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ENHANCING STORAGE CAPACITY OF POTATO SYNTHETIC SEEDS THROUGH THE USE OF SALICYLIC ACID

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Abstract

Solanum tuberosum is one of the most economically important species for food consumption. Because the species is susceptible to various systemic pathogens, in vitro techniques are preferred for storing the germplasm. Synthetic seed technology can be a useful tool in plant conservation, as it combines the advantages of vegetative and generative propagation. Using this technology, in combination with salicylic acid, a plant growth regulator known to mediate the plant response to cold temperatures, this study aims to enhance the tolerance of Solanum tuberosum explants to cold temperatures during in vitro storage. Nodal segments and shoot tips obtained from in vitro cultures of Solanum tuberosum 'Salad Blue' were encapsulated in sodium alginate solutions containing different concentrations of salicylic acid (0; 25 µM, 50 µM, and 75 µM) and stored at 4°C and under dark conditions for 60 days. Synthetic seeds were inoculated on a regeneration medium with 0.3 mg/L IAA and different concentrations of BAP (2 mg/L, 3 mg/L, and 4 mg/L). Even though the growth regulators in the culture medium did not influence the regeneration capacity of the explants, supplementing the alginate matrix with 25 µM salicylic acid increased the storage capacity of the encapsulated explants.

Key words*: artificial seeds, in vitro conservation, slow growth conservation, Solanum tuberosum.*

INTRODUCTION

Synthetic seeds are a type of artificial seeds that are created to emulate the structure of a natural seed. They are a type of slow-growth technique used for short and medium-term *in vitro* conservation of plant germplasm. The term was first introduced in 1977, by Toshio Murashige. Initially, it referred to only encapsulated somatic embryos (Murashige, 1977), but later, Bapat et al. (1987), has expanded the concept to non-embryogenic tissues. Non-embryogenic tissues that can be encapsulated include axillary or terminal buds, nodal segments, cell aggregates, or any other type of tissue that can develop into plants after short and medium periods of storage. Non-embryogenic tissues are preferred because somatic embryos have an asynchronous development and many species are recalcitrant to the process of obtaining them. However, they possess the ability to simultaneously develop shoots and roots, compared to other types of tissues (Ara et al., 2000; Micheli & Standardi, 2016; Magray et al., 2017). The advantages of cold storage in micropropagation are that it can diminish the cost of maintaining germplasm *in vitro*, as it minimizes manual labor and the number of subcultures (West et al., 2006) as well as costs associated with medium components, electricity, and space.

The applications of this technology are various, and include the multiplication of endangered species, elite genotypes, species where seed production is difficult or where the seeds are not true to type, or even commercially important species (Ray & Bhattacharya, 2008; Ghanbarali et al., 2016).

This technology has been successfully applied in *Solanum tuberosum*, by several authors, as it is an alternative that is very convenient to the conventional propagation of this species (Ghanbarali et al., 2016). Conventional conservation of potatoes is represented by storing tubers, which means they have to be grown annually, which is time-consuming (Roque-Borda et al., 2021). Germplasm conservation using true seeds is not an option, since this species is highly heterozygous and it produces seeds that are not true to type. However, since *Solanum tuberosum,* is a species

that originated in South America, in the subtropical biome, its capacity to withstand the low temperatures that are used within this technology, is limited. There are, nevertheless means of increasing the resistance to chilling temperatures in several species.

Cold storage using synthetic seed technology allows to storage of potato shoot tips for 180 days at temperatures of 4°C and 10°C, however, storing them at 4°C can successfully increase the storage time to 270 days, as the shoot tips will progressively turn brown faster at temperatures of 10°C, compared to 4°C (Nyende et al., 2003). Preculture of *Solanum tuberosum* 'Sante' and 'Agria' explants in MS medium supplemented with 10^{-6} M concentration of 24-epibrassinolide before the encapsulation of explants enhances the growth of axillary buds (Ghanbarali et al., 2016). 24-epibrassinolide (EBr) is a type of brassinosteroid, a growth regulator that was observed to influence a range of growth and development processes (Ghanbarali et al., 2016; Planas-Riverola et al., 2019) and to increase the tolerance to different types of abiotic stress, such as salt stress (Alam et al., 2019; Sousa et al., 2022), pesticide stress (Sharma et al., 2016), high and cold temperature stress (Xi et al., 2013; Chen et al., 2019; Fang et al., 2019). Direct sowing into *ex vitro* conditions, in soil, is possible using encapsulated nodal segments, with a survival rate of 57%, if treated with rooting powder before planting (Sarkar & Naik, 1998).

Another growth regulator with an important role in mediating the resistance to low temperatures is salicylic acid. Salicylic acid (S.A.) is renowned not just as a signal molecule mediating plant immunity, but also for its role as a plant growth regulator (Hayat et al., 2007; Rivas-San Vicente & Plasencia, 2011; Li et al., 2022). It has been demonstrated to have a role in mediating the plant response under different types of abiotic stress, for instance, salt stress (Idrees et al., 2012; Jayakannan et al., 2015) cadmium stress (Krantev et al., 2008; Kovács et al., 2014), drought stress (Bandurska & Stroi ski, 2005; Hayat et al., 2008; Chen et al., 2014), cold temperatures stress (Chen et al., 2020; Guo et al., 2023) and biotic stress (Emilda et al., 2020; Li et al., 2022).

There is numerous research that focuses on the influence of salicylic acid on cold temperature tolerance in several economically important species, for example, *Zea mays* (Li et al., 2017; Zhang et al., 2021), *Citrullus lanatus* (Jing-Hua et al., 2008), *Triticum aestivum* (Ignatenko et al., 2019; Wang et al., 2021), *Solanum melongena* (Chen et al., 2011) and *Spinacia oleracea* (Shin et al., 2018). Temperatures of 8°C are low enough to cause an increase in the endogenous levels of salicylic acid in *Cucumis sativus* seedlings (Dong et al., 2014). In wheat, the exogenous application of 100 µM salicylic acid enhances the activity of antioxidant enzymes and the accumulation of proline, increasing tolerance to cold temperatures (Ignatenko et al., 2019).

The exogenous treatment of leaves and roots with a 0.5 µM salicylic acid solution for one day can increase the chilling tolerance in sensitive banana seedlings (Kang et al., 2003).

In fruits, it has been regarded to enhance the tolerance to chilling injury of 'Hayward' kiwifruits by controlling the metabolism of hormones and proline and by maintaining the structure of the cell (Niu et al., 2024). In cucumber, salicylic acid has a critical role in the response of seedlings to chilling tolerance. (Dong et al., 2014) noted that the treatment of cucumber seedlings with inhibitors of salicylic acid biosynthesis will reduce the accumulation of endogenous S.A. and the plants have less tolerance to chilling injury.

Therefore, taking into account the multiple aspects previously reported, the purpose of this study is to observe the response of the explants of *Solanum tuberosum* 'Salad Blue' to the encapsulation technique and to assess the influence of salicylic acid on the cold storage capacity of the encapsulated explants.

MATERIALS AND METHODS

Preparation of encapsulation solutions and culture medium

For the encapsulation of *Solanum tuberosum* 'Salad Blue' explants, 4 variants of sodium alginate solutions were used, and one variant of calcium chloride solution.

The encapsulation matrix consisted of 3% (w/v) sodium alginate and 3% (w/v) D(+) Sucrose, prepared with basal MS macro elements, microelements, and vitamins, as described by (Murashige & Skoog, 1962). Three concentrations were employed to observe salicylic acid's influence on synthetic seeds'

storage capacity: 25; 50 and 75 µM. The composition of each variant of sodium alginate solution is detailed in Table 1.

Table 1. The composition of the sodium alginate solutions used for the encapsulation of explants

No	Code	Composition
1.	V0	MS components, 3% sucrose, 3% sodium alginate and no salicylic acid
2.	V1	MS components, 3% sucrose, 3% sodium alginate, and 25 µM SA
3.	V2	MS components, 3% sucrose, 3% sodium alginate, and 50 µM SA
4.	V3	MS components, 3% sucrose, 3% sodium alginate, and 75 µM SA

Regarding the hardening solution, only one variant of 100 mM $CaCl_2$, prepared in distilled water, was used. The regeneration medium for *Solanum tuberosum* 'Salad Blue' synthetic seeds consisted of basal MS macro elements, microelements, and vitamins, as described by Murashige and Skoog (1962), with 3% (w/v) D(+) Sucrose, 7 g/L Agar, in three variants, with three different concentrations of BAP (2; 3 and 4 mg/L) and 0.3 mg/L IAA, detailed in Table 2.

Table 2. The composition of the culture mediums used for the regeneration of synthetic seeds

No.	Code	Composition
1.	X0	MS components, 3% sucrose, 7 g/L agar, and no growth regulators
2.	X1	MS components, 3% sucrose, 7 g/L agar + 2 mg/L BAP and 0.3 mg/L IAA
3.	X2	MS components, 3% sucrose, 7 g/L agar + 3 mg/L BAP and 0.3 mg/L IAA
4.	X3	MS components, 3% sucrose, 7 g/L agar + 4 mg/L BAP and 0.3 mg/L IAA

The storage medium for low-temperature conservation contained MS salts in quartered concentration and 2.5 % (w/v) D (+) sucrose. The liquid medium was distributed approximately 40 ml in small jars of 100 ml total capacity

The pH of all culture mediums, storage medium, and sodium alginate solution was adjusted to 5.75 and then sterilized in the autoclave at 121°C and 1.1 bar atmospheric pressure for 20 minutes.

Encapsulation of explants

The biological material used for the experiment 'Salad Blue' shoots (Figure 1) from *in vitro* cultures, maintained in the Plant Micropropagation Laboratory of the Research Center for Studies of Food Quality and Agricultural Products from the University of Agronomic Sciences and Veterinary Medicine of Bucharest. Before encapsulation, nodal segments were grown for 2 months on Murashige and Skoog medium (Murashige & Skoog, 1962), without any growth regulators.

Figure 1. *In vitro* obtained shoots of *Solanum tuberosum* 'Salad Blue' used for encapsulation

The *in vitro* grown shoots were cut into approximately 2-3 mm long nodal segments, with at least one axillary bud present (Figure 2), and placed in the sodium alginate solution.

Figure 2. Nodal segments of *Solanum tuberosum* 'Salad Blue' prepared for encapsulation

The explants, together with a small quantity of the sodium alginate solution, were dipped into the calcium chloride solution using a glass pipette. The $CaCl_2$ solution containing the explants was constantly stirred (on a magnetic stirred, at approximately 10 rpm) during the ion exchange process, to allow the formation of isodiametric capsules. After 13 minutes, the encapsulated explants were rinsed three times with sterile distilled water to remove any remains of the $CaCl_2$ solution. After rinsing, the

capsules were dried for a few minutes on sterile filter paper (Figure 3) and then placed in small jars containing the conservation medium.

Figure 3. Encapsulated nodal segments of *Solanum tuberosum* 'Salad Blue'

The storage medium for low-temperature conservation contained MS salts in quartered concentration and 2.5% (w/v) D (+) sucrose. Synthetic seeds were stored in the conservation medium, for 60 days, at 4°C, under dark conditions. After 60 days, the synthetic seeds were inoculated on the regeneration mediums, as detailed in Table 3. The synthetic seeds were transferred into the growing room, at a temperature of 22-25°C, 5023 lx light intensity using white, red, and blue light-emitting diodes (LEDs) and with a 16 hours light and 8 hours darkness photoperiod.

Statistical analysis

For the statistical analysis, The Real Statistics Resource Pack (https://real-statistics.com/) for Excel 2019 was used. Because the sample size was not equal for all variants, the Kurskal-Wallis test was used for the analysis of variance instead of ANOVA.

Table 3. Regeneration medium for *Solanum tuberosum* 'Salad Blue' synthetic seeds with different concentrations of 6-benzylaminopurine (BAP) and Indole-3-acetic acid (IAA)

Medium variant	Growth regulators concentrations	Control - 0 µM S.A. (V0)	Variant 1 - 25 µM S.A (V1)	Variant 2 - 50 µM S.A (V2)	Variant 3 - 75 µM S.A (V3)
X0	BAP (mg/L)	0	2	3	4
	IAA (mg/L)	0	0.3	0.3	0.3
X1	BAP (mg/L)	0	2	3	4
	IAA (mg/L)	0	0.3	0.3	0.3
X2	BAP (mg/L)	0	2	3	4
	IAA (mg/L)	0	0.3	0.3	0.3
X3	BAP (mg/L)	0	2	3	4
	IAA (mg/L)	0	0.3	0.3	0.3

RESULTS AND DISCUSSIONS

Considering the total number of seeds encapsulated in each variant of salicylic acid (V0 - 0 µM, V1 - 25 µM, V2 - 50 µM and V3 - 75 µM), the highest percentage of regeneration was observed in variant V1 with 25 µM salicylic acid (78.72%), followed by the variant V3 with 75 µM salicylic acid (58.33%), V0 - 0 µM salicylic acid (51.06%) and V2 with 50 µM salicylic acid (43.75%), as it can be observed in Figure 4.

Generally, the highest regeneration percentages of 91.67% were achieved in variant V1X1 (25 µM on medium with 2 mg/L 1 BAP and 0.3 mg/L IAA), variant V1X3 (25 µM on medium with 4 mg/L and 0.3 mg/L IAA), and variant V0X0 (0 µM S.A. and no hormones in the regeneration medium), as depicted in Figure 5.

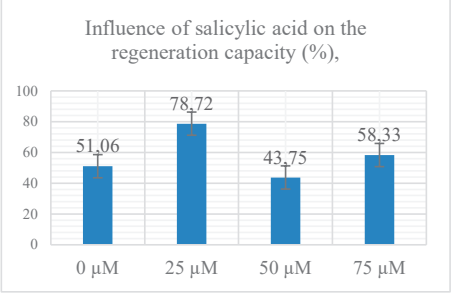

Figure 4. Influence of the concentration of salicylic acid on the regeneration of synthetic seeds observed two weeks after inoculation on medium

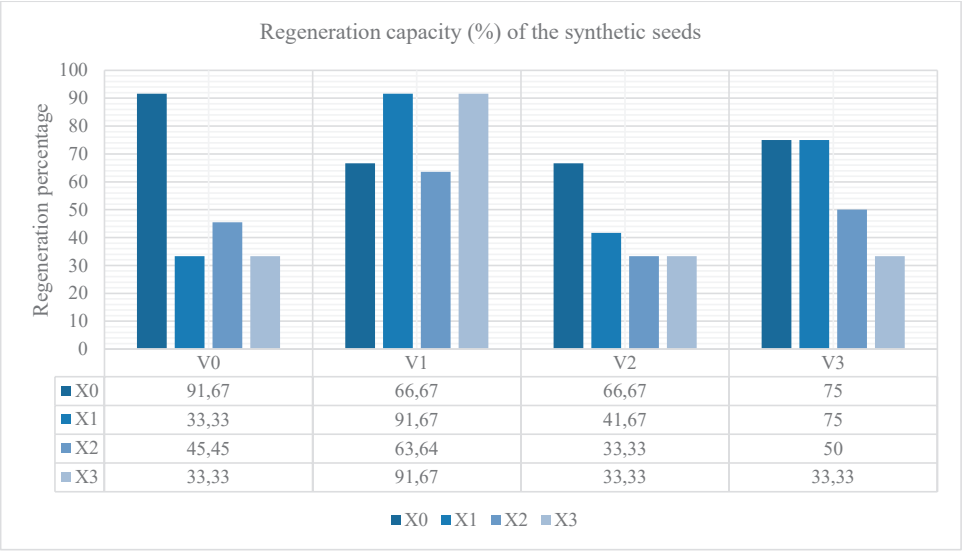

Figure 5. The regeneration capacity (%) of the synthetic seeds, observed two weeks after inoculation on the regeneration medium

Regarding the concentrations of growth regulators present in the growing medium, the highest percentage of regeneration was recorded on X0, the control medium, with no growth regulators (81.25%). Adding IAA and different concentrations of BAP decreased the speed of regeneration from 60.42% in the X1 variant (2 mg/L BAP + 0.3 mg/L IAA) to 47.92% in variant X3 (4 mg/L BAP and 0.3 mg/L IAA) and to 47.83% in variant X2 (mg/L BAP and 0.3 mg/L IAA), accordingly to Figure 6.

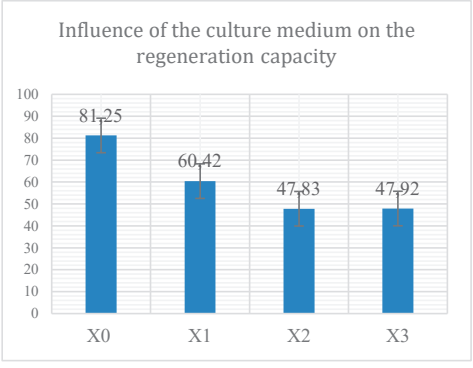

Figure 6. Influence of the concentration of growth regulators on the regeneration of synthetic seeds, observed two weeks after inoculation on medium

It must be noted, that all explants inoculated on the mediums with growth regulators were able to regenerate shoots, but at a very low speed, because during the first month of culture, the tissue at the base of the explants started dedifferentiate and produce callus cells.

Growth of synthetic seeds

Average shoot length (mm)

The highest values regarding the average shoot length seeds were recorded in the variants of synthetic seeds that were sown on hormone-free medium: 56.18 mm (V3X0 - 75 µM S.A.), 54.62 mm (V1X0 - 25 µM S.A.), 54.44 mm (V2X0 - 50 µM) and 49.32 mm (V0X0 - 0 µM). Overall, the lowest average growth values were recorded in the variants that were sown on the mediums with the highest concentrations of BAP (X): 4 mg/L BAP and 0.3 mg/L IAA (Figure 7). Kruskal-Wallis revealed statistical differences between the analyzed variants.

Regarding the overall hormone concentrations of the regeneration medium, the Kruskal-Wallis test revealed significant differences between the four variants, with growth factor declining with increasing hormone concentrations, with the highest value of 54.31 mm on the hormone-free medium (X0) and the lowest value of 22.45 mm on the medium with 4 mg/L BAP and 0.3 mg/L IAA (Figure 8 A). No statistically significant differences were recorded in the variants sown on medium with 2 mg/L BAP and 0.3 mg /L IAA (X1) and 3 mg/L BAP and 0.3 mg/L IAA (X2).

