCUSSIONS

The quality of dermatocosmetic products is determined by appearance (Figure 2), smell and colour.

Figure 2. Freshly made cream

The Table 3 shows the results of organoleptic analyses performed shortly after preparation of the cream.

Table 3. Organoleptic test results

Organoleptic characteristic	Characteristics of freshly made cream
Appearance	Creamy, homogeneous, consistent, no phase separations, spreads easily
Smell	Pleasant, fragrant odour, specific to ylang-ylang oil
Colour	Yellowish-white

The cream had a pleasant appearance, with a creamy consistency and no signs of phase separation.

The odour was pleasant, perfumed, and specific to ylang-ylang oil.

The yellowish-white colour of the cream was due to the ingredients used, mainly burdock and capsicum oils.

For the skin, a balanced **pH**, usually between 5.4-5.9, plays a crucial role in maintaining the natural protective barrier. This barrier helps to protect the skin against bacteria and other harmful microorganisms and to keep moisture in the dermal layers.

The pH is determined to verify if the cream may irritate the skin or mucous membranes. It should be as close as possible to that of the skin. According to F.R. X, it should be between 4.5-8.5.

Considering that normal, balanced skin has a pH of 5.5-6, the value of 5.8, obtained for the freshly prepared cream, shows that it can be safely used on the skin.

The **spreadability** is determined to verify if the the cream will spread well on the skin. Basically, the deformability of the cream is determined by pressing 1 g of cream between two glass plates (142 g per plate).

The results obtained for cream spreadability are presented in Figure 3.

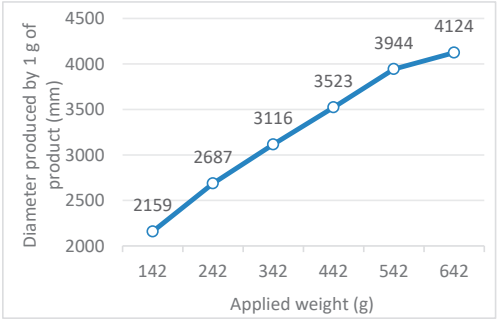

Figure 3. Results obtained for cream spreadability

Testing the **solubility** of the cream in certain solvents showed that it was insoluble in all the solvents we used (Table 4).

Table 4. Solvents for solubility determination

Solvent	Solubility
Methanol	Insoluble
Carbon tetrachloride	Insoluble
Benzen	Insoluble
Petroleum ether	Insoluble
Hot water	Insoluble

The conductivity method was used to determine the **emulsion type**. After applying the protocol, it was determined that the cream allowed the electric current to pass through its mass, thus the emulsion is O/W type.

The **peroxide value** is a parameter that measures the degree of oxidation of cosmetic products and reflects the oxygen content in the form of peroxide (hydroperoxide) in a substance. For oils in particular, it indicates the degree of rancidity.

The peroxide value determined for the cream was in the limits set by the Romanian Pharmacopoeia X (R.P. X, 1998).

The peroxide value was 0.75%, a value close to 0 and very far from 5, the maximum permissible value. The results proved that the ingredients used were fresh and of good quality.

The **stability test** is of major importance for cosmetic products. Due to its complex composition containing ingredients with different degrees of unsaturation, with a mainly organic structure, creams can be altered by temperature variations and the oxygen in the atmosphere. The ingredients may suffer structural alterations, resulting in changes in colour, splitting of some chains, formation of oxygenated groups and free radicals that continue the destructive process.

In case of the analysed cream, the main characteristics of the product did not change significantly. Both appearance and smell remained the same, with no major differences from the initial tests, and the phases did not separate.

The pH did not change after refrigeration, but a decrease of 0.2 units was measured after exposure to 40°C. Even so, the value was still in the optimum range.

Regarding spreadability, no major differences from the initial determinations were observed, with the largest deviations, up to 24 mm, observed after refrigeration.

The peroxide value showed slight increases in stability tests. The results were 0.79, after refrigeration and 0.85, after exposure at 40°C.

CONCLUSIONS

The tests performed on the formulated cream resulted in the following main conclusions:

- ✓ The organoleptic tests demonstrated the quality of the cream. It had a pleasing appearance and colour, creamy consistency, with no signs of phase separations, and the odour was specific to ylang-ylang oil;
- ✓ The pH value of 5.8 showed that the cream can be safely applied on the skin, without the risk of irritation caused by too high or too low acidity;
- ✓ The cream was insoluble in all the tested solvents;
- ✓ The peroxide value confirmed the quality of the cream and the freshness of the ingredients. Even after the stability tests no major increases from the initial values were determined.

The tests and analyses have demonstrated a high quality cosmeceutical formula, homogeneous, with good speadability, with anti-rheumatic properties due especially to the burdock oil, capsicum oil and harpagophytum oil.

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THE ANTIOXIDANT POTENTIAL OF SOME *Mespilus germanica* L. EXTRACTS

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Abstract

This study aimed to evaluate the antioxidant activity and mineral content of medlar fruit (Mespilus germanica L.*) extracts, using different solvents to identify the most effective extraction method. Extracts were prepared using acetone, ethanol, water, and chloroform. Polyphenol content was analyzed using HPLC, minerals were determined using inductively coupled plasma mass spectrometry (ICP-MS), and antioxidant activity was assessed using chemiluminescence assays to determine IC_{50} values. The acetonic extract (MP1) had the highest polyphenol content and strong antioxidant activity (AA% of 64%). The aqueous extract (MP2) was richest in minerals. The ethanolic extract (MP3) balanced polyphenol content and antioxidant activity (AA% of 51%). The chloroform extract (MP4) showed a pro-oxidant effect (AA% of -151%). Acetone is the best solvent for extracting polyphenols, while water is ideal for mineral extraction. The ethanolic extract provides a balanced option. Chloroform extracts should be used with caution due to potential pro-oxidant effects. These findings help optimize extraction methods for health benefits, with potential applications in food and pharmaceuticals.*

Key words*: antioxidant activity, chemical composition, Mespilus germanica* L.*, polar and non-polar extracts.*

INTRODUCTION

Mespilus germanica L., commonly known as medlar, is a fruit-bearing plant belonging to the *Rosaceae* family. Despite its long history of cultivation, its use has declined, making it an underutilized source of bioactive compounds. Recent studies have highlighted its potential as a significant source of antioxidants, which play a crucial role in preventing oxidative stress-related diseases (Popović-Djordjević et al., 2022; Voaides et al., 2021; Żołnierczyk et al., 2021).

The medlar fruit is rich in various bioactive compounds, including phenolics, flavonoids, and organic acids. These compounds contribute to its antioxidant capacity (Nistor et al., 2024; Yunusa, 2021). The study conducted by Katanić Stanković et al. (2022) have identified key phenolic compounds such as gallic acid, quercetin, and caffeic acid in medlar extracts. The total phenolic content (TPC) and total flavonoid content (TFC) in medlar fruits are comparable to other well-known antioxidant sources like blackthorn and hawthorn (Katanić Stanković et al., 2022).

Antioxidant activity of medlar extracts has been assessed using various *in vitro* assays, including DPPH and ABTS radical scavenging activities (Samarakoon et al., 2016). Using these analyses, it is possible to measure the ability of the extracts to neutralize free radicals, thus indicating their potential to decrease oxidative stress. Medlar extracts have shown significant scavenging activities, although they are often modest compared to synthetic antioxidants like BHT (butylated hydroxytoluene) (Rahimi-Nasrabadi et al., 2013).

The study of Katanić Stanković et al. (2022) reported that the DPPH radical scavenging activity of medlar extract was higher than that of several other natural antioxidants. This activity is attributed to the high concentration of phenolic compounds, which are effective hydrogen donors, stabilizing free radicals (Katanić Stanković et al., 2022). Additionally, the ABTS assay results corroborate these findings, further establishing the potent

antioxidant properties of medlar extracts (Görmez et al., 2024).

The antioxidant properties of *Mespilus germanica* extracts are not just limited to neutralizing free radicals but also extend to various health benefits. These extracts have shown potential antidiabetic and anti-inflammatory effects (Kızıltaş et al., 2021). The α-glucosidase inhibitory activity of medlar extracts suggests their possible use in managing diabetes by slowing down carbohydrate digestion and glucose absorption (Żołnierczyk et al., 2023).