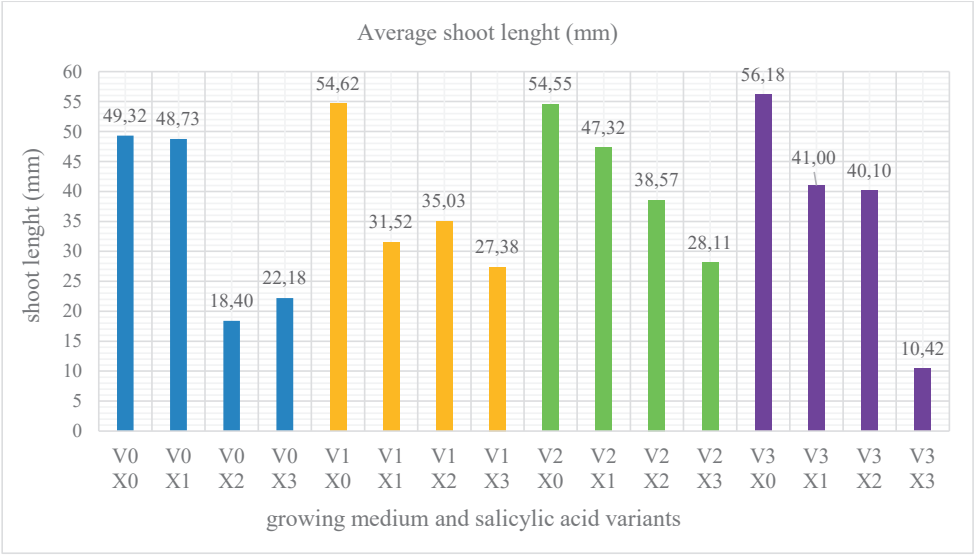

Figure 7. Average length (mm) of the shoots regenerated from the encapsulated explants

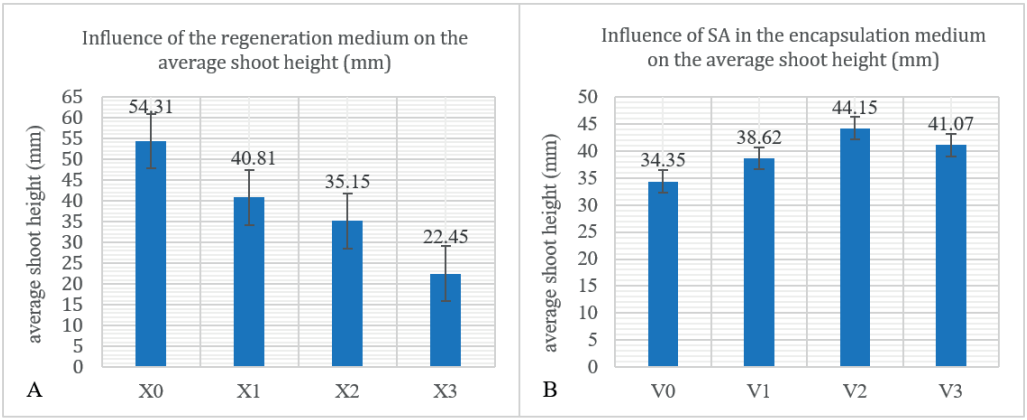

Figure 8. Influence of the growth regulators in the regeneration medium on the average shoot height (A). Influence of the encapsulation matrix on the average shoot height (B)

If analyzed by the concentration of salicylic acid in the medium, the Kruskal-Wallis test reveals a p-value > 0.05, pointing out no statistically significant differences regarding the average shoot length.

The highest average value of 44.15 mm was obtained for the explants encapsulated in the variant with 50 µM SA, followed by 41.07 mm on the variant with 75 µM, 38.62 mm on the variant with 25 µM and the lowest value, 34.35 mm on the control variant.

CONCLUSIONS

Even though it originates from a subtropical biome, *Solanum tuberosum* is a species that has a good potential to be conserved using *in vitro* cold slow-growth techniques and synthetic seed technology. Because conventional conservation of this species through tubers is time-consuming and not economical, and because conservation through seeds is not possible, the development of other conservation protocols is important.

Encapsulation of explants of *Solanum tuberosum* using synthetic seed technology can also ensure that the material that is regenerated from them is free of pests and diseases, and, compared to other *in vitro* conservation methods, such as cryopreservation, it is less expensive and requires less specialized equipment.

Supplementing the alginate matrix with 25 µM salicylic acid increases the regeneration capacity to 78.72% in synthetic seeds of 'Salad Blue'. Sharifeh et al. (2011), obtained similar results, where supplementing the alginate matrix with 25 µM or 50 µM salicylic acid increases the viability of *Helianthus annus* synthetic seeds, after 90 days of storage.

Regarding the regeneration medium, growth regulators are not mandatory for the regeneration of 'Salad Blue' nodal segments, as our study has shown that it decreases the speed of regeneration and the height of the shoots, as the concentrations of BAP and IAA used in this experiment stimulated more the dedifferentiation of cells and callus growth, than the regenerations of shoots.

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IN VIVO TESTING OF LACTIC ACID BACTERIA AS BIOCONTROL AGENTS AGAINST *B. cinerea* ON WHITE GRAPE BERRIES

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Abstract

The aim of this study was to test three strains of lactic acid bacteria (LAB): Lactobacillus fermentum MI204, Lactobacillus plantarum MI207 and Pediococcus pentosaceus MI213 in order to establish their potential to inhibit the development of the gray mold, Botrytis cinerea, on the surface of white grape berries to extend their shelf life during the storage period. The experiments involved the treatment of grape berries with LABs suspension, followed by their artificial contamination with B. cinerea spores. The berries were than stored and sampled every 24 hours for determining the load of B. cinerea and LABs by culture-based techniques and by qPCR. The L. plantarum MI207 strain was shown to be effective in inhibiting the development of the B. cinerea fungus on the surface of white grapes. Furthermore, all three LAB strains were able to maintain their viability and multiply on the surface of the grape berries during the storage period. The qPCR technique proved to be effective in quantifying the genetic material from both B. cinerea and the three strains of LAB in a shorter time compared to the use of the culture-based methods.

Key words: *lactic acid bacteria, Botrytis cinerea, biocontrol, qPCR.*

INTRODUCTION

Grapes are among the most consumed fruits around the world. The main purpose of the global grape production is wine-making, accounting for 57% of total production, while 36% is used for table grapes and 7% for dried grapes. Furthermore, the demand for grapes and grape products is increasing due to the associated health benefits, including reduced risks of cardiovascular diseases, type 2 diabetes, certain cancers, and other chronic complications (Zhou & Raffoul, 2012; Perestrelo et al., 2014; Rathi & Rajput, 2014; Insanu et al., 2021). Yearly, *B. cinerea* causes disastrous losses in grape production, typically reducing yields by 20-30%, and in severe cases losses can reach up to 50% of the total yield (Shen et al., 2021).

To preserve their freshness and quality, grapes require special storage conditions, and the management of humidity and temperature is crucial to prevent infections with different fungi, among which the most common is *Botrytis cinerea*, also known as the gray mold (Fedele et al., 2020). *B. cinerea* can colonize over 500 plant species (Williamson et al., 2007), the most severely affected being vegetables such as cucumbers, tomatoes and zucchini, but also fruits such as strawberries, grapes or raspberries (Cheung et al., 2020).

Table grapes must be kept at low temperatures (0-2°C) and at a relative humidity of 90-95% to prevent dehydration, maintain texture, slow down metabolic processes and prevent the development of mold and other microorganisms. Adherence to proper storage and hygiene practices is essential to prevent and control *Botrytis* infections (Ky et al., 2012; Zhang et al., 2018).

B. cinerea is a spoilage fungus difficult to control, which is why its chemical control remains the main way of inhibiting its development. Classically, the use of synthetic chemical fungicides such as iprodione, fenheaxmid, pyrimethanil, benzimidazoles, triazoles, dithiocarbamates and strobilurines are among the main management method to prevent gray mold (Diguță et al., 2016; Xu et al., 2021; Boiu-Sicuia et al., 2023). However, due to the risk of fungicide resistance or growing food safety concerns associated with fungicide residues on fresh fruits and crops that could harm consumer health in the long term

(AbuQamar et al., 2017), researchers are making increasing efforts to find alternative and safe methods to combat fungal growth.

Hence, biopreservation refers to the extended shelf life and increased safety of food and raw materials obtained by using natural microflora or by using antimicrobial products produced by certain microorganisms (Schnürer & Magnusson, 2005). Among the microorganisms most often used as biological control agents are various species of *Bacillus*: *B. subtilis*, *B. amyloliquefaciens* and *B. velezensis*; *Trichoderma*: *T. atroviride*, *T. harzianum*, *T. asperellum* and *T. gamsii*; and *Pseudomonas* spp. (Sicuia et al., 2015[a]; Sicuia et al., 2015[b]; Mondello et al., 2018; Proca et al., 2020; Lahlali et al., 2022; Boiu-Sicuia et al., 2023; Toma et al., 2023).

Lactic acid bacteria (LAB), mainly known for their probiotic potential (Diguță et al., 2020; Coulibaly et al., 2023; Kouadio et al., 2024), are used as natural biopreservatives in food, in animal feed, in sauerkraut and silage (Voulgari et al., 2010; Badea et al., 2022). Their preservative effect is mainly due to the formation of organic acids and hydrogen peroxide, competition for nutrients and the production of antimicrobial substances (Stiles, 1996; Pristavu et al., 2022).

In recent years, numerous studies have turned their attention to the use of lactic acid bacteria as biological control agents (De Simone et al., 2021; Dopazo et al., 2022; Wafula et al., 2022). LABs are known for their ability to produce numerous antifungal compounds, such as organic acids (lactic, acetic, formic, citric and succinic acid, phenyllactic acid) (Zalán et al., 2010; Yoo et al., 2016; Badea et al., 2022), hydroxyl-fatty acids (Kanauchi, 2019), various protein compounds (Schnürer & Magnusson, 2005) and reuterin (Ortiz-Rivera et al., 2017).

In this context, this study involved the *in vivo* evaluation of three strains of LAB for their potential to inhibit the development of the fungus *B. cinerea* in white grape berries.

MATHERIALS AND METHODS

Microorganisms and growth conditions

Three strains of LAB were used in this study: *Lactobacillus fermentum* MI204, *Lactobacillus plantarum* MI207, and *Pediococcus pentosaceus* MI213. These three strains of LAB have previously been shown by *in vitro* tests to have activity against *B. cinerea*. All of them are belonging to the microorganism collection of UASVM Bucharest. The strains were stored under freezing at -20°C, in MRS broth medium (Man, Rogosa and Sharpe, Oxoid, Limited, Hampshire, United Kingdom) containing 20% glycerol. For the revitalization, the LABs were cultivated in MRS broth medium at 37°C for 24 hours. The fungal strain used was *B. cinerea* MI Aligote Husi, grown on potato dextrose agar (PDA, Alliance Bio Expertise, France) for 2 weeks at 25°C.

Sample preparation

Studied LAB strains were tested for antifungal potential against *B. cinerea* on white grape berries, according to the following protocol.

White table grapes were purchased from a local supermarket, and the berries were separated from the grape brunches by cutting the pedicel, ensuring that it remained attached to the berry. The surface sterilization of the berries was performed by immersion in a 1% sodium hypochlorite solution for 5 minutes, followed by 3 washes with sterile distilled water (Lazo-Javalera et al., 2016). The berries were than sprayed with a sterile suspension of 0.2% sodium carboxymethylcellulose (CMC) and dried under sterile conditions in a laminar flow hood to form a protective coating (Li et al., 2020). Afterwards, the grapes were sprayed with suspensions of viable LABs, and one of the samples was left untreated, representing the control. The berries were then distributed in sterile polypropylene boxes, and a cutting was made on the epicarp area in which *B. cinerea* spores with a concentration of 2.3×10^3 spores mL^{-1} were inoculated. The samples were distributed according to Table 1.

In order to quantify the amount of *B. cinerea* and LABs on the surface of artificially contaminated grapes, three berries were taken in 3 mL of sterile distilled water, after which they were vigorously shaken to retrieve the LABs cells and *B. cinerea* spores and hyphae from their surface. The suspension thus obtained was used in subsequent analyses. Samples were taken at time 0 and after 24, 48 and 72 hours.

Table 1. Working protocol for artificial inoculation with LABs and *B. cinerea* of grape berries

Sample code	MI204	MI207	MI213	*Botrytis cinerea*
		log_{10} cells mL^{-1}		log_{10} spores mL^{-1}
204	8.29	-	-	-
207	-	8.31	-	-
213	-	-	8.49	-
204Bc	8.29	-	-	3.36
207Bc	-	8.31	-	3.36
213Bc	-	-	8.49	3.36
Bc	-	-	-	3.36

Culture-based method to quantify microorganisms

The quantification of microorganisms by classical techniques involves the use of specific culture media. For lactic acid bacteria, we used MRS Agar medium supplemented with cycloheximide, applying the incorporation technique detailed by Diguță & Matei (2020). The plates were incubated at 30°C for 48 hours. The results were expressed as decimal logarithms of colony-forming units per milliliter of sample (log_{10} CFU mL^{-1}).

Quantification of *B. cinerea* and LABs by qPCR

DNA extraction

The microbial DNA was extracted from *B. cinerea* MI Aligote Husi and *L. fermentum* MI204, using a commercial Quick-DNA™ Fungal/Bacterial Miniprep Kit (Zymo Research, Germany) according to the protocol described by the manufacturer. The DNA quantification was performed using a SpectraMax® QuickDrop™ Micro-Volume Spectrophotometer (Molecular Devices, San Jose, CA, USA), then diluted tenfold and quantified by a qPCR Rotor Gene 6000 (Corbett Research, Australia).

qPCR amplification

To create both standard curves, the DNA was diluted tenfold, ranging from 5.50 µg mL^{-1} to 550 fg mL^{-1} for *B. cinerea* and 12.40 µg mL^{-1} to 124 pg mL^{-1} for *L. fermentum*. All dilutions were made in duplicate. Specific primers for *B. cinerea* (Bc3F and Bc3R, according to Carisse et al., 2014) and specific primers or LABs (LacF and LacR, according to Ritchie et al., 2010) were used in this study. The qPCR was performed in a final volume of 25 µL with 5 µL DNA sample, 0.075 µL (0.3 mM) of each specific primers for *B. cinerea* or LABs, 12.5 µL of Maxima SYBER GREEN/ROX qPCR MasterMix (2X, Thermo-Scientific, Baltics, UAB, Vilnius, Lithuania) and water. The qPCR reactions were performed in qPCR Rotor Gene 6000 (Corbett Research, Australia) following the programs: 3 min at 95°C, followed by 40 cycles of 15 s at 95°C and 30 s at 62°C (Al Zaidi et al., 2023). At the end, a melting curve was created by dropping the temperature from 90°C by 0.5°C every 10 s. To quantify the DNA from LAB strains, the program recommended by Uțoiu et al. (2018) was used: 10 min at 95°C for the initial denaturation, followed by 40 cycles of 15 s at 95°C and 60 s at 60°C. The efficiency (E) of the qPCR assay was calculated using the formula $E = (10^{-1/slope} - 1) \times 100$, where the slope was extracted from the curve Ct = f (log Q0), where Ct is the cycle where the fluorescence first occurs, and Q0 is the amount of initial DNA. The efficiency was expressed as a percentage. Subsequently, DNA samples of grapes were submitted to qPCR, and the quantity of *B. cinerea* and LABs DNA was calculated in accordance with the standard curve that was generated for this purpose.

Statistical analysis

The results were obtained by averaging three independent experiments. The results were represented as mean ± standard deviation.

RESULTS AND DISCUSSIONS

Culture-based method to detect LABs

Classical microbial quantification techniques offer a simple and cost-effective means of determining the number of viable microorganisms in a sample by cultivating and counting them on specific culture media. In order to observe the evolution of the number of viable LABs on the surface of grape berries during storage, we utilized the classical method with MRS Agar

culture medium, applying the incorporation technique.

The macroscopic differences between the grape samples treated with LABs suspensions compared to the untreated ones after 72 hours of storage (Figure 1) suggest that the LABs suspensions had an inhibitory effect on *B. cinerea*, the fungus being slightly visible on the surface of the artificially contaminated samples, treated with the LABs suspensions (204Bc, 207Bc and 213Bc) after 72 hours of storage, compared to the untreated control sample (Bc), where the fungus grew at an accelerated rate.

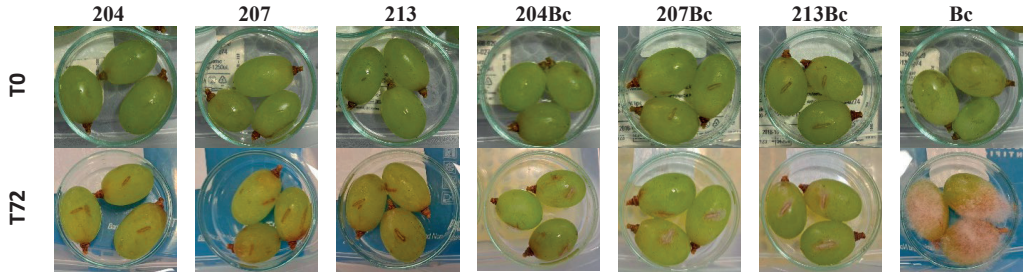

Figure 1. Macroscopic differences between the grape samples treated with LABs suspensions and uncontaminated with *B. cinerea* (204, 207, 213), the grape samples treated with LABs suspensions and artificially contaminated with *B. cinerea* (204Bc, 207Bc, 213Bc) and the grape sample untreated with LABs suspensions and artificially contaminated with *B. cinerea* (Bc), in the initial moment (T0) and after 72 hours (T72)

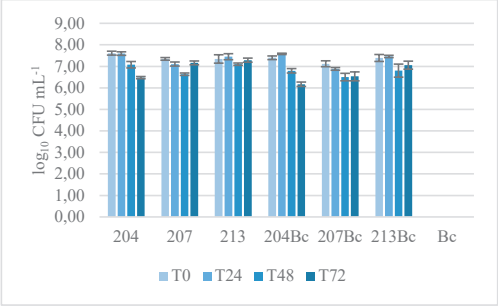

Figure 2. Quantification of LABs by the culture-based method on artificially contaminated grapes

The LABs successfully adapted on the surface of white grapes (Figure 2), maintaining a concentration around 7 $\log_{10}$ CFU mL^{-1} throughout the storage period, grapes being a native source of LABs, thus providing a favorable environment for their development (Franco et al., 2021). Comparable results were obtained by Yin et al. (2022) in a similar experiment on strawberries, where the LABs population remained constant on the surface of the fruits during the 7 days of storage.

PCR-based method to detect *B. cinerea* and LABs

In order to be able to measure the amount of DNA present in a sample using the qPCR technique, it was necessary to create standard curves by diluting the DNA extracted from *B. cinerea*, respectively from *L. fermentum* and amplifying the dilutions by qPCR, following that the Ct values thus obtained, together with the complementary logarithm of the DNA concentrations, to be transformed into standard curves (Figures 3 and 4).

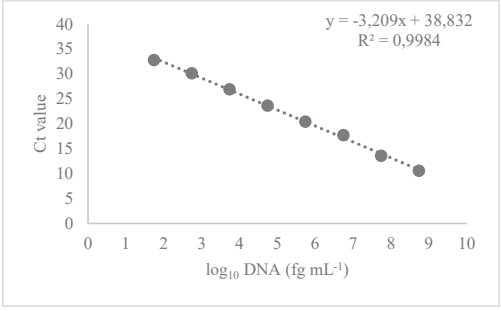

Figure 3. Standard curve generated from the amplification of *B. cinerea* DNA

Both standard curves show a high linearity due to the R^2 values close to 1 (0.999 and 0.995), resulting in a low inter-assay variability. The slopes of the standard curves were -3.209 and -3.454, corresponding to an amplification efficiency of 104.94% and 94.77% respectively, both values falling within the range of 90-110%, limit values established by the literature (Broeders et al., 2014).

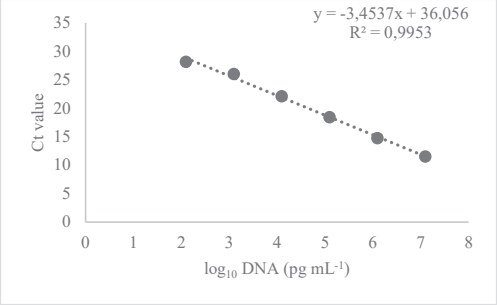

Figure 4. Standard curve generated from the amplification of *L. fermentum* DNA

Under these conditions, the maximum C_t value that could be used for *B. cinerea* was 38, corresponding to a DNA concentration of 1.81 fg, and for LABs, the maximum C_t number was 36, corresponding to a DNA concentration of 1.03 pg.

The qPCR method is a fast and efficient way to quantify DNA, through which we can determine even the lowest concentrations of DNA in a sample. In the present study we applied this method to determine the amount of DNA present on the artificially contaminated grape berries from both *B. cinerea* and the three LAB strains, the results being presented in the following.

The concentration of *B. cinerea* DNA in each grape sample is presented in Figure 5. The results indicate that the LAB strains effectively inhibited the development of *B. cinerea* on the surface of the artificially contaminated grape berries, the DNA concentrations being lower on the artificially contaminated grapes samples treated with LAB strains (204Bc, 207Bc and 213Bc) compared to the untreated control sample (Bc).

It can also be seen that the strain of *L. plantarum* MI207 showed the best efficiency in inhibiting the development of the *B. cinerea* fungus on the surface of the grape berries, the DNA concentration value after 72 hours of storage being $\log_{10}$ 1.32 fg mL^{-1} DNA, compared to $\log_{10}$ 6.49 fg mL^{-1} DNA in the case of the control sample, being also more efficient compared to the other two strains of LAB used in this study, where the DNA concentration after 72 hours of storage was around $\log_{10}$ 3.5 fg mL^{-1} DNA. Recently, similar results were obtained by Chen et al. (2022), who conducted the study in which they demonstrated the effectiveness of the *L. plantarum* CM-3 strain to inhibit the development of the fungus *B. cinerea* on the surface of black grape berries. In another study, the authors demonstrated the effectiveness of the same *L. plantarum* CM-3 strain against *B. cinerea* development on strawberries surface (Chen et al., 2020).

The qPCR method was also used to determine the amount of DNA from lactic acid bacteria on the surface of artificially contaminated grapes to see if they managed to remain viable during storage.

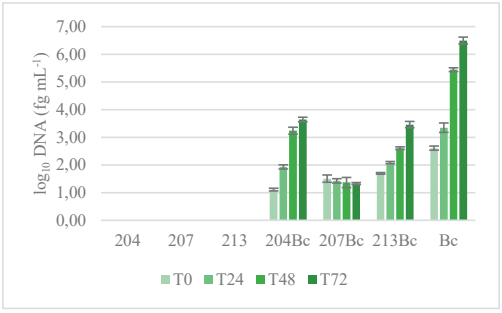

Figure 5. *B. cinerea* DNA concentration detected on the grape samples by the qPCR method, expressed in $\log_{10}$ DNA (fg mL^{-1})

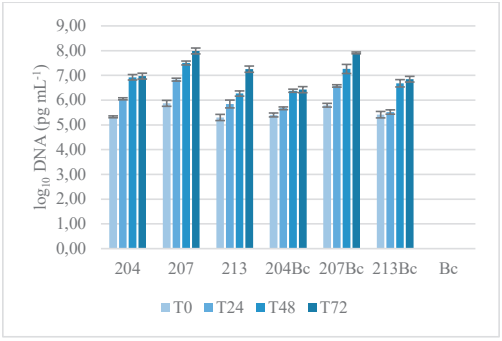

Figure 6. LAB DNA concentration detected on the grape samples by the qPCR method, expressed in $\log_{10}$ DNA (pg mL^{-1})

Compared to the results obtained by quantification using the culture-based method (Figure 2), in the case of DNA quantification by qPCR (Figure 6) we can observe an increase in the concentration of DNA from the LAB strains in the samples, an increase that could be due to the fact that the culture-based method can only quantify cells in a viable state, while the qPCR method quantifies both DNA from viable and non-viable cells.

However, the increase in the concentration of LABs DNA in the samples during the storage period suggests that, indeed, the LAB strains were able to survive and multiply on the surface of the grape berries, producing organic compounds with antifungal effect against *B. cinerea*. According to multiple studies, the qPCR method has been shown to be effective for the detection of LABs in several food matrices (Zwielehner et al., 2008; Kántor et al., 2014; Al-Zaidi et al., 2022; Jérôme et al., 2022). To improve the method and quantify only the amount of DNA from viable cells, in the following studies we can use photoactive dyes such as propidium monoazide (PMA), ethidium monoazide (EMA) or PEMAX, substances capable of binding to the nucleic acids of damaged or dead cells, thus allowing qPCR quantification of only viable cells (Daranas et al., 2018; Shi et al., 2022; Wang et al., 2023).

CONCLUSIONS

Maintaining the nutritional values and sensory properties of perishable fruits susceptible to fungal attack during storage is a significant challenge. The growing interest in organic and unprocessed food products has sparked the interest of scientists in seeking new methods of extending the shelf life of food products, thus avoiding chemical preservation methods. This study demonstrated that the *L. plantarum* MI207 strain effectively inhibited the growth of the fungus *B. cinerea* on the surface of white grapes while also maintaining its viability and multiplying on the grape berries during storage. Furthermore, the qPCR technique proved to be effective in quantifying the genetic material of both *B. cinerea* and the three LAB strains in a shorter time compared to culture-based methods. In this context, LABs offer a promising approach to be used as biocontrol agents, as they are naturally found in foods like fresh vegetables and fruits, are considered safe for human health, are widely utilized in the food industry, and also are producing antimicrobial compounds.

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ADVANCES IN ANTIMICROBIAL CONTROL: CONTRIBUTIONS AND POTENTIAL APPLICATIONS OF ANTAGONISTIC BACTERIA IN THE CONTROL OF MULTIDRUG-RESISTANT PATHOGENIC BACTERIA

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Abstract

Antagonistic bacteria play a crucial role in the fight against multi-drug resistant (MDR) pathogens. They offer an ecological and sustainable alternative to traditional antibiotics, reducing selective pressure and preserving natural microbial ecosystems. In agriculture and livestock farming, their purpose is to inhibit the growth of pathogens through competition for nutrients, producing antimicrobial substances and modulating the immune system. For example, probiotics such as Lactobacillus and Bifidobacterium improve intestinal health and reduce the need for antibiotics in animals. Similarly, bacteria such as Bacillus and Pseudomonas are used to protect plants against various diseases, reducing the need for chemical treatments. In the medical field, antagonistic bacteria are used to prevent and treat various infections, including gastrointestinal and urogenital infections. They also contribute to modulating the gut microbiota and supporting the immune system. However, there are still technical and scientific challenges to be overcome in order to optimize their use, such as understanding the complex interactions between antagonistic bacteria and pathogens, and the stability and efficacy of probiotic formulations.

Key words*: antagonistic, antimicrobial, control, multi-drug resistant, pathogenic.*

INTRODUCTION

The rise of antimicrobial resistance represents one of the most urgent and pressing challenges to global public health in the 21st century (EClinicalMedicine, 2021). Since the discovery of antibiotics, these drugs have played a crucial role in reducing morbidity and mortality from bacterial infections (Laxminarayan et al., 2013; Spellberg et al., 2014). Nevertheless, the excessive and frequently inappropriate use of antibiotics has resulted in the emergence and dissemination of multidrug-resistant (MDR) bacteria, rendering infections more challenging or impossible to treat with current therapeutic modalities (EClinicalMedicine, 2021). Indeed, it is estimated that by 2050, the lack of new antibiotics developed or discovered will result in the extinction of effective antibiotics for treating infections (Rolain et al., 2016) and that deaths from infectious diseases linked to antibiotic resistance will surpass all current causes of death (De Kraker et al., 2016). For example, in North America, the overuse of antibiotics represents a significant public health concern. Approximately 30% of antibiotic prescriptions are deemed unnecessary, contributing to the rising prevalence of drug-resistant infections in the USA (CDC, 2016).

Multidrug-resistant bacteria pose a significant threat in healthcare settings and the community (Reyes et al., 2023), affecting vulnerable populations such as hospitalized patients, the elderly, and immunocompromised individuals. MDR bacteria of greatest concern include methicillin-resistant *Staphylococcus aureus*

(MRSA), carbapenemase-producing *Klebsiella pneumoniae* (KPC), extended-spectrum beta-lactamase-producing *Escherichia coli* (ESBL), multidrug-resistant *Acinetobacter baumannii*, and multidrug-resistant *Pseudomonas aeruginosa*. These pathogens are responsible for a range of serious infections, including urinary tract infections, pneumonia, septicemia, and wound infections (Laxminarayan et al., 2016). The Centers for Disease Control and Prevention (CDC, 2016) estimates that more than 2.8 million cases of infection resulting from antibiotic-resistant bacteria occur annually, with approximately 35,000 deaths attributed to these infections. It is therefore imperative to investigate alternative methodologies for the management of antibiotic-resistant pathogens.

Faced with this growing threat, the scientific and medical community has intensified its efforts to discover and develop new strategies for controlling MDR bacterial infections. In recent years, significant progress has been made in the field of antimicrobial control, with the discovery of novel classes of antibiotics, the development of innovative combination therapies, and the optimization of treatment protocols. Furthermore, there has been a notable shift in focus towards alternative approaches, including the utilization of bacteriophages, peptides, antimicrobial peptides and immunomodulatory therapies.

Moreover, a more comprehensive grasp of the mechanisms underlying antimicrobial resistance has facilitated the development of more targeted therapeutic strategies, allowing for more precise intervention at the molecular level. The continued monitoring of antibiotic usage and the implementation of evidence-based antibiotic stewardship remain pivotal strategies for curbing the dissemination of antibiotic resistance.

The objective of this review article is to provide an overview of recent contributions to the field of antimicrobial control and to discuss the potential applications of these findings in the context of combating multidrug-resistant pathogenic bacteria. By examining scientific advances and technological innovations, this review aims to identify the most promising strategies and remaining challenges in the fight against antibiotic resistance. This review will concentrate on the potential impact of antagonistic bacteria in the development of new therapeutic approaches, thus providing an innovative perspective on how this global public health problem can be overcome. In addition, this study will examine the underlying mechanisms of bacterial resistance and their implications for the development of control strategies. Furthermore, this study details recent advances in research and development of new classes of antibiotics and alternative therapies, evaluates the potential contributions of antagonistic bacteria in the context of new approaches to combating infections caused by MDR bacterial pathogens and identifies potential clinical applications of recent discoveries and discuss future perspectives in the control of MDR bacterial pathogens.

The selection of the articles included in this review was performed based on well-known databases (Scopus, Web of Science, ScienceDirect), using specific key-words ("antibiotic resistance", "multidrug-resistant", "antagonistic bacteria").

The validation of the articles was performed manually, inserting only relevant articles with significant contributions to the field of research, resulting in fulfilling this review in its final form.

CURRENT STATE OF ANTIMICROBIAL RESISTANCE

Europe faces a worrying situation regarding antimicrobial resistance (AMR). Several recent studies show a notable increase in resistance in several common pathogenic bacteria. In a recent study conducted by the European Antimicrobial Resistance Collaborators (2022), it was estimated that 541,000 deaths were associated with bacterial AMR and 133,000 deaths were attributable to bacterial AMR across the WHO European region in 2019. As reported, seven principal pathogens were accountable for approximately 457,000 deaths associated with antimicrobial resistance in 53 European countries. The pathogens are listed in descending order of mortality: *Escherichia coli, Staphylococcus aureus, Klebsiella pneumoniae, Pseudomonas aeruginosa, Enterococcus faecium, Streptococcus pneumoniae* and *Acinetobacter baumannii*. A study by the European Antimicrobial Resistance Collaborators (2022) revealed that methicillin-resistant *S. aureus* was the predominant

pathogen-drug combination associated with AMR-related mortality in 27 countries. Similarly, aminopenicillin-resistant *E. coli* was identified as the leading cause of AMR-related deaths in 47 countries (European Antimicrobial Resistance Collaborators, 2022).

In the Americas region, the emergence of MDR bacteria represents a significant and growing threat to public health. A recent study by the Antimicrobial Resistance Collaborators (2022) demonstrated that 569,000 deaths were attributable to bacterial AMR in the 35 countries of the WHO Region of the Americas in 2019, with an additional 141,000 deaths associated with this antibiotic-resistant bacterial infection. These authors have revealed that lower respiratory tract and chest infections, as a syndrome, were responsible for the largest fatal burden of AMR in the region, with 189,000 deaths (149,000 - 241,000) associated with resistance, followed by bloodstream infections (169,000 deaths) and peritoneal/intra-abdominal infections (118,000 deaths). The six pathogens with the highest mortality rates associated with resistance were identified as *Staphylococcus aureus*, *Escherichia coli*, *Klebsiella pneumoniae*, *Streptococcus pneumoniae*, *Pseudomonas aeruginosa* and *Acinetobacter baumannii* (Antimicrobial Resistance Collaborators, 2022). The combined impact of these pathogens resulted in 452,000 deaths (326,000-608,000) attributed to AMR. Methicillin-resistant *S. aureus* was the predominant pathogen-drug combination in 34 countries in terms of AMR-attributable deaths, while aminopenicillin-resistant *E. coli* was the leading pathogen-drug combination in 15 countries for deaths associated with AMR (Antimicrobial Resistance Collaborators, 2022). Recent studies have indicated that low- and middle-income countries, such as those in Africa, are disproportionately affected by AMR. The findings of the Antimicrobial Resistance Collaborators (2022) in Africa indicate a significant concern, characterized by the prevalence of multiple resistant bacterial strains that present a formidable challenge to public health. The authors estimate that in the WHO African region in 2019, there were 1-05 million deaths (95%) associated with bacterial AMR and 250,000 deaths (192,000-325,000) attributable to bacterial AMR. Additionally, the primary causes of mortality associated with bacterial AMR are lower respiratory tract and chest infections (119,000 deaths, accounting for 48% of all estimated bacterial AMR deaths), bloodstream infections (56,000 deaths, representing 22%), intravenous infections and abdominal diseases (26,000 deaths, or 10%), and tuberculosis (18,000 deaths, or 7%). In addition, seven major pathogens were collectively responsible for 821,000 deaths (636,000-1,051,000) associated with resistance in this region, with four pathogens exceeding 100,000 deaths each. The pathogens in question are *S. pneumoniae*, *K. pneumoniae*, *E. coli* and *S. aureus*. The predominant pathogen-drug combinations identified in 25 and 16 countries, respectively, were third-generation cephalosporin-resistant *K. pneumoniae* and methicillin-resistant *S. aureus*, accounting for 53% and 34% of the entire region (comprising 47 countries) of deaths attributable to AMR (Antimicrobial Resistance Collaborators, 2022). This overview elucidates the gravity of antimicrobial resistance across disparate geographical regions (Table 1), underscoring the imperative for the formulation of global and regional strategies to confront this mounting threat.

Table 1. The incidence of antimicrobial resistance worldwide

Regions	Microorganisms	Associated illnesses	Estimated number of annual deaths	References
Africa	Methicillin-resistant S. *aureus* (MRSA)	Skin infections, pneumonia, septicemia	19,000	WHO (2021)
	Cephalosporin-resistant *E. coli*	Urinary tract infections, septicemia	8,000	CDC Africa (2022)
	Carbapenem-resistant *K. pneumoniae*	Pneumonia, wound infections	10,000	WHO (2020)
	Mycobacterium tuberculosis multiresistant	Tuberculosis	56,000	WHO (2021)
America	Methicillin-resistant S. *aureus* (MRSA)	Skin infections, pneumonia, septicemia	20,000	CDC (2021)
	Cephalosporin-resistant *E. coli*	Urinary tract infections, septicemia	15,000	CDC (2021)
	Carbapenem-resistant *K. pneumoniae*	Pneumonia, wound infections	13,000	CDC (2021)
	Carbapenem-resistant *A. baumannii*	Pneumonia, septicemia	9,000	CDC (2021)
Asia	Methicillin-resistant S. *aureus* (MRSA)	Skin infections, pneumonia, septicemia	70,000	WHO (2021)
	Cephalosporin-resistant *E. coli*	Urinary tract infections, septicemia	50,000	CDC Asia (2022)

	Carbapenem-resistant *K. pneumoniae*	Pneumonia, wound infections	45,000	WHO (2020)
	Carbapenem-resistant *A. baumannii*	Pneumonia, septicemia	40,000	WHO (2021)
	Mycobacterium tuberculosis multiresistant	Tuberculosis	230,000	WHO (2021)
Europa	Methicillin-resistant S. *aureus* (MRSA)	Skin infections, pneumonia, septicemia	25,000	ECDC (2021)
	Cephalosporin-resistant *E. coli*	Urinary tract infections, septicemia	20,000	ECDC (2021)
	Carbapenem-resistant *K. pneumoniae*	Pneumonia, wound infections	17,000	ECDC (2021)
	Carbapenem-resistant *A. baumannii*	Pneumonia, septicemia	10,000	ECDC (2021)
	Mycobacterium tuberculosis multiresistant	Tuberculosis	10,000	WHO (2021)

RESISTANCE MECHANISMS OF PATHOGENIC BACTERIA

The prevalence of resistance is experiencing a considerable increase, particularly in developing countries (Kagambèga et al., 2024). The situation is further complicated by the emergence of MDR, ultradrug resistance (XDR), and, most recently, pandrug resistance (PDR) (Pulingam et al., 2022). It is therefore the view of many authors that MDR should be defined as acquired resistance to at least one agent belonging to three or more antimicrobial categories. Similarly, XDR has been defined as acquired resistance to at least one agent belonging to all but two or fewer antimicrobial categories, while PDR has been defined as acquired resistance to all antimicrobial categories (Basak et al., 2016; Magiorakos et al., 2012).