In another study, medlar extracts demonstrated cytotoxic effects against cancer cell lines, indicating their potential in cancer prevention and therapy. The phenolic compounds in medlar are considered to induce apoptosis in cancer cells, thereby inhibiting their proliferation (Görmez et al., 2024).

The high antioxidant potential of *Mespilus germanica* extracts makes them suitable for various applications in the food and pharmaceutical industries (Tessa et al., 2021). They can be used as natural preservatives to extend the shelf life of food products by preventing oxidation. Moreover, their incorporation into functional foods and nutraceuticals can provide health benefits associated with their antioxidant and antidiabetic properties.

MATERIALS AND METHODS

1. Raw material

Medlar fruits were harvested in October 2022 from the Prahova region. Taxonomic identification was conducted by a team of agronomy specialists from the Faculty of Biotechnologies, USAMV of Bucharest. The *M. germanica* L. fruits were washed with distilled water, dried, and frozen at -20°C. Before use, the fruits were brought to room temperature and then ground in a ceramic mortar. The homogenized samples were used for obtaining polar (water, acetone, ethanol solvents) and non-polar (chloroform solvent) extracts.

2. Preparation of extracts

- 100 grams of homogenized medlar fruit sample were extracted three times consecutively with 200 ml of acetone each time. The combined acetone extracts were concentrated under vacuum and redissolved in 100 ml of 40% ethanol (w/v), yielding 100 ml of extract coded MP1;
- 100 grams of homogenized medlar fruit sample were extracted with 500 ml of distilled water using ultrasound bath extraction for 1 hour at 40°C. The mixture was filtered through filter paper, resulting in 410 ml of aqueous extract. This aqueous extract was concentrated to a residue and redissolved in 100 ml of 40% ethanol, yielding an extract coded MP2;
- 100 grams of homogenized medlar fruit sample were extracted with 500 ml of 70% ethanol using ultrasound bath extraction for 1 hour at 40°C. The mixture was filtered through filter paper, resulting in 460 ml of ethanolic extract. This extract was concentrated to a residue and redissolved in 100 ml of 70% ethanol, yielding an extract coded MP3;
- 100 grams of homogenized medlar fruit sample were extracted with 500 ml of chloroform. The mixture was filtered through filter paper, resulting in 500 ml of chloroform extract. This extract was concentrated to a residue and redissolved in 100 ml of concentrated chloroform, yielding an extract coded MP4.

3. Qualitative determinations

Qualitative analysis aimed to investigate the specific polyphenols from the acetonic and ethanolic extracts, proper for HPLC (high-performance liquid chromatography in the reverse phase) studies; it was used the Elite LaChrom liquid chromatograph equipped with a DAD L2455 detector, a stainless-steel column containing the octadecylsilane stationary phase as the reverse phase. There were used standard solutions at 0.005 g each test sample, as follows: the four standard solutions (gallic acid 95% - *Sigma-Aldrich*, chlorogenic acid >95% - *Sigma-Aldrich*, caffeic acid 99% - *Sigma-Aldrich* and transferulic acid >95% - *Sigma-Aldrich*) were accurately weighed, dissolved in 7 ml of ethanol and added to a 10 ml volumetric flask with the same solvent. The sample solution was diluted 1 ml to 5 ml volumetric flask with ethyl alcohol (Table 1 shows the chromatographic conditions).

Table 1. Chromatographic working conditions

Chromatographic Column	Inertsil ODS3 250 x 4.6 mm
Elution	Gradient
Column Temperature	40 ± 1°C
Flow Rate	1.0 mL/min
Detection	330 nm
Injection Volume	20 µL
Mobile Phase A	1 g/L ortho-phosphoric acid solution dissolved in 1000 mL water, adjusted to *p*H 2.8
Mobile Phase B	Methanol
Mobile Phase A Ratio	70:30
Column Equilibration	30 minutes
Acquisition Time	70 minutes

Table 2. Gradient used in experiments

Time (min)	Mobile phase A (% v/v)	Mobile phase B (% v/v)	Flow rate (ml/min)
0.0	70	30	1.0
60.0	30	70	1.0
60.1	70	30	1.0
70.0	70	30	1.0

After the chromatographic system has been balanced, i.e. the baseline is straight, the reference solution is injected (Table 2 shows the gradient used in experiments).
The test solutions are injected, and the chromatograms are recorded.
The content of the components of interest was calculated with the formula:
Component ″i″, mg/100 ml = Ap x Ce/Ae x 100, where:
Ap: Appearance of the peak of component ″i″ in the chromatogram of the test solution;
Ae: peak area of component ″i″ in the chromatogram of the standard solution;
Ce: the concentration of component ″i″ in the standard solution (mg/ml).

4. Quantitative determinations

Quantitative analyses were performed to investigate the total polyphenols content in the four test extracts: MP1, MP2, MP3, MP4.
Also, it was investigated the content of minerals and trace elements in the four extracts: there was measured the content of magnesium, phosphorus, potassium, calcium, chromium, manganese, iron, copper, zinc and the heavy metals arsenic, cadmium and lead; these determinations were carried out according to a method elaborated and developed in accordance with the general provisions by inductively coupled plasma mass spectrometry (ICP-MS), using the following isotopes: ^{23}Na, ^{24}Mg, ^{31}P, ^{39}K, ^{44}Ca, ^{52}Cr, ^{55}Mn, ^{57}Fe, ^{65}Cu, ^{66}Zn, ^{75}As, ^{114}Cd, ^{208}Pb. The optimal operating conditions are presented in Table 3.

Table 3. Optimal operating conditions of ICP-MS ELAN DRC-e

No. crt.	Parameter	Optimum values selected
1.	RF power for ICP	1250 Watts
2.	Nebulizer Gas Flow	0.96 L/min
3.	Auxiliary Gas Flow	1.20 L/min
4.	Lens Voltage	8.20 (with autolens option)
5.	Integration time	1000 m/s
6.	Dwell time	50 m/s
7.	No. sweeps/reading	20
8.	Mode detector	Dual
9.	No. replies	3

For the determination of the elements by ICP-MS, the samples must be brought to a liquid state; the digestion of the samples is done using a microwave oven, obtaining clear solutions, without loss of elements through evaporation and without contamination. The dry extract samples, accurately weighed, are separated with 8 ml of nitric acid and transferred into a digestion bottle.
The digestion program is one in two steps, for complete mineralization and is presented in Table 4.

Table 4. The digestion program

No. crt.	Power (W)	Preheating time (minutes)	Decomposition time (minutes)	Ventilation
1.	350	20	30	1
2.	550	10	45	3

At the end of the cycle, the mineralized solution is transferred into a 50 ml volumetric flask, this representing the base-stock solution. The sample solution represents the 3:50 ml dilution of the base solution.
- the reference solutions for the calibration curve are in the range: 0.001-1.000 µg/ml, which correspond to the range 1-1000 ppb for Ca, Mg, Zn, K, P, Cr, Mn, Fe, Cu (concentration measurement units given by the software of the ICP – MS device); 0.010-0.080 µg/ml, which corresponds to the range 10-80 ppb for Cd, Pb. Selective isotopes were chosen for each element, depending on the interferences present in the specific matrix of the products: Mg^{24}, P^{31}, K^{39}, Ca^{43}, Cr^{52}, Mn^{55}, Fe^{57}, Cu^{63}, Zn^{66}, Cd^{114}, Pb^{208}.

5. Determination of antioxidant activity

Studies on the antioxidant activity of the four active extracts obtained by processing *M. germanica* L. fruits were conducted using the chemiluminescent (CL) method. As resulted from the previous studies (Neagu et al., 2021; Pirvu et al., 2022; Pirvu et al., 2024), the chemiluminescence method is a fast, sensitive and reproducible chemical method, by which the free radical scavenger potential of a test vegetable extract can be determined, precisely the effectiveness in annihilating the hydroxyl radical from the test environment generated by oxygenated water from the environment; the CL method used is based on the property of luminol to emit (chemi)luminescence in the presence of oxygen free radicals, emitted by oxygenated water from the environment at basic pH (8.6) in the environment provided by the TRIS buffer. The intensity of the chemiluminescence reaction, measured as activity units (u.a.), is recorded every 4 seconds for 60 seconds and is proportional to the amount of free radicals generated by oxygenated water in the environment. The addition of an antioxidant reagent (test sample), for example a plant extract, which contains free radical scavengers leads to the annihilation of a share of hydroxyl free radicals from the environment, therefore a decrease in the intensity of the chemiluminescence reaction (u.a.) is recorded proportional to the concentration of active compounds and their antioxidant effectiveness. The effectiveness of the reaction is measured by comparing it with the negative control sample, which contains the solvent of the test sample.

Thus, by means of the chemiluminescent method, it is possible to analyze:

- the dynamics of the chemiluminescence reaction during 60 seconds, by comparing the undiluted sample with the negative control sample, which contains the solvent of the sample in which the sample was made and involves the serial dilutions;
- the maximum intensity of the antioxidant effect of the test extract (AA%) through comparative measurements 5 seconds after the initiation of the chemiluminescence reaction) - see the formula below:

$$AA\% = \frac{\text{Control sample activity units (a.u.)} - \text{Test sample activity units (a.u.)}}{\text{Control sample activity units (a.u.)}} \times 100$$

- at the same time, the IC_{50} of the test sample by studying a series of serial dilutions from the test sample to capture the inflection point of the chemical reaction, meaning the moment when a maximum antioxidant activity is obtained for a minimum concentration (punctual, the concentration at the halfway point of inflection from which the CL intensity starts to decrease).

Given the ability of the CL method to determine both the antioxidant effect and the pro-oxidant potential of a test sample, this can be considered a pre-pharmacological testing method as it provides an indication of the beneficial or toxic potential of a test product/extract; thus, the method allows a precise selection of the active extracts resulting from the phase of technological works in extractive biotechnologies, which has the effect of reducing the costs and time of subsequent pharmacological testing, allowing to obtain from the very beginning a product with maximized biological potential.

6. Procedure

The test plant extracts are processed as serial dilutions of 6-10 samples (e.g., x1, x2, x4, x8, x16, x32), compared to the negative control series represented by 8-10 serial dilutions of the solvent used to obtain the plant extracts test; the aim of the studies is to calculate the antioxidant activity of the test vegetal extracts, as well as their IC_{50} value, for the quantitative comparison of their effectiveness needed further in the biological and pharmacotoxicological testing; work with samples in triplicate for each point in the serial dilution (n = 3). Punctually, three aliquots of 50 µL of test plant extract (in this case ethanol 50%, v/v) related to each point in the test series are treated (freshly prepared reagent in bi-distilled water); the negative control series is carried out identically, replacing the plant extract with 50 µL ethanol 50% (v/v). The CL reaction is initiated by adding H_2O_2 to the medium, therefore the samples are quickly placed in the measuring device of the chemiluminometer, and the emitted fluorescence, measured as arbitrary units (a.u.), is recorded every 4 seconds for 60 seconds in total, during which it was demonstrated that a constant minimum plateau is reached, which means that the chemiluminescence reaction is consumed.

The results obtained, a.u. during the 60 seconds, respectively a.u. along the series of dilutions, it indicates the effectiveness of the antioxidant activity, scavenger of free radicals of the test plant samples, by comparison with the values obtained for the negative control sample; the sweep of the dilutions of the same series leads to the establishment of the IC_{50}, respectively the moment of inflection in which a maximum antioxidant activity is obtained at the minimum concentration of the test extract is highlighted.

RESULTS AND DISCUSSIONS

1. Analytical results

Polyphenols content

The quantitative content in total polyphenols is presented in Table 5.

Table 5. Quantitative content in total polyphenols

Extract	GAE mg%
ACETONIC MP1 extract	26.70
AQUEOUS MP2 extract	4.90
ETHANOLIC MP3 extract	11.9
CHLOROFORM MP4 extract	1.0

The quantitative analysis of total polyphenols in various extracts is summarized in Table 5. The acetonic extract (MP1) shows the highest concentration of polyphenols, with a value of 26.70 GAE mg %. The ethanolic extract (MP3) has a moderate polyphenol content of 11.9 GAE mg %. The aqueous extract (MP2) contains a lower amount of polyphenols, measuring 4.90 GAE mg %. The chloroform extract (MP4) exhibits the lowest polyphenol content, with only 1.0 GAE mg %.

This data indicates that the acetonic extraction method is the most effective for extracting polyphenols from the samples, while the chloroform method is the least effective.

The qualitative content of polyphenol-carboxylic acids and flavonoids (HPLC) are presented in Table 6.

The qualitative content of polyphenol-carboxylic acids and flavonoids (HPLC) is presented in Table 6.

Table 6. Qualitative content of polyphenol-carboxylic acids and flavonoids (HPLC)

Compound mg/100 ml	**ETHANOLIC Extract MP3**
Gallic acid	0.2727
Chlorogenic acid	0.381
Compound mg/100 ml	**ACETONIC MP1 Extract**
Gallic acid	0.2727
Chlorogenic acid	0.381
Caffeic acid	0.960
Transferulic acid	0.0467

Both extracts contain gallic acid and chlorogenic acid in the same amounts. The acetonic extract (MP1) also includes caffeic acid and ferulic acid, which are not present in the ethanolic extract (MP3).

The presence of increased amounts of caffeic acid and chlorogenic acid was found in the extracts standardized in 40% ethanol.

This data indicates that the acetonic extract (MP1) has a more diverse profile of polyphenol-carboxylic acids and flavonoids compared to the ethanolic extract (MP3).

Mineral and trace elements content

The mineral and trace elements content in the ethanolic, acetonic, and aqueous extracts of medlar (*Mespilus germanica* L.) reveal significant variations in the concentration of these elements across different extraction methods in Table 7.

Table 7. Mineral and trace elements content in the ethanolic, acetonic, and aqueous extracts of medlar

No. crt.	Elements	MEDLAR ACETONIC EXTRACT (MP1)	MEDLAR AQUEOUS EXTRACT (MP2)	MEDLAR ETHANOLIC EXTRACT (MP3)
1.	Mg µg/g)	288.33	386.09	298.27
2.	P (µg/g)	1017.04	1342.20	1121.02
3.	K (µg/g)	3403.08	5683.61	4698.06
4.	Ca (µg/g)	170.45	414.13	404.81
5.	Cr (µg/g)	3.82	3.74	4.55
6.	Mn µg/g)	1.29	2.23	1.805
7.	Fe (µg/g)	88.07	112.15	57.80
8.	Cu (µg/g)	2.48	3.41	3.25
9.	Zn (µg/g)	72.73	21.99	62.165
10.	Cd (µg/g)	0.142	0.10	0.10
11.	Pb (µg/g)	0.27	0.22	0.14

Magnesium (Mg) shows the highest concentration in the aqueous extract at 386.09 µg/g, followed by the ethanolic extract at 298.27 µg/g and the acetonic extract at 288.33 µg/g.

Phosphorus (P) is most abundant in the aqueous extract, containing 1342.20 µg/g, with the ethanolic extract having 1121.02 µg/g and the acetonic extract 1017.04 µg/g. Potassium (K) also has its highest concentration in the aqueous extract at 5683.61 µg/g, followed by the ethanolic extract at 4698.06 µg/g and the acetonic extract at 3403.08 µg/g.
Calcium (Ca) is found in the highest amount in the aqueous extract, with 414.13 µg/g, compared to 404.81 µg/g in the ethanolic extract and 170.45 µg/g in the acetonic extract. Chromium (Cr) has similar concentrations across all extracts, with the highest in the ethanolic extract at 4.55 µg/g. Manganese (Mn) is most concentrated in the aqueous extract at 2.23 µg/g, followed by the ethanolic extract at 1.805 µg/g and the acetonic extract at 1.29 µg/g.
Iron (Fe) is most abundant in the aqueous extract at 112.15 µg/g, with the acetonic extract containing 88.07 µg/g and the ethanolic extract 57.80 µg/g. Copper (Cu) shows the highest concentration in the aqueous extract at 3.41 µg/g, followed by the ethanolic extract at 3.25 µg/g and the acetonic extract at 2.48 µg/g.
Zinc (Zn) is most concentrated in the acetonic extract at 72.73 µg/g, with the ethanolic extract containing 62.165 µg/g and the aqueous extract 21.99 µg/g.
Both cadmium (Cd) and lead (Pb) are found in low concentrations across all extracts, with slight variations.
This data highlights the varying effectiveness of different extraction methods in concentrating specific minerals and trace elements from medlar fruits. The aqueous extract generally shows higher concentrations of most elements, making it potentially more beneficial for mineral extraction.