AMR is a phenomenon that manifests itself through various complex mechanisms, which enable bacteria to survive and multiply despite the presence of antibiotics. These mechanisms can be intrinsic or acquired and include alterations to the antibiotic target, enzymatic inactivation of the antibiotic, modification of membrane permeability, and activation of efflux pumps (Abushaheen et al., 2020).

Modification of the antibiotic target

Bacteria are capable of developing mutations in the genes that encode target proteins of antibiotics. This process results in a reduction in the affinity of the antibiotic for its target (Sharmila Devi et al., 2024). In general, mutation-driven resistance mechanisms emerge as a consequence of alterations in antimicrobial agents, which may occur through three main mechanisms: (i) reduced drug absorption, (ii) activation of efflux mechanisms, or (iii) modifications in metabolic pathways (Samreen et al., 2021). For example, resistance to fluoroquinolones is frequently attributed to mutations in the *gyrA* and *parC* genes, which encode the subunits of the enzymes DNA gyrase and topoisomerase IV (Muylaert & Mainil, 2013). Moreover, a substantial body of evidence from numerous studies has demonstrated that alterations in codons 513, 526, or 531 lead to high resistance to rifampicin, whereas alterations in positions 511 and 533 result in reduced resistance in *M. tuberculosis* (Ohno et al., 1996; Somoskovi et al., 2001).

Enzymatic inactivation of the antibiotic

Bacteria can produce enzymes that degrade or chemically modify the antibiotic, rendering it ineffective (Pulingam et al., 2022). The authors define this as an enzymatic process during which the active antibiotic molecule is rendered inactive by enzymes produced by resistant bacterial cells. For example, hydrolysis is one of the most studied inactivation mechanisms. Davies (1994) demonstrated that *E. coli*, *K. pneumoniae* and *Enterobacter* spp. produce β-lactamase, which is capable of hydrolysing the β-lactam ring of penicillin, cephalosporin and carbapenem. In addition to hydrolysis, the redox process represents a further mechanism of drug inactivation, whereby a drug molecule is oxidised or reduced (Wright, 2005).

Change in membrane permeability

Bacteria can modify their outer membrane to reduce entry of the antibiotic (Abushaheen et al., 2020). Porin, an outer membrane protein (OMP), is regarded as the gateway for antibiotics, including tetracyclines and β-lactams, which gain access to *E. coli* via OmpF, and carbapenems, which enter *P. aeruginosa* through OmpD (Ramirez & Tolmasky, 2010). These researchers demonstrated that structural alteration or even functional elimination of porin genes resulted in diminished influx, which hinders antibiotic penetration into gram-negative bacteria.

Efflux pumps

Bacteria are capable of actively expelling antibiotics from the cell via efflux pumps, thereby reducing the intracellular concentration of the antibiotic. This mechanism is regarded as

the most prevalent among bacteria (Varela et al., 2013; Sharmila Devi et al., 2024) and is a subject of significant research interest (Pulingam et al., 2022). In their 2011 study, Fiamegos et al. demonstrated that efflux pumps can exhibit specificity towards a particular antibiotic, or alternatively, extrude a range of structurally and functionally diverse antibiotics (multidrug efflux pumps, or MEPs) (Fiamegos et al., 2011). According to many authors, macrolides, β-lactams, fluoroquinolones, oxazolidinones, fourth generation cephalosporins and carbapenems are the main classes of antibiotics known to be effused by intrinsic bacterial efflux pumps (Li and Nikaido, 2009; Li et al., 2015). For example, RND (resistance-nodulation-division) family efflux pump systems in *Pseudomonas aeruginosa* expel various antibiotics, including fluoroquinolones, aminoglycosides, and β-lactams (Tetard, 2021).

Enzymatic modification of the antibiotic

Bacteria can produce enzymes that chemically change the antibiotic, making the antibiotic less effective. This mechanism is observed in Gram-positive and Gram-negative bacteria (Abushaheen et al., 2020). This is due to the fact that in enzymatic modification, the addition of acetyl, adenyl or phosphate groups from bacterial enzymes to a specific site of antibiotics occurs with the intention of chemically modifying them and inactivating the antimicrobial agents, thus rendering them unable to bind at the target site (Abushaheen et al., 2020). Additionally, the work of Ramirez & Tolmasky (2010) showed that phosphorylation occurs in macrolides, while acetylation/adenylation and/or phosphorylation occurs in aminoglycosides. For example, aminoglycoside-modifying enzymes, including acetyltransferases, adenylyltransferases, and phosphotransferases, modify aminoglycosides, rendering these antibiotics incapable of binding to bacterial ribosomes (Wright, 1999).

RECENT ADVANCES IN ANTIMICROBIAL CONTROL

Recent advances in antimicrobial control have been driven by the pressing need to combat antibiotic resistance and improve infection management. These advances can be classified into three principal categories: (i) the development of novel antimicrobials, (ii) innovative technologies and (iii) the utilisation of adjuvants and synergies. In recent years, there has been a notable focus on the discovery and development of entirely new classes of antibiotics. Molecules such as teixobactin have been discovered that inhibit cell wall synthesis by binding to a highly conserved motif in lipid II (the precursor of peptidoglycan) and lipid III (the precursor of cell wall teichoic acid). These molecules represent a previously unexploited avenue of research (Ling et al., 2015). Ling et al. (2015) demonstrated that teixobactin exhibits remarkable bactericidal efficacy against *S. aureus*, with a superior capacity to vancomycin in eradicating late exponential phase populations (Ling et al., 2015).

Bacteriophages

The utilisation of bacteriophages, viruses that infect bacteria, has demonstrated considerable potential, particularly in the context of the treatment of multidrug-resistant infections (Wittebole et al., 2014). Phage therapy can be adapted to target specific pathogenic bacteria without affecting the host microbiota. Abedon et al. (2011a; 2011b) investigated the potential of phages as a means of combating pathogenic bacteria. Johri et al. (2021) demonstrated the potent antibacterial and anti-infectious efficacy of Intesti and Fersis phage cocktails, as well as "staphylococcal phages", in the management of chronic bacterial prostatitis. Moreover, additional research has demonstrated the efficacy of a phage cocktail in the treatment of urinary tract infections caused by *K. pneumoniae* following the failure of various antibiotic treatments (Bao et al., 2020). In Belgium and France, Jault et al. (2019) employed a topical cocktail comprising 12 phages (designated PP1131) in the treatment of burns caused by *P. aeruginosa*.

Enzibiotics

Additionally, considerable effort has been dedicated to investigating the potential of phage-encoded enzymes, also known as enzybiotics, as standalone antibacterial agents (Abedon et al., 2011a; 2011b). Indeed, enzymes such as endolysins are produced in a recombinant manner in a pure form and applied outside of bacterial cells (De Maesschalck et al., 2020; Gerstmans et al., 2020). Recent research has demonstrated that endolysins, which are peptidoglycan hydrolases, are capable of acting

within bacterial cells (Abdelrahman et al., 2021; Schmelcher & Loessner, 2021; Murray et al., 2021; Linden et al., 2021). Furthermore, they have been shown to directly cause osmotic lysis in the case of Gram-positive bacteria (Briers et al., 2014; Briers & Lavigne, 2015). Nevertheless, in order to endolysins act on Gram-negative bacteria, a modification must be undergone in order for them to reach the cell wall (Briers et al., 2014; Briers & Lavigne, 2015). Moreover, these enzymes are employed by phages at the conclusion of their lytic infection cycle to dismantle the bacterial cell wall and facilitate the release of newly produced virions (Young and Wang, 2006). The utilization of endolysins is highly advantageous due to their non-toxic nature, rapid action, efficient killing of targeted bacteria and their inherent difficulty for bacteria to combat (Jun et al., 2017; Blasco et al., 2020; Fowler et al., 2020).

Nanobactericides against MDR bacteria

Nanotechnology has emerged as a plausible revolutionary tool for the prioritisation of novel and effective therapeutic options (Baker and Perianova, 2019; Chakraborty et al., 2022). To develop the most effective alternatives for drug-resistant pathogens, extensive research has been conducted into the use of nanoparticles in the fight against multi-resistant bacteria. The work of Jin and He (2011) demonstrated that the combination of magnesium oxide nanoparticles with nisin and zinc oxide nanoparticles exhibited antibacterial activity against *Escherichia coli* O157:H7 and *Salmonella* species.

It is established that nanobactericides are effective against a wide range of pathogens (Baker and Perianova, 2019). A substantial number of research has been conducted with the aim of elucidating the mode of action of nanobactericides. For example, Pal et al. (2007) demonstrated that nanobactericides can interact with cytoplasmic components and nucleic acids, inhibiting respiratory chain enzymes and interfering with the membrane permeability of complex I dehydrogenase. Other research has demonstrated that nanobactericides are capable of producing reactive oxygen species (ROS), inhibiting respiratory enzymes, producing ATP, creating pitting, and leading to the disruption of membrane integrity and cell membrane rupture, which ultimately results in pathogen death (Syed et al., 2018).

ANTAGONISTIC BACTERIA: MECHANISMS OF ACTION

Antagonistic bacteria are microorganisms that inhibit the growth or survival of other bacteria, particularly pathogens. This action can be exerted in various ways, including the production of antimicrobial substances, competition for resources and interference with pathogen communication mechanisms. This review will focus on the production of antimicrobial substances.

Production of antimicrobial compounds

Antagonistic bacteria are capable of producing a diverse range of antimicrobial compounds that are effective in inhibiting the growth of pathogens. Such substances include bacteriocins, natural antibiotics and lytic enzymes.

Bacteriocins: These are peptides or proteins that are produced by bacteria with the purpose of inhibiting or killing other bacteria. They frequently act by disrupting the cell membrane or essential functions of the target cell. For example, Abanoz and Kunduhoglu (2018) demonstrated that the bacteriocin KT11, produced by *E. faecalis* KT11, isolated from traditional Kargı Tulum cheese, exhibited antagonistic efficacy against a variety of Gram-positive and Gram-negative bacteria, including vancomycin- and/or methicillin-resistant bacteria. A further study conducted in India demonstrated that the bacteriocin produced by *L. plantarum* LA21, isolated from fermented foods, exhibited antibacterial activity against pathogenic bacteria, including *B. pumilus*, *B. amyloliquefaciens*, *S. aureus* and *L. monocytogenes* (Leslie et al., 2021).

Natural antibiotics and bioactive compounds: A considerable number of drugs derived from natural products are produced by microbes or by their interaction with hosts (Newman and Cragg, 2020). For example, maclamicin has demonstrated potent antimicrobial activity against Gram-positive bacteria, while lobophorin F, isolated from *Streptomyces* spp., has exhibited significant antibacterial activity. Furthermore, plantacyclin B21AG, a circular bacteriocin produced by *L. plantarum*,

demonstrated high thermostability and broad antagonistic activity (Golneshin et al., 2020).

Actinobacteria: Actinomycetes, in particular, are a highly prolific source of bioactive secondary metabolites. Approximately 45% of the 23,000 or so bioactive secondary metabolites produced by microbial diversity are attributed to actinobacteria (Ara et al., 2012; Sathi et al., 2001; Sirbu et al., 2023). Genera such as *Streptomyces* are responsible for the production of a multitude of bioactive compounds, a considerable proportion of which are antibiotics (Bérdy, 2005; Ganesan et al., 2017). For example, Kumar et al. (2014) evaluated isolates for their antagonistic activity against various pathogens and found that some strains, such as SCA 7, exhibited strong antimicrobial activity.

Cyclic lipopeptides: Recent studies have demonstrated that cyclic lipopeptides produced by *Bacillus amyloliquefaciens* exhibit potent antimicrobial activity. These lipopeptides can be classified into three families: surfactin, iturin and fengycin (Wong et al., 2008). For example, Xu et al. (2014) demonstrated the antibacterial activity of lipopeptides produced by *Bacillus amyloliquefaciens* M1 against multi-resistant *Vibrio* spp. isolated from diseased marine animals.

CONTRIBUTIONS OF ANTAGONISTIC BACTERIA IN THE CONTROL OF MULTI-RESISTANT PATHOGENIC BACTERIA

Recent studies have demonstrated the numerous advantages that antagonistic bacteria offer over traditional antibiotics and novel therapeutic approaches. Antagonistic bacteria frequently act by producing natural inhibitory substances, which impede the development of resistance mechanisms in pathogens. Mora et al. (2020) highlight that antagonistic bacteria reduce the likelihood of resistance developing, as they do not exert the same selective pressure on pathogens as traditional antibiotics. Moreover, Hibbing et al. (2018) posit that antagonistic bacteria facilitate a healthy equilibrium within the microbiota by impeding the proliferation of pathogens while fostering the growth of beneficial microorganisms. This is in contrast to antibiotics, which frequently also destroy beneficial bacteria, thus disturbing the microbiological equilibrium. In a study conducted by Collins et al. (2019), it was demonstrated that antagonistic bacteria can exert long-lasting effects on the prevention of infection. For example, the colonization of the gut with probiotics can prevent the establishment of pathogens, thereby reducing the incidence of recurrent infections. Furthermore, other authors have proposed an ecological approach to the utilization of antagonistic bacteria. Rodriguez et al. (2021) highlight that the utilization of antagonistic bacteria represents a more ecological and sustainable approach, assisting in the reduction of antibiotic usage in the environment and the preservation of natural microbial ecosystems.

Use in agriculture and livestock farming

Antagonistic bacteria play a pivotal role in curbing the reliance on antibiotics in livestock and agricultural production. These beneficial microorganisms can inhibit the growth of pathogens through a variety of mechanisms, including competition for nutrients, the production of antimicrobial substances, and the modulation of the host immune system. In a study conducted by Markowiak and Śliżewska (2018), it was demonstrated that the administration of probiotics to animal diets can enhance gut health and fortify immune defences, consequently reducing the necessity for antibiotics. Probiotics, including *Lactobacillus* and *Bifidobacterium*, are frequently employed in this context (Chaucheyras-Durand and Durand, 2010). Patterson and Burkholder (2003) demonstrated that the addition of probiotics to poultry feed can result in a reduction in the colonization of pathogens such as *Salmonella* and *Campylobacter*. This results in a reduction in infections and the necessity for antibiotic use. In a relatively recent study, Compant et al. (2019) have emphasized the potential of antagonistic bacteria for the biocontrol of pathogens in agricultural crops. For example, *Bacillus* and *Pseudomonas* strains can be applied to protect plants against various diseases, thereby reducing the need for chemical and antibiotic treatments (Compant et al., 2019). Nayak (2020) explored the use of probiotics in aquaculture to prevent bacterial infections in fish. Probiotics can improve the gut health of fish and inhibit the growth of aquatic pathogens, thereby reducing reliance on antibiotics.

The use of antagonistic bacteria represents a promising avenue for reducing the reliance on

antibiotics in livestock and agricultural production. Their capacity to enhance intestinal wellbeing, avert infections, stimulate the immune system and regulate pathogens in crops and aquaculture is well documented. Recent research indicates that the application of these beneficial microorganisms can not only enhance the health and productivity of animals and plants, but also serve as a pivotal strategy in combating antimicrobial resistance.

Clinical and medical applications

The use of antagonistic bacteria in clinical and medical applications is becoming increasingly prevalent due to their numerous health benefits. Over the past decade, a substantial number of research has demonstrated that beneficial microorganisms, commonly referred to as probiotics, can play a pivotal role in the prevention and treatment of various diseases by modulating the gut microbiota, inhibiting pathogens, and enhancing immune system function. Goldstein et al. (2017) demonstrated that specific strains of *Lactobacillus* and *Bifidobacterium* can prevent and treat gastrointestinal infections, including antibiotic-associated diarrhoea and viral gastroenteritis. Sartor (2020) conducted a study examining the efficacy of probiotics in the management of inflammatory bowel disease, including Crohn's disease and ulcerative colitis. Probiotics have been demonstrated to modulate the immune response, reduce inflammation and improve clinical symptoms in patients with inflammatory bowel disease (IBD). In a study conducted by Stapleton et al. (2019), it was demonstrated that the administration of probiotics, particularly *Lactobacillus rhamnosus* GR-1 and *Lactobacillus reuteri* RC-14, can prevent urogenital infections in women, including recurrent urinary tract infections and bacterial vaginosis. In a study by Arrieta et al. (2018), it was demonstrated that probiotics can play a role in the prevention and management of allergies and asthma. Probiotics have been demonstrated to facilitate the education of the immune system in early life, thereby reducing the risk of developing allergic diseases. Cani et al. (2019) demonstrated that probiotics can influence metabolism and be beneficial in the treatment of metabolic diseases such as obesity and type 2 diabetes. Probiotics have been demonstrated to modulate the gut microbiota, enhance insulin sensitivity and reduce metabolic inflammation. Morrow et al. (2012) evaluated the efficacy of probiotics in preventing nosocomial infections, such as *Clostridioides difficile* infections, in hospitalised patients. Probiotics have been demonstrated to reduce the colonization of resistant pathogens and to decrease the incidence of healthcare-associated infections.

Antagonistic bacteria, or probiotics, have the potential to provide effective solutions for a range of clinical and medical conditions. Their capacity to modulate the microbiota, inhibit pathogens and enhance the immune system renders them invaluable instruments in the prevention and treatment of gastrointestinal infections, inflammatory bowel disease, urogenital infections, allergies, metabolic diseases and nosocomial infections. Recent research has highlighted the significance and efficacy of these beneficial microorganisms in the medical field.