2. Antioxidant activity

Figures 1-4 (a, b) show the dynamics of the chemiluminescence reaction and the free radical scavenger effect of the active extracts obtained in the phase of technological studies on medlar fruits.

The results of the research on MP1 - Extract in ethanol 40% from acetonic extract

Figure 1.a illustrates the dynamics of the chemiluminescence reaction for the MP1 test extract (represented by the blue bars) in comparison to the negative control sample (represented by the green line).
The negative control sample shows a decreasing trend in chemiluminescence intensity over time, starting from around 15.000 a.u. and gradually declining to about 900 a.u. in 60 seconds. This indicates a reduction in reactive species over the duration of the test.

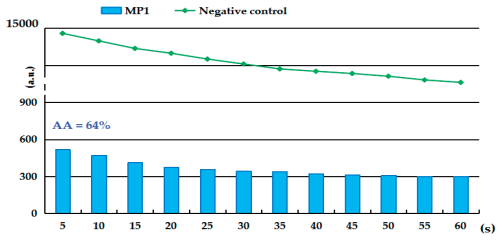

Figure 1.a. The dynamics of the chemiluminescence reaction of the MP1 test extract compared to the negative control sample (green line); AA% calculation

In contrast, the MP1 extract shows a significantly lower and relatively stable chemiluminescence intensity, starting at around 600 a.u. and maintaining values between 300 and 600 a.u. throughout the 60-second period. The consistent low intensity for the MP1 extract suggests it has a strong ability to inhibit the chemiluminescence reaction, indicating potential antioxidant properties.
The antioxidant activity (AA%) of the MP1 extract is calculated to be 64%. This percentage represents the inhibition of chemiluminescence by the extract compared to the negative control. The high AA% value further supports the effectiveness of the MP1 extract in neutralizing reactive species.
This analysis highlights the potential of the MP1 extract as an effective antioxidant agent, capable of significantly reducing the presence of reactive species in the chemiluminescence reaction.

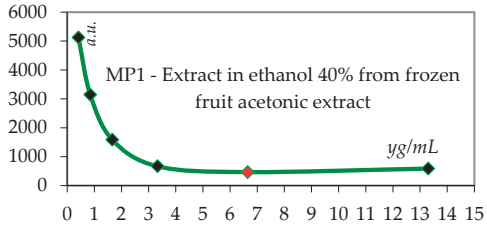

Figure 1.b. The antioxidant activity of the MP1 extract along the dilution series (A); IC_{50} calculation

Figure 1.b shows the antioxidant activity of the MP1 extract across a dilution series. The graph demonstrates how the chemiluminescence intensity decreases as the concentration of the extract increases, indicating its antioxidant activity.
The data points on the graph indicate a sharp decline in chemiluminescence intensity from 5000 a.u. to around 500 a.u. as the concentration of the extract increases from 0 to approximately 7 µg/mL. Beyond this point, the intensity levels off, suggesting that higher concentrations of the extract do not significantly further decrease the chemiluminescence intensity. This plateau indicates that the maximum antioxidant effect has been reached.
The IC_{50} value, represented by the red diamond on the graph, is the concentration of the extract at which the chemiluminescence intensity is reduced by 50% compared to the control. The IC_{50} value provides a quantitative measure of the extract's potency as an antioxidant.
This analysis highlights the effectiveness of the MP1 extract in inhibiting chemiluminescence, with its IC_{50} value serving as a key indicator of its antioxidant capacity.

The results of the research on MP2 - Extract in ethanol 40% from aqueous extract

Figure 2.a shows the dynamics of the chemiluminescence reaction for the MP2 extract (represented by the orange bars) in comparison to the negative control sample (represented by the green line).

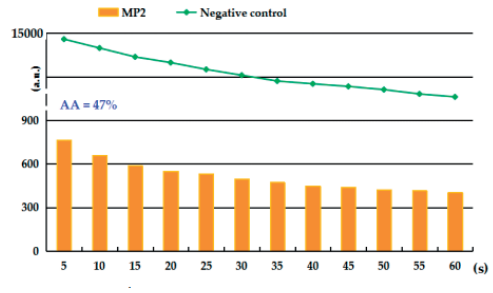

Figure 2.a. The dynamics of the chemiluminescence reaction of the MP2 extract compared to the negative control sample (green line); AA% calculation

The negative control sample shows a decreasing trend in chemiluminescence intensity over time, starting at around 15.000 a.u. and gradually declining to approximately 900 a.u. in 60 seconds. This decrease indicates the natural decay of reactive species over time.
The extract MP2 shows a significantly lower and relatively stable chemiluminescence intensity compared to the control. The intensity starts around 900 a.u. at 5 seconds and remains between 300 and 600 a.u. throughout the 60-second period. This stability and lower intensity suggest that the MP2 extract has a moderate ability to inhibit the chemiluminescence reaction, indicating its antioxidant properties.
The antioxidant activity (AA%) of the MP2 extract is calculated to be 47%. This percentage represents the inhibition of chemiluminescence by the extract compared to the negative control.

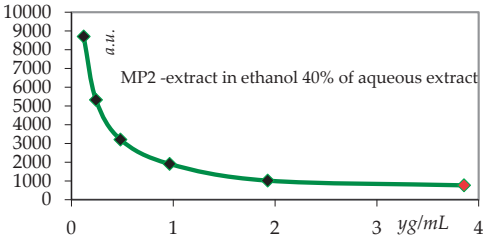

Figure 2.b. The antioxidant activity of the MP2 extract along the dilution series (A); IC_{50} calculation

Figure 2.b illustrates the antioxidant activity of the MP2 extract along a dilution series. The graph shows how the chemiluminescence intensity decreases as the concentration of the extract increases, indicating its antioxidant activity.
The data points on the graph show a steep decline in chemiluminescence intensity from about 9000 a.u. to approximately 1000 a.u. as the concentration of the extract increases from 0 to around 3 µg GAE/mL. This suggests that the extract has a strong ability to reduce reactive species at higher concentrations.
The IC_{50} value, represented by the red diamond on the graph, is the concentration at which the chemiluminescence intensity is reduced by 50% compared to the control.

Research results on MP3 - 40% ethanol extract from 70% ethanol extract

Figure 3.a illustrates the dynamics of the chemiluminescence reaction for the MP3 extract (represented by the purple bars) in comparison to the negative control sample (represented by the green line).

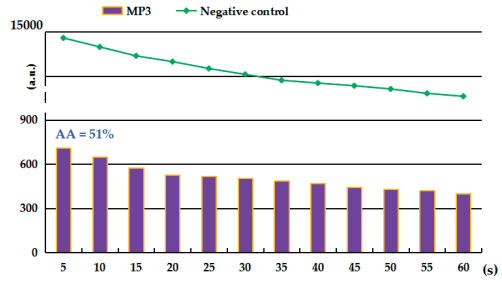

Figure 3.a. The dynamics of the chemiluminescence reaction of the MP3 extract compared to the negative control sample (green line); AA% calculation

The negative control sample shows a decreasing trend in chemiluminescence intensity over time, starting at around 15.000 a.u. and gradually declining to approximately 900 a.u. in 60 seconds. This decrease indicates the natural decay of reactive species over the course of the experiment.

In contrast, the MP3 extract maintains a significantly lower and relatively stable chemiluminescence intensity. The intensity starts around 600 a.u. at 5 seconds and remains between 300 and 600 a.u. throughout the 60-second period. This stability and lower intensity suggest that the MP3 extract has a good ability to inhibit the chemiluminescence reaction, indicating its antioxidant properties.

The antioxidant activity (AA%) of the MP3 extract is calculated to be 51%. This percentage represents the inhibition of chemiluminescence by the extract compared to the negative control.

Figure 3.b shows the antioxidant activity of the MP3 extract along a dilution series. The graph indicates how the chemiluminescence intensity decreases as the concentration of the extract increases, demonstrating its antioxidant activity.

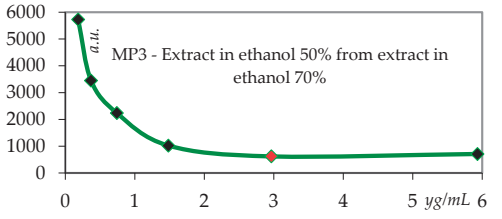

Figure 3.b. The antioxidant activity of the MP3 extract along the dilution series (A); IC_{50} calculation

The data points on the graph show a steep decline in chemiluminescence intensity from about 5000 a.u. to approximately 1000 a.u. as the concentration of the extract increases from 0 to around 3 µg GAE/mL. Beyond this point, the intensity levels off, suggesting that higher concentrations of the extract do not significantly further decrease the chemiluminescence intensity. This plateau indicates that the maximum antioxidant effect has been reached.

The IC_{50} value, represented by the red diamond on the graph, is the concentration at which the chemiluminescence intensity is reduced by 50% compared to the control.

The results of the research on MP4 - Extract in ethanol 40% of the extract in chloroform

Figure 4.a illustrates the dynamics of the chemiluminescence reaction for the MP4 extract (represented by the red bars) in comparison to the negative control sample (represented by the green line).

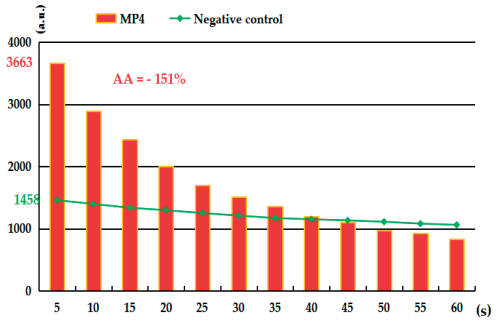

Figure 4.a. The dynamics of the chemiluminescence reaction of the MP4 extract in comparison with the negative control sample (green line); AA% calculation

The negative control sample shows a consistent decrease in chemiluminescence intensity over time, starting at approximately 1458 a.u. and gradually declining to around 1000 a.u. in 60 seconds. This decrease indicates the natural decay of reactive species over time.

In contrast, the extract MP4 shows a significantly higher chemiluminescence intensity initially, starting at around 3663 a.u. at 5 seconds. The intensity decreases over time but remains substantially higher than the negative control throughout the 60-second period. The MP4 extract exhibits a different/specific behavior where its intensity starts high and gradually declines, which suggests it may be acting as a pro-oxidant rather than an antioxidant in this specific setup.

The antioxidant activity (AA%) of the MP4 extract is calculated to be -151%. This negative percentage indicates that the MP4 extract actually increases the chemiluminescence intensity compared to the negative control, suggesting a pro-oxidant effect rather than an antioxidant effect.

Figure 4.b illustrates the antioxidant activity of the MP4 extract along a dilution series. The graph shows how the chemiluminescence intensity decreases as the concentration of the extract increases, indicating its antioxidant activity.

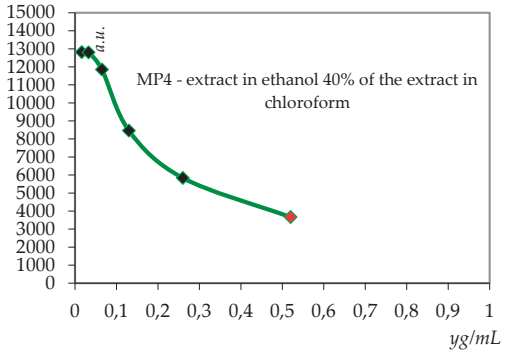

Figure 4.b. The antioxidant activity of the MP4 extract along the dilution series (A); IC_{50} calculation

The data points on the graph show a steep decline in chemiluminescence intensity from about 13.000 a.u. to approximately 4000 a.u. as the concentration of the extract increases from 0 to around 0.5 µg GAE/mL. This decline suggests that the extract has some ability to reduce reactive species at higher concentrations, but the initial high values and the form of the curve may still reflect pro-oxidant activity, as indicated in Figure 4.a.

The IC_{50} value, represented by the red diamond on the graph, is the concentration at which the chemiluminescence intensity is reduced by 50% compared to the control.

Overall, the results of our study on medlar fruit extracts align well with existing literature on the antioxidant activity and polyphenol content of plant-based extracts. The high polyphenol content found in our acetonic extract (MP1) and its diverse profile of polyphenolic compounds, including caffeic and ferulic acids, are consistent with findings from other studies that highlight acetone as an effective solvent for extracting bioactive compounds from plant materials (Katanić Stanković et al., 2022).

Moreover, our study's observation that the acetonic extract demonstrates strong antioxidant activity is supported by similar research on polyphenolic plant extracts. Studies have shown that polyphenols, particularly those extracted with organic solvents like acetone, possess significant antioxidant properties due to their ability to neutralize free radicals and inhibit oxidative stress (Stagos, 2020; Nieto, 2020).

Neagu et al. (2021) investigated the antiproliferative activity of ethanolic extracts from *Stokesia laevis*, highlighting that ethanolic extracts, due to their high content of bioactive compounds, are effective in therapeutic applications. This supports our conclusions regarding the ethanolic extract (MP3), which demonstrated significant antioxidant activity and a high polyphenol content, suggesting potential applications in health-related fields.

In contrast, the pro-oxidant effect observed in our chloroform extract (MP4) is notable and emphasizes the need for careful selection of extraction solvents. This phenomenon, while less commonly reported, is not unprecedented and suggests that certain solvents might extract compounds that can either neutralize or exacerbate oxidative stress, depending on the conditions (Stanković et al., 2022; Stagos, 2020).

Furthermore, the higher mineral content found in the aqueous extract (MP2) of medlar fruit supports the idea that water is particularly effective in extracting minerals, as corroborated by studies on mineral and trace element extraction from plant materials (Stanković et al., 2022).

Pirvu et al. (2022) investigated the effects of laser irradiation on the antioxidant activity of *Plantago lanceolata* aqueous extracts. This study can be related to our findings on the aqueous extract (MP2) of medlar fruits, especially regarding the antioxidant properties. Both studies emphasize the influence of different extraction and treatment methods on the antioxidant potential of plant extracts, providing a broader context for your results.

Pirvu et al. (2024) explored the chemical and biological attributes of fruits after their shelf life, showing that although the bioactivity of some compounds may decline, certain treatments can

maintain or even enhance their stability. These findings are relevant for discussions on the use of medlar fruit extracts in food and pharmaceutical products, highlighting the importance of optimising storage and processing conditions to maximise antioxidant benefits.
These findings reinforce the importance of selecting appropriate solvents based on the desired bioactive compounds and the intended application, whether for antioxidant purposes or mineral extraction in food and pharmaceutical industries.

CONCLUSIONS

The study investigated the antioxidant activity and extraction efficiency of medlar fruit extracts using various solvents. Polyphenol content, mineral composition, and antioxidant capacity were analyzed across acetonic, ethanolic, aqueous, and chloroform extracts. The acetonic extract (MP1) had the highest polyphenol content and showed the most diverse range of polyphenolic compounds, making it the most effective solvent for polyphenol extraction. The aqueous extract (MP2) was richest in essential minerals like magnesium and potassium. Antioxidant activity tests revealed that the acetonic extract had the strongest activity, while the chloroform extract exhibited a pro-oxidant effect, raising concerns about its use. The ethanolic extract offered a balanced profile of polyphenol content and antioxidant activity, making it suitable for potential health applications. The study recommends acetone for polyphenol extraction and water for mineral extraction, while advising caution with chloroform due to its potential pro-oxidant effects. These findings contribute to optimizing extraction methods for medlar fruits in the food and pharmaceutical industries.