Challenges and obstacles to overcome

The utilisation of antagonistic bacteria presents a number of significant advantages, although it is not without inherent challenges. Sanders et al. (2018) emphasise that the stability and viability of antagonistic bacteria during storage and delivery represent a significant challenge. For probiotics to be effective, they must survive the conditions of manufacture, storage and passage through the gastrointestinal tract. Zmora et al. (2018) have demonstrated that the interaction between probiotics and the resident gut microbiota may be complex and unpredictable. The efficacy of some probiotics may be constrained by their inability to colonise effectively or to persist in the gut, where they may be eliminated by the existing microbiota. In their 2015 paper, Doré et al. addressed concerns regarding the safety of probiotics, particularly in vulnerable populations such as those with immune system deficiencies. While probiotics are typically regarded as safe, there have been isolated reports of adverse effects, including opportunistic infections, in rare instances. Reid et al. (2019) emphasise that the effects of antagonistic bacteria may be specific to certain strains and hosts. It is therefore challenging to make generalized claims about the benefits of probiotics, and a personalized approach may be required to optimize efficacy. As Hill et al. (2014) observe, the precise mechanisms of

action of antagonistic bacteria remain incompletely understood. A more comprehensive grasp of the interrelationships between probiotics, hosts, and pathogens is essential to enhance the efficacy of antagonistic bacteria. Fontana and Bermudez-Brito (2019) addressed the regulatory challenges associated with the marketing of probiotics. There are discrepancies in quality standards, regulatory frameworks and health claims across different regions, which present challenges to the universal approval and acceptance of probiotic products.

Despite the considerable benefits that antagonistic bacteria offer to human and animal health, a number of technical and scientific constraints must be addressed to fully realize their potential. The stability and viability of probiotics, complex interactions with existing microbiota, safety concerns, specificity of effect, incomplete mechanisms of action, and regulatory challenges are all areas requiring ongoing research and improvement. Advances in these fields will facilitate the optimal exploitation of antagonistic bacteria while mitigating associated risks.

CONCLUSIONS

Antagonistic bacteria represent a promising advance in controlling multi-resistant pathogens and reducing the use of antibiotics. Their applications in agriculture, livestock farming, and medicine are showing encouraging results in terms of intestinal health, preventing infections and stimulating the immune system. Nevertheless, further research is needed to overcome technical and scientific limitations and maximise their potential. The integration of antagonistic bacteria into current practices could thus play a crucial role in the fight against antimicrobial resistance, contributing to a more sustainable and healthy future.

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SENSORY ANALYSIS EVALUATION STUDY OF FOREIGN AND NATIONAL APRICOT VARIETIES

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Abstract

This research aimed to investigate the significant consumer indicators in apricot fruit by different groups of Romanian and foreign cultivars. The sensory analysis was carried out in three harvest seasons during 2021-2023, and the method used was questionnaires (tasting sheets). The analyzed parameters were: the appearance of the fruit given the size (1-3), form (1-3), color (1-3); pulp firmness (1-4), pulp juiciness (1-5), taste (1-6), and flavor (1-4). The evaluation of the size and shape of the fruits in the Romanian and foreign cultivars showed that in both batches, in general, the fruits had very good ratings and were very well appreciated. The fruit color index varied between 2 and 3 in both studied groups. Pulp firmness analysis showed that the uniformity of this index was higher in the foreign cultivars. Examining fruit taste and pulp juiciness showed that the fruits of both groups were almost in the same range and had similar grades. The best taste among the foreign cultivars was observed in Pisina/M29C and among the Romanian cultivars in Amiral and Orizont.

Key words*: Flavor, sensory analysis, panel test.*

INTRODUCTION

Apricot (*Prunus armeniaca* L.) belongs to the *Rosaceae* family (Ahmadi et al., 2008). Apricots became endemic in large areas such as Iran, Turkey, Afghanistan, the Middle East, and East China more than 5,000 years ago (Faust et al., 1998; Buttner, 2001). This fruit grows in areas with moderate climates (Topcu & Uzundumlu, 2010; Ucar & Engindeniz, 2018). Apricot is one of the fruit trees cultivated on a high scale in southern Europe. One of the main reasons for diverse research to create new varieties consistent with European climatic conditions is the high nutritional value of apricots and products produced based on this fruit (Iordanescu et al., 2018). The nutritional value of this fruit is high (Iordanescu & Micu, 2012; Hegedus et al., 2010; Ali et al., 2011), and from the distant past, it has been used in home remedies (Kan & Bostan, 2010). Apricot is used in fresh, dried, and processed forms (Altindag et al., 2006; Özdoğru et al., 2015). Improving fruit's sensory characteristics is an important factor in increasing the sense of pleasure of tasting fruit (Joanna et al., 2019). Rootstock influences cultivar growth characteristics, fruit size, plant performance, and growth under stress conditions (Zhebentyayeva et al., 2012). It should be mentioned that other factors such as variety, geographical conditions of cultivation place, tree cultivation system, and fruit ripening process also affect the characteristics of the fruit (Mratinic et al., 2011; Iordanescu et al., 2012; Milosevic et al., 2010; Campbell et al., 2011; Leccese et al., 2010; Ayour et al., 2017). Understanding the sensory characteristics that affect consumers' choice of fruit significantly increases their marketability (Dawson & Healy, 2018). In sensory evaluation, responses to product features perceived by the senses are analyzed and interpreted (Stone & Sidel, 2004). Sensory characterization is used as an analytical test in breeding programs (Lawless & Heymann, 2010). In strawberries, sensory analysis showed that local cultivars were selected for color, foreign samples for aroma, and self-produced samples for flavor, texture, and overall (Hasna et al., 2022). The study of some sensory characteristics in apricots showed that some physicochemical characteristics of the fruit positively correlated with the sensory characteristics (Lespinasse et al., 2006). The study of eight apricot cultivars in Italy found that the overall quality of the fruit has a positive correlation with its taste, sweetness, and juiciness. In addition, fruits with a proper

sugar-to-acid ratio balance were more appreciated (Valentini et al., 2006). Since the characteristics related to the fruit significantly affect its marketability and its choice by the consumer, this research investigated the sensory factors of fruits of foreign and Romanian cultivars by different parameters.

MATERIALS AND METHODS

The sensory analysis of the quality of apricot fruits obtained in the Experimental Fruit Field of the Faculty of Horticulture was carried out in three harvest seasons during 2021-2023. The method used was questionnaires (tasting sheet) in which several 224 people participated (Tables 1, 2, and 3).

Table 1 Participants distribution to the questionnaire by gender

No.	Gender	No	%
1	Male	102	45.54
2	Women	122	54.46
	Total	224	100%

Table 2. Participants distribution to the questionnaire by age

No.	Age	No.	%
1	10-20	8	3.57
2	21-30	56	25.00
3	31-40	50	22.32
4	41-50	55	24.55
5	51-60	37	16.52
6	Over 60 year	18	8.04
	Total	224	100%

Table 3. The analyzed groups by employment status

No.	Occupation	No.	%
1	Professor	26	11.61
2	Assist. Univ.	5	2.23
3	Researcher	13	5.80
4	PhD	7	3.13
5	Master stud.	5	2.23
6	Engineer	22	9.82
7	Lawyer	22	9.82
8	Employee	79	35.27
9	Student	28	12.50
10	Retired	5	2.23
11	No answer	12	5.36
	Total	224	100%

To evaluate the fruits, the answers were scored using the scoring scale with an interval from 1-6, where 1 was the lowest value and 6 was the highest. The analyzed parameters were: the appearance of the fruit given the size (1-3), form (1-3), color (1-3); pulp firmness (1-4), pulp juiciness (1-5), taste (1-6), and flavor (1-4). 16 foreign and 15 Romanian cultivars were tasted. The analysis included the 'Early orange' cultivar, in the Romanian group, being in the same cultivation plot.

RESULTS AND DISCUSSIONS

Fruit size

The evaluation of the fruit size in the Romanian and the foreign cultivars in the research showed that the fruits were generally well and very well appreciated in both batches. Primaya/SJA (Trident) (2.82), Primaya/SJA (Bi-Baum) (2.85), Vitillo/M29C (2.79), Medflo/M29C (2.75), Pisana/M29C (2.85), Delice/M29C (2.87), and Bergeron/M29C (2.72) had been the better evaluated fruits compared to other cultivars. In the Romanian cultivars, the size of the fruits was appreciated more in the cultivars Amiral (2.72), Hybrids (2.73), and Orizont (2.84) compared to other cultivars (Figure 1).

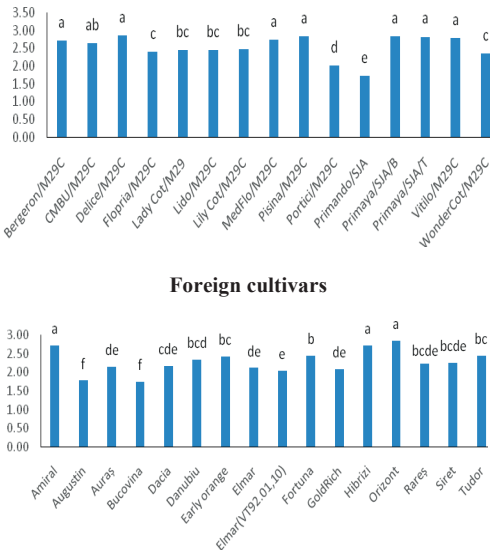


Figure 1. Fruit size evaluation in tested cultivars (the scores, represented on the Y-axis, were statistically compared, using ANOVA and Duncan test for $p \leq 0.5$) (*except Early orange)

Fruit shape

The evaluation of the fruit shape in the foreign and Romanian cultivars showed that all the

varieties examined received good and very good ratings. The best appreciations were attributed to the cultivars Primaya/SJA (Trident) (2.79), Vitillo/M29C (2.71), Medflo/M29C (2.78), and Pisina/M29C (2.70). Among the Romanian cultivars, the most appreciated for this attribute was the cultivar Orizont (2.82), followed by Amiral (2.64) (Figure 2).

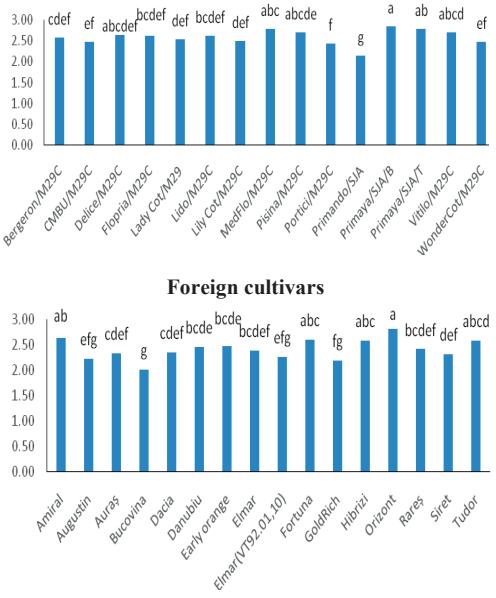


Figure 2. Fruit shape evaluation in the studied and Romanian cultivars
(the scores, represented on the Y-axis, were statistically compared, using ANOVA and Duncan test for p≤0.5)
(*except Early orange)

Fruit color

The fruit color evaluation results showed close values between the cultivars tested, with some differences. The best appreciations were for the cultivars Delice/M29C (2.74), Flopria/M29C (2.75), Lido/M29C (2.74), and Primaya/SJA/T (2.74). Primando/SJA (1.62) was the lowest-rated of these cultivars. In the Romanian cultivars, the best evaluation was at Orizont (2.78) and the smallest at Bucovina (2.12) and, respectively, Dacia (2.10). Generally, this index was between 2 and 3 in the foreign and Romanian cultivars (Figure 3).

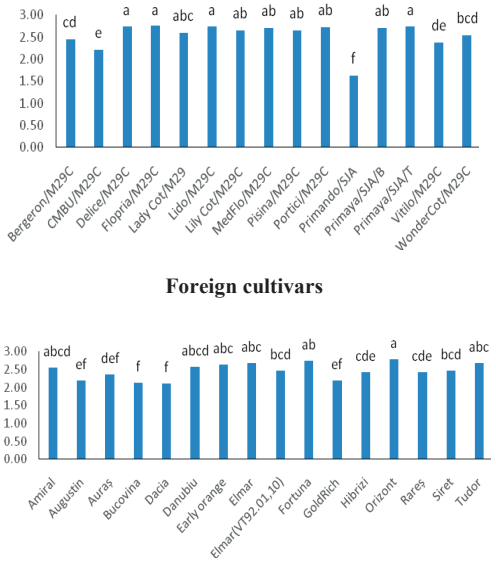
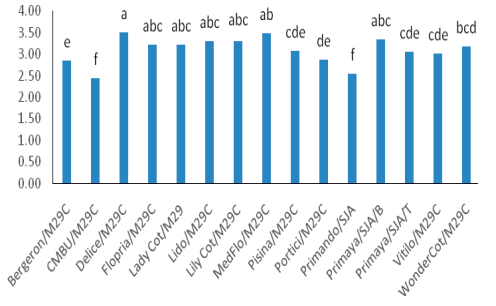
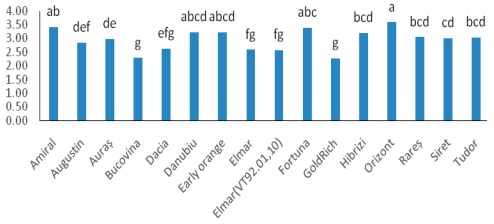


Figure 3. Fruit color evaluation in the studied and Romanian cultivars
(the scores, represented on the Y-axis, were statistically compared, using ANOVA and Duncan test for p≤0.5)
(*except Early orange)

Pulp firmness evaluation

The examination of this parameter in apricot cultivars showed that the best appreciations were for the cultivar Delice/M29C (3.51), and the least appreciated being CMBU/M29C (2.43) and Primando/SJA (2.55). The value of this index in other cultivars of this group was higher than 3. The study of Romanian cultivars found that the highest value of this index was in the cultivar Orizont at the rate of 3.58 (Figure 4).





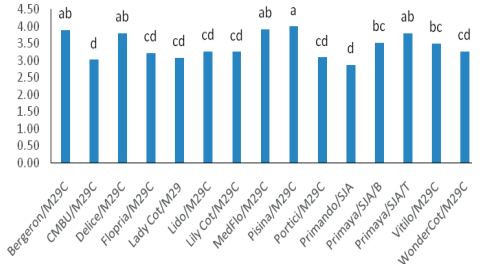
Romanian cultivars (*)

Figure 4. Fruit pulp firmness evaluation in studied and Romanian cultivars
(the scores, represented on the Y-axis, were statistically compared, using ANOVA and Duncan test for p≤0.5)
(*except Early orange)

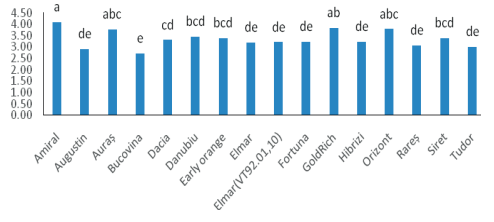
Pulp juiciness evaluation

In terms of pulp juiciness evaluation, the results determined that the highest value was attributed to the cultivar Pisina/M29C (4.00).

The lowest value of this index was observed in cultivars Primando/SJA (2.87) and CMBU/M29C (3.02). This index was higher than 3 and lower than 4 in other cultivars in this group. Among the Romanian cultivars, it was established that the Amiral cultivar had the highest index value (4.09). This index was less than 2 in Augustin (2.91) and Bucovina (2.72). Other cultivars were placed in the same range and less than 4 (Figure 5).



Foreign cultivars

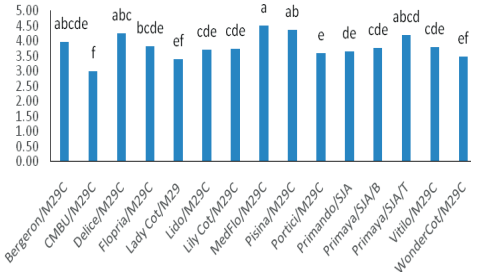


Romanian cultivars (*)

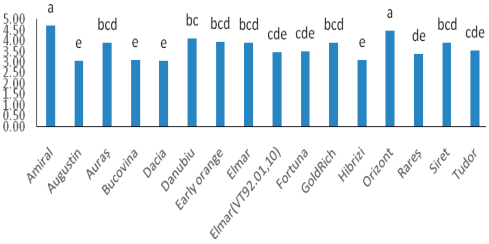
Figure 5. Fruit pulp juiciness evaluation in the studied cultivars
(the scores, represented on the Y-axis, were statistically compared, using ANOVA and Duncan test for p≤0.5)
(*except Early orange)

Fruit taste evaluation

Examining the fruits of different apricot cultivars from the two studied groups, it was found that the fruits of both groups were almost in the same range and had similar grades. In the cultivars studied, only four cultivars, including Delice/M29C (4.25), MedFlo/M29C (4.50), Pisina/M29C (4.37), and Primaya/SJA (Trident) (4.19), had a rating higher than 4. Other cultivars had a value lower than 3 and higher than 2. The evaluation of the taste index of the Romanian cultivars also showed that in this group, the cultivars Danubiu (4.10) and Orizont (4.45) had a higher value compared to other foreign cultivars (Figure 6).



Foreign cultivars



Romanian cultivars (*)

Figure 6. Fruit taste evaluation in the studied cultivars
(the scores, represented on the Y-axis, were statistically compared, using ANOVA and Duncan test for p≤0.5)
(*except Early orange)

Flavor evaluation

Following the analysis of the flavor index of the studied cultivars, it was found that the highest value of this index was observed in Pisina /M29C (3.45), followed by Medflo/M29C (3.16). The aroma index value for other cultivars studied in this group was less than 3. It should be noted that the fruits of the cultivar CMBU/M29C had the lowest value of this index (2.39). In the Romanian cultivars, only four cultivars, Amiral (3.37) and Auraș (3.11), Danubiu (3.06), and

Orizont (3.23), obtained a higher index value compared to other cultivars (Figure 7).

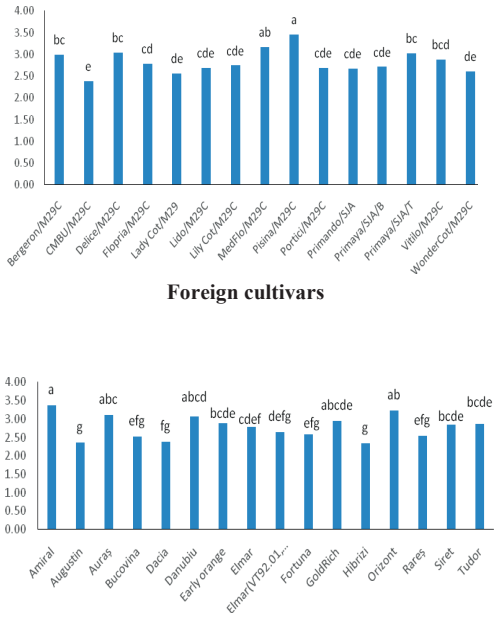


Figure 7. Fruit flavor evaluation in the studied and Romanian cultivars
(the scores, represented on the Y-axis, were statistically compared, using ANOVA and Duncan test for p≤0.5)
(*except Early orange)

Classification of the tested varieties according to the evaluated parameters

By analyzing the cultivar groups based on the studied traits, it was found that they were divided into four separate groups. The cultivars Portici/M29C, Pisina/M29C, Bergeron/M29C, CMBU/M29C, and Delice/M29C were placed in a group; the cultivars Lido/M29C and Medflo/M29C in another group; while Flopria/M29C, Wonder Cot/M29C, and Rubista/M29C in another separate group and finally Primaya/SJA (Bi-Baum), Lilly Cot/M29C, Primaya/SJA (Trident), and Lady Cot/M29C were grouped. The study of Romanian cultivars also showed that the cultivars Elmar (VT92.01,10), Siret, Tudor, Orizont, Hibrizi, and Amiral were classified into a group, while cultivars Augustin and Bucovina in another group, as well Fortuna, Rareș in a group and the cultivars Danubiu, Elmar and Auraș in a group. The Cultivar Dacia stood out through the ratings it received, distancing itself from the others (Figure 8).