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PRELIMINARY STUDIES REGARDING THE ANTIMICROBIAL PROPERTIES OF SOME BIOPRODUCTS DERIVED FROM *Zingiber officinale* AND *Curcuma longa*

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Abstract

The study aimed to evaluate the antimicrobial activity of certain bioproducts obtained from turmeric and ginger rhizomes, respectively, four essential oils (EO) purchased from NJoy and Oleya and four bioproducts obtained through chloroform and ethanol extraction. The results showed that the EO of ginger (NJoy) exhibits moderate antimicrobial activity against E. coli and significant antimicrobial activity on S. aureus, C. albicans, and C. parapsilosis; the EO of turmeric (Oleya) exhibit moderate antimicrobial activity for E. coli and C. albicans. The bioproduct, derived from the extract of turmeric in chloroform, has a significant antimicrobial effect on C. albicans and C. parapsilopsis. The alcoholic extracts of turmeric and ginger exhibit local antimicrobial effects on E. coli and no antimicrobial activity for C. albicans, C. parapsilopsis, or S. aureus. In conclusion, the preliminary tests achieved in vitro indicate significant differences between the tested bioproducts, some of these showing high potential for specific antimicrobial applications,

Key words*: Zigiber officinale*, *Curcuma longa, antimicrobial properties.*

INTRODUCTION

The rhizomes of *Curcuma longa* (turmeric) and *Zingiber officinale* (ginger) have gained considerable attention over time due to their uses in traditional medicine (Daily et al., 2016). Turmeric rhizomes, known for their intense aroma and colour, are often used as a spice. Ginger rhizomes, with their mildly spicy taste, are utilised as a seasoning or in the formulation of various tea blends (Christine et al., 2021). Both spices not only enhance the flavour of food but also offer health benefits due to their antioxidant, antitumour, and antimicrobial properties. Although numerous scientific articles present the therapeutic potential of turmeric and ginger, their antimicrobial activities against specific pathogenic microorganisms, such as *S. aureus*, *E. coli*, *C. albicans* and *C. parapsilosis*, remain insufficiently studied. The incidence of infections caused by these microorganisms is significant. For instance, *Stapylococcus aureus* frequently causes skin and soft tissue infections (Carolus et al., 2019). *Escherichia coli*, a Gram-negative bacteria, is associated with urinary tract infections and gastrointestinal diseases (Kaper et al., 2004). *Candida albicans* and *Candida parapsilosis* are the opportunistic fungal species linked to invasive candidiasis (Pappas et al., 2018; Silva et al., 2012). The present study aimed to evaluate the antimicrobial activities of certain bioproducts derived from turmeric and ginger rhizomes, specifically essential oils obtained through hydrodistillation and bioproducts obtained by extraction in selective solvents such as ethanol or chloroform.

MATERIALS AND METHODS

Bioproducts of ginger and turmeric

The essential oils of turmeric (EO-2.1; EO-2.2) and ginger (EO-1.1; EO-1.2) were purchased from the Romanian market at a local medicinal plant store (Plafar, Bucharest, Romania). The chemical composition of the essential oils used in the studies was determined by gas chromatography coupled with mass spectrometry (GC-MS, CLARUS 500, PERKIN ELMER, Waltham, USA) and is presented in

Table 1. The extracts (bioproducts) were obtained from fresh ginger and turmeric rhizomes, purchased from the Romanian market (organic products grown under ecological agriculture in Peru and Brazil), through maceration in 96% ethanol (GE = ginger extract in ethanol; TE = turmeric extract in ethanol) and chloroform (GC = ginger extract in chloroform; TC = turmeric extract in chloroform), using a plant-to-solvent mass ratio of 1: 2. Fresh turmeric and ginger rhizomes were washed, peeled, and cut into small pieces, forming cubes of approximately 2.5 mm. These pieces were placed in a black glass bottle with the corresponding solvent. The four containers were stored in a dark place at room temperature for two weeks, with manual agitation every two days. After two weeks, the clear liquid phase was removed from the bottles and filtered through 150 mm filter paper (Prat Dumas France, Solantis, Bucharest, Romania). The ethanolic extract was used unmodified. For the chloroform extract, the solvent was evaporated using a rotary evaporator at 50°C, and the remaining residue was dissolved in a minimal amount of dimethyl sulfoxide. In each extract obtained, the total polyphenol content was assessed using the Folin-Ciocalteu method (Agbor et al., 2014), using the gallic acid as the reference. The coding and polyphenol content for each extract are presented in Table 1. All the extracts were stored in darkness in refrigerator at 5ºC.

Table 1. Characteristics of bioproducts derived from *Zingiber officinale* and *Curcuma longa*

Plants	Bioproduct type	Code	Polyphenols content (mg GAE/L ± ST. DEV.	Source	Observație
Zingiber officinale (Ginger rhizoms) Brazil, Peru	Essential Oil NJoy	E.O 1.1	-	Romanian market	Voucher specimen deposited in the ICCF herbarium with the identifier name: Ziof21
	Essential Oil Oleya	E.O 1.2	-		
	Alcoholic extract (etanol)	GE	91.00±1.82		
	Crude extract derived from chloroform extraction, condiționed in DMSO (dimethyl sulfoxide)	GC	398.00±7.56		
Curcuma longa (Turmeric rhizoms) Brazil, Peru	Essential Oil Oleya	E.O 2.1	-		Voucher specimen deposited in the ICCF herbarium with the identifier name: Culo21
	Essential Oil NJoy	E.O 2.2	-		
	Alcoholic extract (ethanol)	TE	1980.00±37.62		
	Crude extract obtained în chloroform, and conditioned in DMSO	TC	12.48±0.12		

Microorganisms

The antimicrobial activity of the essential oils and of extracts of turmeric and ginger was tested using the Kirby-Bauer diffusive disks methodology on the following microorganisms: *Escherichia coli* (ATCC 25922), *Staphylococcus aureus* (ATCC 25923), *Candida albicans* (ATCC 10231), and *Candida parapsilosis* (ATCC 22019), following the methodology outlined by Schroder et al. (2022) and Albisoru et al. (2024).

Methodology used for antimicrobial activity

For reactivation, the microorganisms were inoculated in Petri plates with TSA (Tryptone Soy Agar, Sigma Aldrich. Darmstadt, Germany) medium for bacteria and PDA (Potato Dextrose Agar, Sigma Aldrich, Darmstadt, Germany) medium for *Candida* sp. and incubated at 37°C for 24 and 48 hours, respectively. After that, a suspension of each microorganism was prepared in sterile saline serum (0.9% NaCl), containing 10^8 CFU/mL. This suspension was evenly spread across the surface of each Petri dish (Φ = 90 mm) using a sterile cotton swab. For bacteria, Mueller Hinton agarised (Sigma Aldrich, Darmstadt, Germany) plates were used, and for *Candida* sp., PDA medium was employed. On the surface of each Petri dish inoculated with the test microorganism, 3-5 sterile cellulose discs

(Φ = 6 mm) were placed and impregnated with 30 µL of a 1 % essential oil solution (diluted in sterile propylene glycol) or 30 µL of each extract. The resulting plates were incubated at 37°C for 24 hours for bacteria and 48 hours for yeasts, after which the inhibition zone diameters were measured. For comparison, standardised discs impregnated with a specific antibiotic for each tested microorganism were used: ampicillin (AMP25) for *S. aureus*, gentamicin (Gen10) for *E. coli*, and clotrimazole (CT10.8) for *Candida* sp. (Sigma Aldrich, Darmstadt, Germany BioRad Lab, Hercules, CA, USA) (Babeanu et al., 2022; Voicu et al., 2022; Fotina et al., 2024; Mohammed et al., 2023; Marchidan et al., 2023).

Statistical Analysis

The experimental data obtained were aquired from (3-5) determinations and were reported as the mean inhibition zone diameter (notation Φ), with the corresponding standard deviation (STDEV).

RESULTS AND DISCUSSIONS

Characterisation of the essential oils

The major compounds from the essential oils of turmeric and ginger from NJoy are shown in Figure 1.

Ar-turmerone represents the major compound found in turmeric oil, accounting for 44.61% of the mixture, followed by turmerone (23.91%) and curlone (19.92%).

The major compound from ginger essential oil is geraniol (40.43%), followed by nerol (21.86%) and geraniol acetate (7.15%).

The major compounds in the turmeric and ginger essential oils from Oleya are presented in Figure 2. As can be observed, the major compound in Oleya's turmeric oil is Ar-turmerone (37.35%).

In the essential oil of ginger, the main compounds found by GC MS analysis are zingiberene (47.31%) and cedrene (29.13%).