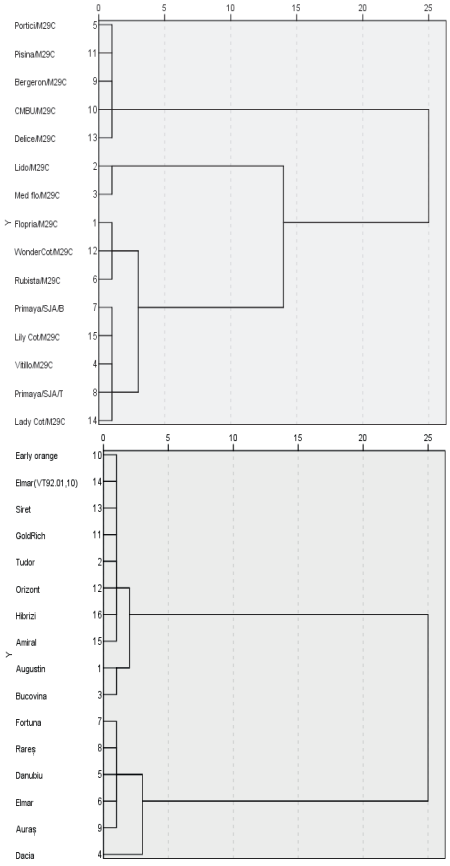


Figure 8. Cluster analysis of the studied cultivars

Discussions

One of the primary things that attract the consumer's attention is the fruit's appearance and quality, which significantly affects its marketability and selection (Gatti et al., 2009). Furthermore, fruit quality, influenced by sensory properties such as aroma, texture, appearance and taste, nutritional value, and chemical compounds, is one of the critical factors in the acceptance of different apricot cultivars by consumers (Abbott et al., 2006). As a result, evaluating sensory characteristics in apricots is one of the practical and essential tools in describing and introducing different cultivars. Several studies have evaluated the sensory parameters of apricots (Infante et al., 2008; Defilippi et al., 2009; Robini et al., 2006; Infante et al., 2006). In these studies, it has

been stated that there is a correlation between the sensory indicators of the fruit and the physical and chemical characteristics. Factors such as fruit appearance, texture, color, and taste during ripening determine the final quality of the fruit. Therefore, sensory evaluation of apricot fruit should be done at the right time of fruit ripening according to the right balance between sugar and acid and the right quality of the fruit texture (Egea et al., 2007). This research investigated sensory indicators such as fruit size, shape, color, flavor, taste, pulp firmness, and pulp juiciness. The results showed that the appreciation of the fruit size in the Romanian cultivars was somewhat lower than in the foreign cultivars. Primando/SJA had the lowest value among the foreign cultivars. In the research of Azodanlou et al. (2003), the parameters of flavor, acidity, juiciness, sweetness, flesh firmness, and aroma were used to evaluate the sensory properties of apricots. In the research conducted in Spain on two different genotypes of apricots, it was found that the sensory evaluation of the fruit by assessors can determine the best ripening time of the fruit (Egea et al., 2006). Valentini et al. (2006) found that the overall quality of apricot fruit correlates with flavor, juiciness, and sweetness. It has been stated that using sensory evaluation is the best option for juiciness, melting, and floury texture (Lespinasse et al., 2006).

CONCLUSIONS

In general, it can be considered that sensory features allow the introduction and comparison of varieties. The research results showed the difference in sensory characteristics between foreign and Romanian cultivars. Furthermore, the results showed that Primando/SJA obtained lower scores concerning fruit size, shape, color, pulp firmness, and pulp juiciness indices among the foreign cultivars. It should be mentioned that the lowest scores of fruit taste and flavor in foreign cultivars was observed in the CMBU/M29C cultivar. In Romanian cultivars, Amiral and Orizont cultivars registered the highest values in most sensory analysis parameters evaluated in this study.

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COMPARATIVE ANALYSIS OF BRAZZEIN PRODUCTION IN *IN VIVO* AND *IN VITRO* SYSTEMS

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Abstract

Brazzein, a highly potent sweetener derived from the fruit of the African plant Pentadiplandra brazzeana, has garnered attention due to its potential as a low-calorie alternative to sugar. This study presents a comparison between in vivo and in vitro systems for the production of brazzein, focusing on yield, cost-effectiveness, and sustainability. Utilizing genetically modified organisms (GMOs) in in vivo systems, specifically engineered yeast and bacteria, we explored the scalability and efficiency of brazzein production. Conversely, in vitro systems involved cell-free synthesis, highlighting the control over production conditions and the reduced risk of contamination. Economic analysis revealed that while in vivo systems benefit from lower initial investment costs and higher production rates, in vitro systems may offer long-term sustainability and lower environmental impact, attributed to reduced resource consumption and waste generation. This study provides critical insights into the feasibility of scaling brazzein production for commercial use, evaluating the pros and cons of each system. Further research into genetic engineering and optimization of culture conditions could enhance the efficiency and yield of brazzein production, contributing to the development of healthier sweetening options for the global market.

Key words*: brazzein, cell-free protein synthesis, in vitro, in vivo, production, sweet protein, Tx-Tl.*

INTRODUCTION

Brazzein is one of the five sweet-tasting plant proteins including mabinlin, neoculin, thaumatin, and monellin. Although there is a sixth sweet-tasting protein called pentadin, it is not characterized yet. These plant proteins are found in the tropical forests of Africa or South Asia. Pentadin and Brazzein are largely found in Cameroon, Gabon, Congo, and West African tropical forests.

The seeds are dispersed through primates who eat the fruits and spread the seeds with their feces. Among the tropical forests, evolutionary pressure is caused by the primate preference for the sweetest fruits, causing an increase in their sugar content (Koveshnikova et al., 2023). Among all the sweet plant proteins, Brazzein is considered the most desirable due to its low lingering off-taste, hence it being the most interesting plant protein.

Brazzein was identified for the first time by Ming and Hellekant back in 1994 as a new thermostable and sweet protein that is easily derived from the *P. brazzeana* fruit (Lynch et al., 2023). Its thermostability was tested through incubation at 98°C for 2 hours and 80°C for 4.5 hours. Nevertheless, the findings confirm that Brazzein does not lose its natural sweetness, an indication of its protein stability even at high temperatures (Koveshnikova et al., 2023). Moreover, the thermostability and sweetness of Brazzein are just some it's features. It is also known to demonstrate an isoelectric point of 5.4 and high-water solubility.

It is extracted from the *P. brazzeana* fruits through buffer solutions including phosphate buffer, which are precipitated with ammonium sulfate (Figure 1).

Source	Extraction	Isolation	Purification	Characterization	Main Results
P. brazzeana	0.1 M phosphate buffer at pH 7.0 containing 5% glycerol, 0.1 mM DTT, 20 mL PMSF, 0.1 mM EDTA and 0.5% (*w*/*v*) PVP at 4 °C	Protein precipitation with ammonium sulfate 30% and 85%	Ion-exchange chromatography using a CM-Sepharose CLdB column (gradient: NaCl of 0.1 to 0.4 M in 20 mM sodium citrate at pH 3.6)	SDS- PAGE; ESI-MS; sequence determination by S-Pyridylethylation and S-carboxymethylation of brazzein and peptide fragment separation by RT-HPLC.	Brazzein is a single-chain polypeptide; the molecular weights obtained by SDS-PAGE and ESI-MS were 6.5 KDa and 6.473 KDa, respectively; C-terminal is a tyrosine; 8 cysteines out of 54 residues.

Figure 1. Methods to extract, isolate and characterize Brazzein (Saraiva et al., 2023)

Nevertheless, growing the plant is considered quite challenging, hence the extraction and production of Brazzein is quite expensive. Multiple studies have applied the technological production of Brazzein by using genetic engineering through bacteria, transgenic plants, yeasts, and animals. Once there is extraction of the protein, it is expressed and isolated, then purified and characterized. There are multiple methods of characterization, purification, and isolation of Brazzein.

Brazzein is defined as a 6.5kDa sweet-tasting protein that has four disulfide bonds. Its sweetness is considered quite close to the sweetness of sucrose. However, it has high sweetness potency. It is also known for its high solubility and exceptional thermostability over a wide range of pH values, vital for most of its food applications (Bains et al., 2021). The long history of human consumption of Brazzein and its lack of bitterness makes it a preference by most people to other natural sweeteners. Additionally, the high-water solubility, pH stability, and extreme temperature tolerability of Brazzein make it preferred in the majority of food applications. For instance, there are identified seven sweet-tasting proteins besides Brazzein including monellin, neoculin, mabinlin, thaumatin, lysozyme, and pentadin.

For a long time, Brazzein has been utilized in safely sweetening food by the African natives, an indication that it has no health risks associated with it. However, the Food and Drug Administration in the United States must approve any food before it is consumed in the country. Through the rigorous tests mandated by the Food and Drug Administration, the people had to be guaranteed the safety of consuming Brazzein (Lynch et al., 2023). There is a need for extensive research in the possible acute toxicity areas to allergens or toxins and the protein breakdown tests concerning digestive enzyme therapy (Gatea et al., 2021). Therefore, the Food and Drug Administration has tested the protein's bioactivity *in vivo* and *in vitro*. Despite the protein showing a minimum 45% similarity to the antifungal drug drosomycin and antimicrobial agent defensin, the protein has antifungal and antimicrobial activities. Owing to the structural similarity of the Brazzein and defensin-like proteins, often assumed to be allergens, several people have raised concerns regarding the possible allergenicity of Brazzein.

MATERIALS AND METHODS

There are methodologies applied in the *in vitro* and *in vivo* systems of production for Brazzein. In the paper, the methodology largely involved a description of the search terms, the findings of the study, and the databases and processes followed in the *in vivo* and *in vitro* production of Brazzein plant protein.

The methodology had a replicable and comprehensive search process, that detailed the sources.

In the collection of the data for the paper, there was a selection of the study, excretion and various synthesis methods were the data that was looked upon.

Twelve journal articles and publications were used in the study for the comparative analysis of *in vitro* and *in vivo* production of Brazzein.

In Vitro Production of Brazzein

Cell-free transcription-translation (TXTL) is a versatile technology for the construction, characterization, and interrogation of genetically programmed biomolecular systems done outside the organisms.

In vitro, the production of Brazzein requires recapitulation of the gene expression by providing unparalleled flexibility to design, engineer, and analyze quantitatively the impacts of physical, chemical, and genetic contexts on the biochemical systems function.

Saraiva et al. (2023) point out that it involves steps such as preparation of cell lysates, reaction set-up, transcription and translation, and purification.

In the preparation step, cell lysates containing cellular components including tRNAs, ribosomes, and amino acids will be prepared alongside transcriptional and translational factors.

The reaction setup step will involve assembling the Tx-Tl reaction mixture through a combination of the cell lysate with a DNA encoding Brazzein, involving other components including cofactors, salts, and energy sources.

The transcription and translation step will involve the transcription of the DNA into mRNA, then it is translated by ribosomes in the cell lysate to produce Brazzein.
The last step of the purification of Brazzein from the reaction mixture involves purification techniques including chromatography.

In Vivo Production of Brazzein

In vivo, a system of production applies the genetically modified organisms especially bacteria, such as *Escherina coli* and yeasts. More specifically, the techniques of genetic engineering were applied in the introduction of genes encoding the Brazzein plant protein into the genomes of the host organisms, allowing the production of the needed sweet protein (McElwain et al., 2022).

Bacterial production of Brazzein

In vivo, the production of Brazzein involves bacterial production through genetic engineering techniques that modify the bacteria through steps such as inserting the Brazzein gene in the bacteria, optimized fermentation process, and purification of the Brazzein produced.
The most commonly used bacterium for the recombinant production of Brazzein plant protein is *E. coli*. Its success was first achieved in 2000. Nevertheless, the expression of the protein using this organism was limited by the requirement of an additional phase of removing the fused protein; and the majority of the plant protein produced needed additional chemical refolding steps.
Lactic acid bacteria including *Lactococcus lactis* are considered more advantageous and recognized as safe, creating a massive opportunity for the agro-industry to apply easily for the production of plant proteins such as Brazzein. *L. lactis* is useful for the production of Brazzein but at a low scale due to the possible lack of detection, and the Brazzein produced is not as sweet as that produced through *E. coli*.
In the expression of Brazzein, *E. coli* is designed, expressed, and synthesized at 30 degrees Celsius for the soluble form of Brazzein.
(Zhang et al., 2023) note that there are instances when Brazzein has been expressed in *L. lactis* through a nisin-controlled expression system for the production of the plant protein.
The fermentation process has to be optimized for proper secretion and expression of recombinant Brazzein in bacteria.
The optimization ensures that conditions of controlled fermentation are vital in the improvement of the production of the Brazzein produced.
The purification process will aim to clean up multiple biomolecules and large debris such as bacterial cells (Han et al., 2022).
The purification involves 2 hours of treatment at 80°C, precipitation of 30-80% ammonium sulfate, and ion exchange chromatography through diethylaminoethyl or carboxymethyl Sepharose columns.

Yeast production of Brazzein

Successful attempts have been made in the expression of Brazzein through yeast. *Saccharomyces cerevisiae* was used in the first expression of Brazzein. However, antibodies were used to confirm the identity of the expressed protein but there was no more characterization of the recombinant Brazzein.
Although there were multiple attempts of yeasts used in the production of Brazzein, the researchers settled for the use of *Kluyveromyces lactis* due to its intrinsic robustness. Especially the purified Brazzein, considered identical to the natural Brazzein plant protein was generated.
The synthetic gene encoding Brazzein was expressed as a sweet protein in the yeast *K. lactis*. To optimize expression and extracellular secretion for expression in soluble and active forms, the Brazzein gene can be designed based on the yeast codon preference. There will be specific steps involved in the yeast production of Brazzein. The major steps will be the cloning of genetics, fermentation, and purification.
The cloning of the Brazzein gene will be done in a secretion vector such as p KLAC2, containing yeast prepropeptide signal. The genes *KIERO1* and *KIPDI* will be overexpressed in the *K. lactis* yeast, enhancing the secretion of Brazzein.
Saraiva et al. (2023) explain that the fermentation step will involve applying a chemically defined medium to optimize the cell

growth and Brazzein production phases. For industrial production of Brazzein, the *K. lactis* yeast will be preferred due to its safety and suitability. Controlled conditions for fermentation will be vital in maximizing the production and secretion of Brazzein.

The last step will be the purification of the produced Brazzein through techniques such as ultrafiltration for obtaining highly pure Brazzein. The fermentation and purification process must be optimized for the commercial production of Brazzein.

RESULTS AND DISCUSSIONS

In Vivo Production of Brazzein

The restriction of the Brazzein production as well as the location of the plant, multiple methods have been explored regarding its production. The most common systems of producing Brazzein have been *in vivo* and *in vitro* systems of production. However, the best preferred natural method of producing Brazzein is bio diversion. The *in vivo* system of producing Brazzein is largely reliant on the application of certain genetically modified organisms including engineered bacteria and yeast. According to Meilina et al. (2021), the organisms secrete and express the targeted Brazzein protein. In the production of Brazzein through an *in vivo* system, the yeast specifically *K. lactis* has been used as a primary host for the production of recombinant Brazzein. Most people have preferred the application of *K. lactis* yeast for the mass production of Brazzein protein due to its high rate of production and purity from the production while maintaining lower costs of production compared to other production systems.

However, there have been cases of researchers going for *E. coli* in the production of Brazzein. It is largely applied as a bacterial host, especially for the production of heterologous proteins. The preference for using *E. coli* in the production of Brazzein is supported by the good expression of *E. coli*'s IPTG dependence, indicating the suitability of applying bacterial systems in the production. Although *E. coli* is considered the first biotransformation, back in 2000, additional biotransformation tests have confirmed that *E. coli* has a lower level of sweetness compared to the original plant (Komolov et al., 2023). Later, there was production of Brazzein through *Pichia pastoris*, reaching yields of 120 mg/L of Brazzein produced in 6 hours. This system of production did not show robustness since there was sometimes a generation of (104 mg/L after 6 hours) sweet Brazzein using *Kluyveromyces* in a cultured medium. Other organisms have since been used in the production of Brazzein such as the *Bacillus licheniformis* which is preferred due to its affordable costs, high secretion, and quick growth. Other peculiar applied mediums for the production of Brazzein in biotransformation include rice, maize, and lettuce based media.

In the attempts to produce an active and soluble form of Brazzein through the secretory expression system of *K. lactis* yeast, there have been ineffective results due to the inaccurate disulfide bonds formation. Additional studies have explored other microbial system of producing Brazzein. For instance, there has been production of Brazzein within corn kernels in an attempt to optimize the production and leverage the proprietary technology.

In Vitro Production of Brazzein

The *in vivo* approaches in the production of Brazzein have been complemented through an *in vitro* system of production. In this system of producing Brazzein, there is the application of cell-free protein synthesis (CFPS). Meilina et al. (2021) describe it as a technology of production that required the involvement of the S30-buffer cell extract of *E. coli*, chimeric RNA polymerase of T7 bacteriophage, and a multicopy plasmid vector with the Brazzein gene inserted in the process. It is such a successful process especially in the synthesis of Brazzein synthesis, producing a 2 mg/ml Brazzein mixture, higher than the common systems of production that use whole-cell expression. These technology platforms are preferred due to their ability to solve the complex challenges in the production of plant proteins such as Brazzein.