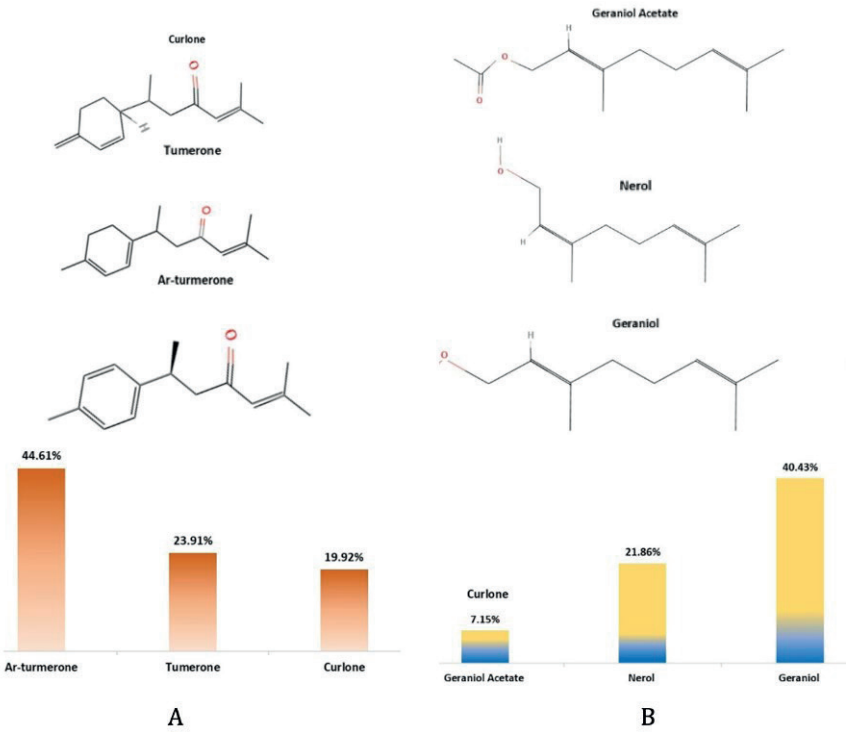

Figure 1. Major compounds present in NJoy turmeric (A) and ginger (B) essential oils

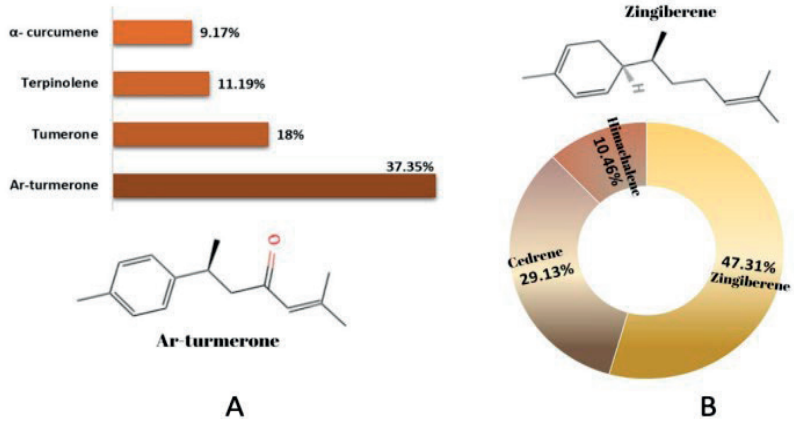

Figure 2. Major compounds present in Oleya's turmeric (A) and ginger (B) essential oils

Characterisation of the turmeric and ginger extracts

The main components of the bioproducts obtained by ethanol or chloroform extraction were quantified using the NIST data bases (Table 2) after the analysed samples were normalised. In the turmeric ethanolic extract, the major chemical compounds found by GC-MS analysis are curlone (35.43%) and Ar-turmerone (16.39%). In the ethanolic ginger extract, the major compounds are (1S,5S)-2-methyl-5-((R)-6-methylhept-5-en-2yl) bicyclo [3.1.0]hex-2-ene(7-epi-sesquithujene) (25.41%) 3-(1,5-dimethyl-4-hexenyl)-6-methylene-,[S-(R*,S*)]-cyclohexene(β-sesquiphellandrene) (12,28%) și 1-(1,5-Dimethyl-4-hexenyl)-4-methyl-benzene (α-curcumene) (10.15%). In the Table 3 are presented the major compounds identified in the chloroform extracts obtained from fresh rhizomes of turmeric and ginger. The turmeric extract contains tumerone (32.9%), Ar-tumerone (16.9%), and curlone (16.1%). The ginger extract contains trans-α-bergamotene (21.2%), benzene, 1-(1,5-dimethyl-4-hexenyl)-4-methyl (15.2%), and (E)-β-farnesene (11%). Regarding the polyphenolic compound content, it was found that the extract obtained from turmeric rhizomes in ethanol contains the highest amount of polyphenols (1980±37.62 mg GAE/L), followed by the extract obtained from ginger rhizomes in chloroform (398±7.56 mg GAE/L) (Table 1).

Table 2. Major compounds in bioproducts obtained by extraction from turmeric and ginger rhizomes in ethanol

	Compounds	Relative concentration, %	Retention time, min
Turmeric	Tumerone	35.43	15.57
	Curlone	19.90	15.84
	Ar-turmerone	16.39	15.52
Ginger	(1S,5S)-2-methyl-5-((R)-6-methylhept-5-en-2-yl)bicyclo[3.1.0]hex-2-ene (7-epi-Sesquithujene)	25.41	14.15
	3-(1,5-Dimethyl-4-hexenyl)-6-methylene-[S-(R*,S*)]-cyclohexene (β-Sesquiphellandrene)	12.28	14.42
	1-(1,5-Dimethyl-4-hexenyl)-4-methyl-benzene (α-Curcumene)	10.15	14.04

Table 3. Major compounds in bioproducts obtained by extraction from turmeric and ginger rhizomes in chloroform

	Compounds	Relative concentrations, %	Retenţion time, min
Turmeric	Tumerone	32.9	15.54
	Ar Tumerone, izomer	16.9	15.61
	Curlone	16.1	16.09
Ginger	Trans, α- bergamotene	21.2	12.99
	Benzene, 1-(1,5-dimethyl-4-hexenyl)-4-methyl	15.2	12.83
	(E)-β -Farnesene	11.0	13.42

Antimicrobial Activity

The preliminary study results revealed the following:

a) For *E. coli*, the essential ginger oil from NJoy (E.O-1.1) and the essential turmeric oil from Oleya (E.O-2.1) demonstrated significant antimicrobial activity, with average inhibition zone diameters Φ = 18.33 mm and Φ = 13.67 mm, respectively. The other tested essential oils exhibited only local antimicrobial activity, characterised by average inhibition zone diameter below 7.67 mm. The bioproduct obtained from turmeric rhizomes in chloroform showed no antimicrobial activity (Figure 3).

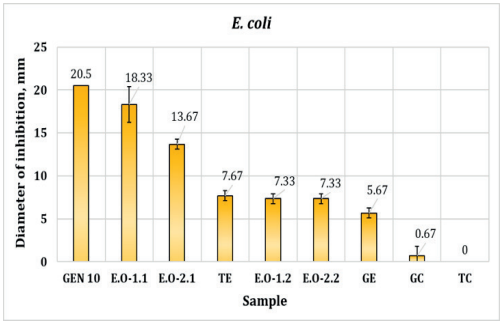

Figure 3. Antimicrobial activity of turmeric and ginger bioproducts on *E. coli*

b) In the case of *S. aureus*, the antimicrobial activity was lower. The essential ginger oil from NJoy (E.O-1.1) exhibited moderate antimicrobial activity, with an average inhibition zone diameter of 10.67 mm. The essential turmeric oil from Oleya (E.O-2.1), the essential ginger oil from Oleya (E.O-1.2), and the ethanolic ginger extract showed only local antimicrobial activity, with average inhibition zone diameters Φ=7.67 mm, Φ=6 mm, and respectively Φ=0.33 mm. The essential turmeric oil from NJoy (E.O-2.2), the ethanolic turmeric extract (TE), the turmeric extract derived from chloroform (TC), and the chloroform ginger extract (GC) do not exhibit antimicrobial activities (Figure 4).

c) For *C. albicans*, the essential ginger oil from NJoy (E.O-1.1) exhibited significant antimicrobial activity, with an average inhibition zone diameter Φ = 18 mm, followed by the essential turmeric oil from Oleya (E.O-2.1) with Φ = 13 mm, and the essential oil of ginger from Oleya (E.O-1.2) with Φ = 12.67 mm.

The turmeric extract derived from chloroform (TC), the essential oil of turmeric NJoy (E.O-2.2), and the chloroform ginger extract (GC) displayed only local antimicrobial activity, with average inhibition zone diameters Φ = 10 mm, Φ = 9.67 mm, and Φ = 3 mm, respectively. The ethanolic extract of turmeric and ginger does not exhibit antimicrobial activity (Figure 5).

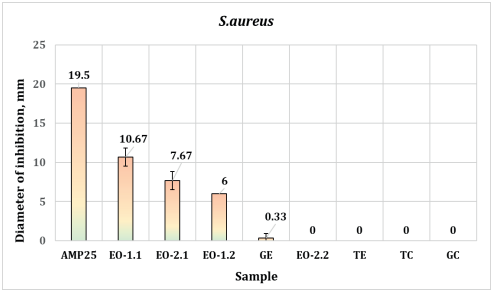

Figure 4. Antimicrobial activity of turmeric and ginger bioproducts on *S. aureus*.

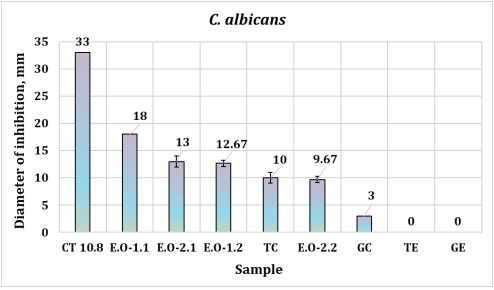

Figure 5: Antimicrobial activity of turmeric and ginger bioproducts on *C. albicans*

d) In the case of *Candida parapsilosis*, the essential ginger oil from NJoy (E.O-1.1) and the turmeric extract derived from chloroform exhibited significant antimicrobial activity, with average inhibition zone diameters Φ = 18.33 mm and Φ = 16.67 mm, respectively. The ethanolic turmeric extract showed only local antimicrobial activity, with an average inhibition zone diameter Φ = 2 mm. The other bioproducts do not exhibit antimicrobial activity for *Candida parapsilosis* (Figure 6).

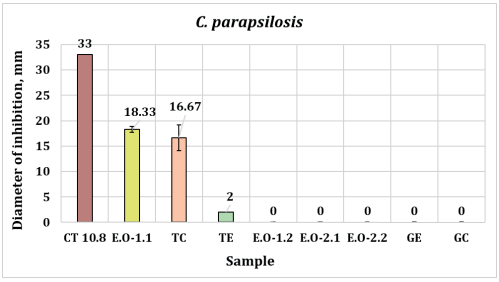

Figure 6. Antimicrobial activity of turmeric and ginger bioproducts on *C. parapsilosis.*

The analysis of turmeric essential oils highlighted the predominant presence of Ar-turmerone. These findings are supported by other researchers who have identified Ar-turmerone as the main compound in turmeric essential oil. For instance, Poudel et al. (2022) detected 65 constituents in the essential oil of *C. longa*, with Ar-turmerone being the dominant compound, reaching a concentration of 25.5% in the system. Another study by Pino et al., (2018) confirmed that Ar-turmerone was the most abundant compound (45.5%) in the analysed essential oil. For ginger essential oils, the composition varies between the two suppliers. The essential oil from NJoy contains geraniol as the major compound, followed by nerol. In contrast, the essential oil from Oleya has zingiberene as the dominant compound, followed by cedrene. Variations in the composition of essential oils from different sources have also been reported by Al-Dhahli et al., (2020). Studies performed on essential oils obtained from two different sources (Chinese ginger and Saudi ginger) revealed differences in their chemical composition. Chinese ginger essential oil had α-zingiberene as the dominant compound, whereas in Saudi ginger essential oil, the primary component was Ar-curcumene. The authors emphasise that such variations are common in essential oils and can be attributed to factors such as the genetic diversity of plants, climatic conditions, and extraction techniques. Regarding bioproducts obtained through solvent extractions, the turmeric rhizome extracts in ethanol and chloroform were found to contain tumerone and curlone as the predominant compounds. These results align with findings reported by Raje et al. (2015), who identified tumerone and curlone as the major constituents in turmeric extracts. In terms of antimicrobial activity against *Escherichia coli,* the essential ginger oil from NJoy (E.O-1.1) exhibited the highest antimicrobial activity, with an average inhibition zone diameter of 18.33 mm. This result is consistent with other studies (Silva et al., 2018; Imamović et al., 2021), where inhibition diameters of 19 mm and 23.67 mm, respectively, were reported for ginger essential oil on *E. coli*. The significant antimicrobial efficacy of NJoy ginger essential oil may be attributed to its high geraniol content, a finding also supported by studies conducted by Mączka et al., (2020). The mechanism of action of geraniol is based on its ability to dissolve lipids in the microbial membrane, leading to loss of cellular integrity and microbial cell death. Other researchers (Odo et al., 2023; Gonçalves et al., 2019) have reported a local antimicrobial activity for alcoholic extracts and turmeric essential oil against *E. coli,* with average inhibition zone diameters Φ = 7 mm and Φ = 8 mm, respectively. These findings are consistent with the results obtained in this study for biopreparations TE and E.O-2.2. For *S. aureus,* the essential ginger oil from NJoy (E.O-1.1) exhibited the highest antimicrobial activity, with an average inhibition zone diameter Φ = 10.67 mm, a value comparable to those reported by other studies (Sharma et al., 2016; Njobdi et al., 2018). The results suggest that most of the tested essential oils exhibit moderate antimicrobial activity against *S. aureus*. Exposure to the essential turmeric oil from Oleya (E.O-2.1) led to the appearance of an inhibition zone Φ = 7.67 mm, a value which indicates a local antimicrobial activity on *S. aureus*, similar to the value reported by Gonçalves and collaborators (Gonçalves et al., 2019) (Φ = 8.3 mm). This similarity may be attributed to the common

presence of Ar-turmerone and α-curcumene in turmeric essential oils.

Exposure *Candida sp*., to the essential ginger oil (E.O-1.1), containing geraniol and nerol as major compounds, resulted in the appearance of significant antimicrobial activity. This observation is supported by other studies, which indicate the antifungal effect of these compounds. For instance, da Silva et al. (2024) demonstrated that geraniol exhibits significant antifungal activity on *Candida sp*., inhibiting microorganism growth at concentrations between 110 µg/mL and 883 µg/mL. Sharma et al. (2018) showed that the geraniol strongly inhibits *Candida sp.* growth, by preventing processes of biofilm formation, cell adhesion, and secretion of hydrolytic enzymes. Tian et al. (2017) evaluated the activity of nerol (a major compound in the essential oil used in this study, respectively E.O-1.1) and reported its involvement in both early and late apoptosis of *C. albicans* cells. Regarding the antimicrobial activity of biopreparations obtained through alcohol extraction, it was observed that these types of bioproducts generally do not exhibit antimicrobial activity for *C. parapsilosis*, *C. albicans*, or *S. aureus*. In the case of *Escherichia coli,* the alcoholic extracts obtained from ginger or turmeric rhizomes exhibit a local antimicrobial activity

CONCLUSIONS

Studies perforrmed regarding the antimicrobial properties of various bioproducts derived from turmeric and ginger rhizomes, revealed distinct activities. During the preliminary tests, it was found that the essential ginger oil from NJoy (E.O-1.1) exhibited moderate or significant antimicrobial activity against all tested microorganisms, namely: *E. coli* (moderate antimicrobial activity), *S. aureus* (significant antimicrobial activity), *C. albicans* (significant antimicrobial activity), *C. parapsilosis* (significant antimicrobial activity). The essential turmeric oil from Oleya (E.O-2.1) showed moderate antimicrobial activity on microorganisms such as *E. coli* and *C. albicans*. Among the bioproducts obtained by solvent extractions, the bioproduct obtained through extraction with chloroform and turmeric rhizomes, which contain 398 mg GAE/L, exhibit significant antimicrobial activity against *C. parapsilosis* and *C. albicans*. The bioproducts obtained by extraction from alcoholic media and turmeric or ginger rhizomes exhibit local antimicrobial activity on *E. coli* but do not exhibit antimicrobial activity on *C. albicans*, *C. parapsilosis*, or *S. aureus*.

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