Environmental Impacts and Sustainability of the Production Systems

The viability of the production systems of plant proteins such as Brazzein is important in

comparing the two systems. More studies confirm that *in vivo* systems of producing Brazzein, particularly through the application of genetically modified organisms such as genetically engineered bacteria and yeast, can easily provide the much-needed cost-effective and scalable production. Nevertheless, multiple researchers have raised their concerns over *in vivo* systems of producing Brazzein through genetically modified organisms. Most of the concerns are environmental concerns regarding the impact the production has on the environment and the regulatory oversight related to genetically modified organisms.

In the case of the in vitro system of production, the researchers have argued that it is a sustainable system of production that should be supported. The *in vitro* approach of production leveraged cell-free technology which is crucial in the minimization of resource wastages and consumption, such as energy and water (Zagorskaya & Deineko, 2021). Moreover, the approach is vital in the reduction of possible waste generation during the process of Brazzein production. *In vivo* approaches for the production of Brazzein such as production in corn kernels demonstrate the potential of the method to enhance environmental friendliness and sustainability. Therefore, through the application of cultivated crops in the production of Brazzein, the production process is easily scaled up as the unused corn biomass on the farms is applied in the value chain such as for fuel, food, or feed.

Cost Effectiveness and Rate of Production in The Two Production Systems

The two approaches to production are cost-effective by reducing the rate of production costs. For instance, the *in vivo* systems proved to have lower costs on the investment especially due to their leverage of the present production and infrastructure facilities. The *in vitro* approach of production was found to offer much-needed long-term sustainability and potential reduction of the operating costs due to their optimization for efficient utilization of resources and reduction of the waste generated from the production.

The two approaches of *in vivo* and *in vitro* production systems for Brazzein, it is vital to note that they all demonstrate a high rate of production as well as reduced costs of production. According to Leal et al. (2021), in the case of the *in vivo* system of producing Brazzein, particularly the system of *K. lactis* expression, there was mass production of Brazzein of the highest purity level as well as a high rate of production supported by low costs of production. The approach has leveraged the inherent capabilities of living cells to secrete and express Brazzein as the target protein. This approach has heavily contributed to the scalability and efficiency of the *in vivo* system of Brazzein production.

Contrastingly, the *in vitro* production of Brazzein through the CFPS systems is believed to have realized a tremendous volumetric rate of production, even beyond the maximum values realized in earlier attempts of whole cell expression production systems. More specifically, the technology of cell-free protein synthesis is seen to provide the benefit of higher control on the conditions of Brazzein production. This rate of production potentially allows more efficiency regarding scaling up as well as reduction of the contamination risks.

Food Application

Using the two systems of producing Brazzein, food industries can use the produced Brazzein. Endemic consumption of Brazzzein as a sweetening agent or raw fruits is considered such as a long-term ethnobotanical heritage. Being a plant protein, Brazzein is considered a low-calorie sweetener and has a low potential for causing gastrointestinal distress in humans.

However, there is a need for additional research on the industrial scale-up for the application of Brazzein. The long history of consumption, good sensory properties, and high sweetness potency of Brazzein make it a promising natural sweetener. The food companies understand that Brazzein offers physicochemical properties including extreme pH stability, high solubility in water, and extreme temperature stability, all considered vital in food applications.

Extensive research should investigate the means of fighting the challenge of obtaining sweet-tasting plant protein from its very natural source. The recent efforts in the overexpression of Brazzein through multiple heterologous systems such as yeast, bacteria, plants, and

animals. The expression systems of yeast are considered quite efficient and ensure economical expression for the secretion of Brazzein (Lynch et al., 2023). The secreted protein through yeast is easy to purify and has similar characteristics to natural protein.

More importantly, the high rate of production of Brazzein through biotechnology constantly paves the way for food applications, since Brazzein is a low-calorie and high-intensity sweetener alternative.

CONCLUSIONS

As revealed in the paper, the two approaches of production have their limitations and strengths. *In vitro* systems of Brazzein production, utilizing the cell-free protein synthesis technology, there is the benefit of enhanced control over conditions of Brazzein production, reduced contamination risks, and demonstrates the potential of an environmentally friendly and sustainable process of production. The cell-free protein synthesis technology applied in the *in vitro* systems of production guarantees high volumetric production of the plant protein, demonstrating its potential as a viable option for *in vivo* methods of Brazzein production.

Conversely, *in vivo* systems of production of Brazzein through engineered bacteria and yeasts, there is the potential for increased production and low costs of production for Brazzein. The efficiency and scalability of the *in vivo* system of production for Brazzein make it preferred for commercial-scale production.

All in all, companies interested in mass production of Brazzein will base their decision on whether to use *in vitro* or *in vivo* production systems on multiple factors.

For instance, the companies will consider sustainability, cost-effectiveness, and expected yield in production. However, there is a need for additional development and research on *in vivo* and *in vitro* systems of Brazzein production.

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VALORIZATION OF VEGETAL BY-PRODUCTS IN NEW MATRICES FOR THE IMPROVED FOOD SUSTAINABILITY SYSTEMS

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Abstract

This reviw paper is based on a bibliographic study of over 70 articles published in the period 2001-2023 and presents the need for the valorization of agro-industrial by-products, a review of the main research directions for the valorization of plant by-products for the purpose of better environmental sustainability, as well as the most used methods of valorization of this nutritionally valuable waste. The recycling of waste in the cascade represents the engine of the circular bioeconomy that supports finding solutions to problems related to the sustainability of the environment, economic, ethical, social. The methods and procedures for valorizing plant waste are diversified depending on the type of waste and aim to develop new sustainable food system on the consumer benefits. Vegetal by-products still contain valuable bioactive compounds such as fibers, vitamins, minerals, which can contribute to obtaining new products with added value and can become good sources of raw materials for the food industry.

Key words*: vegetal by-products, food waste, food sustainability, processing methods, trends and perspectives.*

INTRODUCTION

Agriculture and the food industry are the most representative branches of the agro-food industry in Europe (Palvic et al., 2023), and an important source of organic waste in the form of food waste (Raksasat et al., 2020). Inadequate management of the waste resulting from the primary processing of raw materials of plant origin, as well as the waste resulting from the processing of products results in the quantitative formation of waste, which leads to environmental pollution (Awasthi et al., 2021).
The waste resulting from the food industry produces pollution (Ilyas et al., 2021), being responsible for approximately 26% of global greenhouse gas emissions (Zioga et al., 2022). The global problem of waste could be solved by implementing zero-waste production technologies, using different methods aimed at the valorization of plant-based waste by emphasizing the importance of raw materials and achieving sustainable development objectives (Sabater et al., 2021; Liu et al., 2021).
Earth represents an important source of natural resources that, in connection with the activities of operators in different sectors, generate a vast range of waste (Srivastava et al., 2023).
The use of by-products in new matrices promote the circular and sustainable economy by implementing innovative methods and strategic approaches for the recovery of valuable products and the minimization of waste through several methods (Stanciu et al., 2023).
The review aims to identify the main directions of valorization of various by-products of plant origin, resulting from agriculture and processing in the food industry, for the purpose of environmental sustainability.

THE CONTENT OF PLANT BY-PRODUCTS IN BIOACTIVE COMPOUNDS

Food waste is composed of complex ingredients (vitamins, mineral salts, fibers, bioactive substances) that have been removed from the original material (Galanakis, 2012). Fruits, vegetables and other food products are rich in dietary carbohydrates, bioactive phytochemicals that provide health benefits in addition to basic nutrition (Liu, 2013; Dranca & Oroian, 2018).
They represent important sources of bioactive compounds that contribute to the prevention of

degenerative diseases (diabetes, Alzheimer, Parkinson) (Renard, 2018).

The presence of carotenoids in waste of vegetal origin offers wide opportunities to sustainable productions, due to their bioactive properties: antioxidant role, antitumor role, etc. (Cassani et al., 2022). Following the industrial processing of eggplants, secondary products are obtained (eggplant skins, etc.) which are thrown away without further recovery.

Eggplant skin is an important source of anthocyanins, with antioxidant, antimicrobial and antitumor properties (Karimi et al., 2021).

The industrial processing of sugar beet to obtain sugar releases large amounts of sugar beet pulp, waste that can be considered a valuable by-product as a source of hemicellulose, cellulose, pectin (Usmani et al., 2023).

Melon (*Cucumis melo* L.) extracts, especially the peel, contain phytochemical compounds with antimicrobial, antiviral, antioxidant, anti-inflammatory, antidiabetic effects, etc. (Gómez-Garcia et al., 2020).

Avocado processing leads to the generation of impressive amounts of pits that represent 13%-18% of the fruit's mass. Avocado seeds contain starch (66.3%), protein (4.9%), arabiose (4.12%), pentosans (3.3%) (Tesfaye et al., 2022). Sari et al. (2022) studied the processing of almonds, walnuts, cashews is done with the production of large amounts of waste rich in protein (45-55%), carbohydrates (30-35%), fiber, etc. (Sari et al., 2022).

By-products resulting from wine and olive oil industry are rich in bioactive compounds which may be used in obtaining novel functional food products (Balli et al., 2021). Olive waste contains phenolic compounds, their concentration being influenced by the growing area and variety. The phenolic fraction consists of different groups: phenolic acids, phenolic alcohols, flavonoids and secoiridoids (Veneziani et al., 2017).

Studies conducted in the last 15-20 years have shown that grape pomace could be a potential source of bioactive compounds (Chowdhary et al., 2021).

White grape pomace (unfermented waste) represents 10% to 30% of the crushed grape mass. The main macromolecules detected in grape pomace are polyphenols, proteins, etc. (Canalejo et al., 2021). In Table 1are presented some information on bioactive compounds found in grapes are given.

Table 1. Reporting of polyphenols from grapes/juice in the literature (Source: Majeedet et al., 2023)

Compound name	Fruit grapes	References
Quertin	Grape	Rockenbach et al., 2011; Bruno Romani et al., 2021; Gomez-Mejia et al., 2022.
Catecchin	Grape	Ferri et al., 2017; Rockenbach et al., 2011; Meini et al., 2019; Deshales et al., 2022; Ferri et al., 2017.
Catechin gallate	Grape (*Vitis vinifiera* L.)/Pomace	Ferri et al., 2017; Perez-Navarro et al., 2019.
Epicatechin gallate	Grape (*Vitis vinifiera* L.)	Ferri et al., 2017; Ivanova et al., 2011; Arts et al., 2000; Perez-Navarro et al., 2019.
Gallic acid	Grape (*Vitis vinifiera* L.)	Pedras et al., 2020; Meini et al., 2019; Alvareda et al., 2019; Bruno R. et al., 2021; Ferrari et al. 2017.
Malvidin-3-glucoside	Grape (*Vitis vinifiera* L.)	Perez-Navarro et al., 2019; Assayed & Adb El-Aty, 2009; Kammerer et al., 2004.
Malvidin-3-O-glucoside	Grape	Meini et al., 2019
Chlorogenic acid	Grape	Karunanidhi et al., 2013; Ferri et al., 2027.

TRENDS IN VALORIZATION OF VEGETABLE BY-PRODUCTS

About 1.3 billion tons of waste and by-products are produced annually (Gottardi et al., 2021). The valorization of products and by-products by ensuring food quality and safety is an important aspect in the evaluation of valorization strategies (Socas-Rodrigues et al., 2021).

The valorization of waste resulting from the food industry aims to stimulate innovative technologies in order to improve the use of resources in the cascade (Javourez et al., 2021). Due to the presence of carotenoids and their bioactive properties, waste of vegetal origin provides wide opportunities for sustainable productions, being able to be used to color fruit juices, pasta, meat, etc. (Cassani et al., 2022).

Vegetable and fruit co-products contain high concentrations of bioactive compounds, which makes the extraction of bioactive substances a capitalization strategy (Renard Chaterine, 2018). Anthocyanins from eggplant peel are an important alternative of synthetic additives that can be used to extend the shelf life of food products (Karimi et al., 2021).

The research paper "Geophagy: The earth-eaters of lower Southwestern Australia" recommends further studies for the red root vegetable (*Haemodorum spicatum*), which is part of the Haemodoraceae family, and which grows mainly in Western Australia. The bulbs, stem, leaves and seeds of the vegetable produce natural dyes: red, pink, purple and green. Red pigments (Hemocorina) are responsible for the spicy and hot flavor of the bulbs (Macintyre & Dobson, 2017).

The red pigment of *Haemodorum spicatum* can be considered as a sustainable alternative substitute to replace artificial dyes with natural dyes for increasing the demand for environmentally friendly, non-toxic and healthy eco-dye foods. (Liang et al., 2023). Sugar beet pulp has been mainly used as feed in animal nutrition, recent developments indicate the utilization of sugar beet pulp to obtain biofuels, biohydrogen, platform chemicals such as: alcohols, microbial enzymes, lactic acid, citric acid, proteins unicellular and biodegradable plastic materials (Usmani et al., 2023).

By-products resulting from different processing stages such as molasses, starch, fruits, fresh vegetables, etc. can be used in order to develop new food matrices thus obtaining edible bioproducts (lactic acid, cellular proteins). Contaminated, altered, expired waste can be used in some anaerobic digestion (Awasthi et al., 2022; Awasthi et al., 2022b).

In the food sector, pectin is used as a gelling or thickening agent because of the health benefits and its role as a health-promoting functional agent or fat substitute. The isolation of pectin from plant material or agro-food by-products also has non-food applications that include the pharmaceutical, medical industry (Dranca & Oroian, 2018).

The processing of melons (*Cucumis melo* L.) results in seeds and peels that contain important bioactive compounds, which are of interest in the food, pharmaceutical and cosmetic industries. These findings strengthen the support of researchers in analyzing the functional and nutritional potential of these wastes and the formulation of new functional foods (Gómez-Garcia et al., 2020).

Throwing avocado pits in the landfill or incinerating them has the effect of polluting the environment, which is why studies and researches aimed at overcoming technical-economic barriers are encouraged in order to develop and sell new products, as well as to establish model biorefineries (Tesfaye et al., 2022).

The techno-economic feasibility of biorefineries is the basis of the exploitation of avocado kernels, aiming at a circular bioeconomy (Rodriguez-Martinez et al., 2022). The processing of walnuts, almonds, cashews, hazelnuts, produces large amounts of waste that could be used to produce bioactive peptides, biofuels, etc. (Sari et al., 2022).

The by-products resulting from the processing of peanuts are a valuable source of nutrients, natural antioxidants, antimicrobials.

Antioxidants are used in food to prevent oxidative reactions and undesirable effects on food quality during processing and storage (Lorenzo et al., 2018).

Grape pomace is the main by-product of the wine industry. New applications related to waste from the wine industry led to different sectors: agriculture, animal husbandry, cosmetics, pharmaceuticals, as well as the bioenergy recovery sector regarding the potential for health and a sustainable environment (Chowdhary et al., 2021), and an attractive option of transforming biodegradable waste into stable products, excellent sources of plant growth or soil fertilizer (Xu et al., 2022).

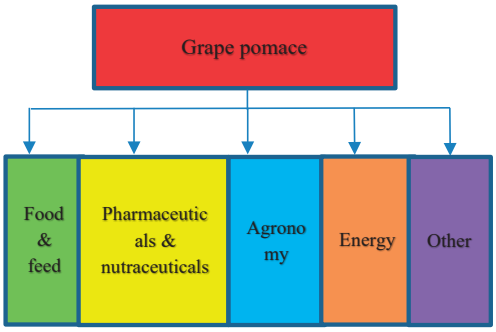

Figure 1. Application of grape pomace (Madaddian et al., 2022)

Grape seeds contain polyphenols, such as flavanols, flavonols, anthocyanins and stilbenes, being mainly located in the skin, while others, such as catechins and procyanidins, are only present in the seeds in concentrations that vary according to terrain, variety, etc. (Moro et al., 2021).

Studies show that the use of 7% of grape pomace or olive residue in the profile of some pasta leads to their fortification, showing good resistance to cooking, an optimal texture after cooking, increasing the nutritional profile of the pasta through the amount of phenolic compounds and fibers (Balli et al., 2021).

Wheat, barley are the dominant crops in countries with a medium and high level of development. By grinding wheat, rolled barley, bran or straw are obtained as secondary products, rich in protein and dietary fiber (Galanakis, 2012).

Over the years, studies have been carried out to enrich the nutritional composition of bread, by adding different flours or ingredients with large amounts of bioactive compounds with health benefits (Stanciu et al., 2023).

Figure 2. Sorana organic seabuckthorn - fresh, pomace and in form of powder (Source: Popa et al., 2022)

The use of sea buckthorn powder as an ingredient has led to promising experimental results regarding bread making (Popa et al., 2022).

Agro-industrial by-products, from bakery products, etc., are used as a source of cheap fermentation contributing to improving the economy and increasing productivity (Arya et al., 2022).

The earliest forms of biotechnology date back several thousand years, before the discovery of the existence of microbes. Microbes are widely used as living organisms, indispensable due to their involvement in the realization of bio productions such as food additives, cosmetic products, valuable chemical products, biopharmaceutical products, etc. (Pfeifer et al., 2021).

MATERIALS, METHODS AND TECHNIQUES

For this research I analyzed Scopus, the Elsevier database and SprinerLink Journals and entered key words such as "sustainability" or "vegetable by products". I analyzed the data from various reviews to have an overall picture of the methods used for the valorization of vegetable and animal by-products for the purpose of environmental sustainability.

It is estimated that the world population will reach approximately 10 billion people in 2050, which means an increase in the amount of food and/or biomass to be processed, but also a high amount of waste (Vicente et al., 2023).

Food waste is usually organic residues generated by the processing of raw materials into food, while a by-product is obtained as a result of the manufacturing process of the main product, often with a market value (Tiwari & Khawas, 2021; Rodriques-Ramos et al., 2022).

The full recovery of eggplant waste, through the extraction of anthocyanins and pectin, is carried out by different methods and techniques developed and improved, with ultrasound and microwaves (Karimi et al., 2021). The pressed drupes resulting as waste after obtaining the oils could be exploited by hydrolysis in order to obtain therapeutic benefits that would have a higher value than the current one as animal feed (Sari et al., 2022). A review indicates the need to capitalize on indigenous African leafy vegetables, altered by bacteria pseudomonadaceae, enterobacteriaceae, etc., fermentation being deepened in this case as a form of cheap capitalization. The controlled fermentation carried out with the help of lactic acid bacteria leads to the improvement of the nutritional content and the extension of the shelf life of the vegetables (Misci et al., 2022).

Anaerobic digestion technology is becoming more and more important due to its contribution to the sustainability of the environment and the circular bioeconomy (Tavera-Ruiz et al., 2023), anaerobic digestion being a way of valorizing agricultural waste (Nagarajan A. et al, 2023).

Pretreatment of melon skins, peanut skins, etc., is a necessary step in obtaining value-added products (Ajayi & Lateff, 2023).

Biorefineries are new state-of-the-art concept where multiple by-products are manufactured from renewable feedstock (Kasani et al., 2022). The waste streams generated by the food industry, especially the sugar industry, make it possible to replace plastic materials with ecological bioplastics in the form of polyhydroxyalkanoates, which represent an attractive alternative due to their biodegradability and biocompostability properties (Saratale et al., 2021).

Organic waste from the food and agricultural industry represents a major source of recoverable biomass for energy (Akbi et al., 2017). The main raw material for renewable energy production is biomass waste, which can contribute to reducing traditional energy sources and changing climate conditions (Usmani et al., 2023).

Manea (2017) studied the use of carrot juice, beetroot or pomace powder in some meat products to improve color (Manea, 2017). Composition of grape pomace varies considerably between different varieties (Ribeiro et al., 2015; Gongalves et al., 2017). The pomace powder was obtained after pressing the grapes and separating the juice. The remaining residue was dried at 20-25˚C, then ground with an electric stirrer. After obtaining, the powder was stored in hermetically sealed glass containers protected from sunlight and heat protected from sunlight and heat (Manea, 2017). In the Table 2 various processing methods of grape pomace are presented to obtain bioactive compounds.

Table 2. Processing grape pomace by different methods and obtaining by-products with added value (Source: Ilyas et al., 2021)

Initial processing	Traditional & conventional methods	By-products with added value
Grape Pomace	**Methods Traditional:**	
	Soxhlet extraction Maceration Reflux extraction	Phenolic compounds
	Methods Conventional:	
	Solid-Liquid extraction	Flavonoids
	Supercritical fluid extraction	Antioxidants
	Accelerated solvent extraction	Anthocyanin
	Dynamic superheated liquid extraction	Hemicellulose
	Ultraound assited extraction	Polyphenols
	Pulsed electric field extraction	Acetaldehydes
	Enzyme assisted extraction	

TRENDS AND PERSPECTIVES

Waste management represents a potential solution towards the circular economy in the food system (Jurgilevich A. et al., 2016; Lavelli, 2021). The circular economy is the main element of the Green Deal, food traceability being a key element of environmental sustainability, the goal being the production of healthy and environmentally friendly foods (Tesfaye et al., 2022).

The current trends regarding the recovery of waste from the food sector, but also the finding of innovative solutions regarding the partial or total replacement of animal proteins with vegetable proteins are evaluated by scientists from the food sector, which contribute to the need to find answers to some ethical demands, pro-health and last but not least environmental sustainability (Kotecka-Majchrzak et al., 2020). The current trend of plant-based dairy and meat alternatives has the potential to contribute to environmental sustainability by reducing gas emissions and food systems (Giacalone et al., 2022), and technological innovation approaches essentially have ways to optimize food quality according to consumer requirements and preferences, environmental sustainability (Abecassis et al., 2018).

CONFLICT OF INTEREST

Both authors declare no conflict of intertest.

CONCLUSIONS

The concept of valorization of agri-food waste is based on the realization of a circular bioeconomy, starting from the valorization of food by-products in new food matrices, continuing with research, the development of

innovative technologies, the creation of new food products with added value.

The vegetable waste obtained from the processing of raw materials of vegetable origin contains important bioactive compounds, which are of interest in the food, pharmaceutical and cosmetic industries. These findings strengthen support for researchers in analyzing the functional and nutritional potential of these wastes and in formulating new functional foods such as meat analogs.

Research and innovative technologies support environmental sustainability by finding alternatives, such as meat analogues, soy-based foods enjoying a promising future on the international market.

In the context of the cascading valorization of raw materials, but also of environmental sustainability, ecological agriculture represents an alternative that offers farmers the opportunity to satisfy the demand for ecological products, etc. The studies and research aim at overcoming the technical-economic barriers in order to develop and sell new products, as well as the establishment of model biorefineries.

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HEAVY METAL CONTAMINATION IN SOIL AND ITS ACCUMULATION IN HOME GROWN TOMATO (*Solanum lycopersicum* L.) FRUITS

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Abstract

The paper aimed to assess the hygienic quality of soils in home gardens and the safety of consumption of tomatoes grown in these soils. Fruits of three tomato cultivars, namely Radana, Tornado F1, and Cherolla F1 from four localities of Slovakia (Sabinov, Žilina, Oravská Lesná, Hliník nad Hronom) along with soil samples were analysed using Varian AA 240FS/240Z atomic absorption spectrometer. Based on the results, all soils can be classified as highly contaminated, extremely polluted, and of high to very high risk. Results showed that monitored cultivars are not bioaccumulators of analysed risk elements, however, the content of Pb and Cd exceeded the limits set by Commission Regulation (EU) 2023/915 in some of the samples. While the results of the health risk assessment showed that consumption of monitored cultivars does not pose a risk to the consumers, it is important to take other dietary sources of risk elements into account, since monitored tomatoes alone could contribute up to 5.7% of the provisional monthly intake of Cd.

Key words*: cadmium, lead, risk elements, Solanum lycopersicum L., tomato*

INTRODUCTION

Tomatoes, fruits of *Solanum lycopersicum* L., are one of the most widely grown and commonly consumed vegetables, utilized in a variety of processed food products, including sauces, ketchup, purees, pastes, soups, juices, and juice blends, as well as whole or diced, sliced, quartered, or stewed canned tomatoes. They are popular due to their taste, availability, affordability, and recognized health benefits (Salehi et al., 2019; Ali et al., 2020). According to FAO/WHO, tomatoes were the most-produced vegetable, with more than 186 million tons produced in 2022, and also the most-consumed vegetable with 22.66 kg consumed per capita in 2021.

Tomatoes are important not only because of the quantity consumed but also because of their high nutritional value and positive effects on human health (Ilić et al., 2014). They are a valuable source of essential nutrients and bioactive compounds, such as carotenoids, phenolics, and glycoalkaloids (Rao et al., 2018; Wang et al., 2022). A negative correlation was found between tomato consumption and the mortality rates from all causes, and the mortality rates from coronary heart disease, cerebrovascular illness, gastric cancer, and prostate cancer (Li et al., 2021).

Because of the abovementioned benefits, and low maintenance cultivation requirements, home growing of tomatoes is fairly popular. However, there is currently a major global issue regarding the contamination of soil and crops with heavy metals. Vegetable contamination is gaining a lot of attention as awareness of the potential health risks grows (Gupta et al., 2021). In addition to a health risk, the presence of heavy metals can also negatively impact the amount of nutrients, such as lycopene and ascorbic acid, in tomatoes (Collins et al., 2022).

Due to the popularity of tomatoes, and the pressing issue of heavy metal contamination, this manuscript addresses the content of heavy metals in the soils and its subsequent accumulation in homegrown tomato fruits.

MATERIALS AND METHODS

Localities

Samples were grown in home gardens in 4 localities in Slovak Republic: Sabinov (1), Žilina (2), Oravská Lesná (3), and Hliník nad Hronom (4)

Sampling
Three tomato cultivars, namely Radana (R), Tornádo F1 (T), and Cherolla F1 (Ch) were conventionally cultivated. Samples were collected by hand, in a state of full ripeness.
Soil samples were taken at 10 cm into the GeoSampler paedological probe (Thermo Fisher Scientific, Hampton, NH, USA). Air dryed samples were ground with a VEB Thurm ZG 1 grinding machine (Stahlbau Magdeburg GmbH, Magdeburg, Germany) to fine earth (0.125 mm average particle size).

Soil sample preparation
Total heavy metals in soil were extracted in the *aqua regia* (2.5 mL 65% HNO_3 Suprapur® (Merck, Darmstadt, Germany) and 7.5 mL 37% HCl Suprapur® (Merck, Darmstadt, Germany) at 160°C for 15 minutes using MarsX-press5 microwave digestion apparatus (CEM Corp., Matthews, NC, USA). After mineralization, samples were filtered through Filtrak 390 quantitative filter paper (Munktell, GmbH, Bärenstein, Germany) and diluted with deionized water (0.054 $\mu S.cm^{-1}$) to a volume of 100 mL.
The bioavailable forms of heavy metals were extracted by 20 g of dried soil sample in 50 mL of NH_4NO_3 (c = 1 $mol.L^{-1}$, Merck, Darmstadt, Germany) for 2 h using a Unimax 2010 horizontal shaker (Heidolph Instrument, GmbH, Schwabach, Germany).
After extraction, the samples were filtered through Filtrak 390 quantitative filter paper (Munktell, GmbH, Bärenstein, Germany), and 0.5 mL of HNO_3 was added.

Plant sample preparation
Homogenized dried samples were mineralized in a mixture of 5 mL of HNO_3 Suprapur® (Merck, Darmstadt, Germany) and 5 mL of deionized water (0.054 $\mu S.cm^{-1}$) using a Mars Xpress 5 closed microwave digestion system (CEM Corp., Matthews, NC, USA), at 160 °C for 15 min. and maintaining it at constant temperature for 10 min.
After digestion, samples were filtered through Filtrak 390 quantitative filter paper (Munktell, GmbH, Bärenstein, Germany) and filled to a volume of 50 mL with deionized water.

Determination of heavy metals in samples
Heavy metals in soil and plant samples were determined according to Vollmanova et al. (2014) by Flame AAS method (Fe, Mn, Zn, Cu, Co, Ni, Cr) using VARIAN AASpectra DUO 240FS atomic absorption spectrophotometer, and Graphite Furnace AAS method (Cd, Pb) using VARIAN AASpectra DUO 240Z atomic absorption spectrophotometer (Varian, Ltd., Mulgrave, VIC, AUS).

Bioaccumulation factor
To determine the ability of the plant to uptake heavy metal from the substrate to their fruits, the bioaccumulation factor was calculated as the ratio of the heavy metal content in the plant and the heavy metal content in the soil.

Environmental risk assessment
To evaluate the contamination of monitored soils, the Degree of contamination (Cdeg), pollution load index (PLI), and potential ecological risk factor (PERF) were calculated according to Čeryová et al., (2023)

Health risk assessment
To evaluate the risks arising from the consumption of monitored tomatoes, % of intake was calculated for adult humans weighting 70 kg using the average consumption of tomatoes (22.66 kg per capita in the year 2021), and tolerable intakes set by WHO and EFSA: 56 mg of Fe, 8 mg of Mn, 0.3-1 mg of Zn, 35 mg of Cu, 0.6 mg of Co, 0.91 mg of Ni, 56 mg of Cr per day, and 1.75 mg of Cd per month.

Statistical analysis
Statistical analyses were performed using XLSTAT (Lumivero, 2024).

RESULTS AND DISCUSSIONS

Heavy metal content in soil samples
Total heavy metal content and content of bioavailable forms of heavy metals in monitored soils are expressed in Table 1 and Table 2. The limit value of total Cd content was exceeded in all soil samples. The limit value of total Zn content was exceeded in soil samples from Sabinov, Oravská Lesná, and Hliník nad Hronom. The critical value of mobile forms of Cd and Pb was exceeded in all soil samples.

Table 1. Total heavy metal content in monitored soils (mg/kg)

Locality	Fe	Mn	Zn	Cu	Co	Ni	Cr	Pb	Cd
1	21307±2214	967±112	345±51.5	28.1±3.33	9.73±1.12	38.2±0.42	32.5±0.33	35.7±0.28	2.42±0.02
2	25779±23547	1153±125	74.0±11.3	26.5±2.81	12.7±1.30	46.9±0.55	30.5±0.33	37.3±0.41	3.39±0.04
3	17978±1528	1468±158	890±99.9	36.9±4.69	8.60±0.95	32.7±0.28	35.5±0.32	46.4±0.44	4.42±0.04
4	17427±1855	616±79	162±18.1	57.7±7.53	7.60±0.71	19.9±0.18	21.8±0.28	47.2±0.40	2.84±0.03
Limit value*			150	60		50		70	0.7

*According to Act No 220/2004, valid in Slovak Republic

Table 2. Bioavailable heavy metal content in monitored soils (mg/kg)

Locality	Fe	Mn	Zn	Cu	Co	Ni	Cr	Pb	Cd
1	0.39±0.03	0.26±0.02	0.23±0.03	0.15±0.01	0.16±0.02	0.30±0.04	0.06±0.007	0.53±0.06	0.18±0.02
2	0.22±0.02	0.15±0.01	0.15±0.02	0.06±0.01	0.17±0.02	0.29±0.03	0.04±0.005	0.52±0.04	0.20±0.02
3	0.69±0.08	2.04±0.02	0.40±0.03	0.40±0.05	0.22±0.02	0.42±0.04	0.05±0.005	0.68±0.06	0.24±0.03
4	0.17±0.02	0.24±0.02	0.20±0.02	0.11±0.13	0.11±0.01	0.22±0.02	0.01±0.001	0.31±0.04	0.13±0.01
Critical value*			2	1		1.5		0.1	0.1

*According to Act No 220/2004. valid in Slovak Republic.

Exceeded levels of total and mobile forms of Cd were also reported in other localities of Slovakia (Fazekašová et al., 2021; Lidiková et al., 2021a; Lidiková et al., 2021b; Musilová et al., 2021; Musilová et al., 2022). According to Fazekašová et al. (2021), Slovakia has many so-called geochemical anomalies, which are notably high in Cd.

Table 3. Environmental risk assessment of monitored soils

Locality	Cdeg	PLI	PERI
1	109.7	3.7	276.0
2	126.8	3.6	370.0
3	144.5	4.7	488.3
4	83.6	3.1	319.5

Based on the environmental risk assessment (Table 3), monitored soils can be defined as highly contaminated, extremely polluted, and of moderate to considerable risk.

Heavy metal content in plant samples

The contents of determined heavy metals in monitored samples are shown in Table 4. The content of Fe ranged from 2.47 to 5.57 mg.kg^{-1}. The content of Mn ranged from 0.72 to 1.55 mg.kg^{-1}. The content of Zn ranged from 0.09 to 0.32. The content of Cu ranged from 0.28 to 0.91. The content of Co ranged from 0.01 to 0.05. The content of Ni ranged from 0.02 to 0.17. The content of Cr ranged from 0.01 to 0.05. The content of Pb ranged from 0.02 to 0.17. The content of Cd ranged from 0.01 to 0.08. Maximum levels of Pb were exceeded in all cultivars from Žilina, in cultivars Radana and Cherolla F1 from Sabinov, and in cultivars Tornado F1 and Cherolla F1 from Hliník and Hronom. Maximum levels of Cd were exceeded in all cultivars from Sabinov and Žilina, in cultivar Radana from Oravská Lesná, and in cultivar Cherolla F1 from Hliník nad Hronom.

Musilová et al. (2022) determined heavy metals in tomatoes grown in the Spiš region of Slovakia and reported similar levels of Mn, Ni, and Pb (0.91-1.32, 0.03-0.15, 0.02-0.16 mg. kg^{-1}), higher levels of Fe, Zn, and Cu (5.41-14.7, 1.23-1.41, 1.10-1.81 mg.kg^{-1}) but lower levels of Cd (BDL). Suárez et al. (2007) reported that the content of Fe, Cu, Zn, and Mn in tomatoes is affected by the cultivar, cultivation method, period of sampling, and region of production. They reported 1.67-2.62 mg Fe.kg^{-1}, 0.18-0.34 mg Cu.kg^{-1}, 0.60- 1.06 mg Zn.kg^{-1}, and 0.55-1.53 mg Mn.kg^{-1} in different tomato cultivars. According to Vélez-Terreros et al. (2021) Cd, Cr, Ni, and Pb concentrations were higher in conventional tomatoes. On the other hand, Rossi et al. (2008) reported, that organic tomatoes had higher Cd and Pb levels but a lower Cu content.

Table 4. Content of heavy metals in monitored samples (mg/kg fresh weight)

Locality	Cultivar	Fe	Mn	Zn	Cu	Co	Ni	Cr	Pb	Cd
1	R	2.47±0.31 a	0.83±0.12^{ab}	0.09±0.002^{a}	0.28±0.03 a	0.04±0.006 fg	0.05±0.008 abc	0.03±0.004^{bc}	0.09±0.01^{a} bc	0.08±0.01^{b}
	T	3.19±0.41 abc	0.89±0.08^{b}	0.13±0.02 abc	0.45±0.04 b	0.01±0.001 a	0.02±0.002 a	0.03±0.003^{b}	0.03±0.003 abc	0.08±0.01^{b}
	Ch	4.07±0.37 cde	1.19±0.13^{b}	0.14±0.01 abc	0.47±0.05 b	0.02±0.003 bc	0.05±0.006 abc	0.03±0.004^{bc}	0.12±0.02^{a} bc	0.06±0.01^{a} b
2	R	4.68±0.51 def	1±0.12^{ab}	0.19±0.01 abc	0.68±0.07 bc	0.02±0.002 sb	0.05±0.005 abc	0.03±0.004^{b}	0.14±0.02^{b} c	0.07±0.01^{a} b
	T	5.57±0.67 f	1.54±0.17^{ab}	0.32±0.05 c	0.91±0.11 e	0.03±0.002 bcd	0.07±0.007 abc	0.03±0.003^{b}	0.17±0.02^{c}	0.08±0.01^{b}
	Ch	4.14±0.46 cde	1.55±0.22^{ab}	0.28±0.07 c	0.59±0.06 bcd	0.03±0.003 cde	0.03±0.004 ab	0.03±0.004^{bc}	0.17±0.02^{c}	0.06±0.01^{a} b
3	R	4.97±0.6^{ef}	1.16±0.13^{ab}	0.23±0.04 bc	0.7±0.08^{cd}	0.04±0.004 fg	0.04±0.004 abc	0.01±0.001^{a}	0.03±0.004 abc	0.05±0.01^{a} b
	T	3.61±0.33 bcd	0.90±0.09^{ab}	0.17±0.03 abc	0.65±0.08 cd	0.04±0.004 ef	0.03±0.003 abc	0.01±0.002^{a}	0.02±0.003 a	0.02±0.002^{ab}
	Ch	3.81±0.46 cd	1.05±0.14^{ab}	0.15±0.02 abc	0.46±0.05 b	0.03±0.004 de	0.17±0.019 c	0.02±0.002^{a}	0.02±0.003 ab	0.02±0.001^{a}
4	R	2.67±0.29 ab	0.78±0.09^{a}	0.13±0.01 abc	0.54±0.06 bc	0.03±0.003 de	0.03±0.003 ab	0.03±0.003^{b}	0.04±0.005 abc	0.01±0.001^{a}
	T	4.01±0.36 cde	0.82±0.07^{ab}	0.15±0.02 abc	0.72±0.08 d	0.04±0.004 ef	0.07±0.008 bc	0.04±0.005^{cd}	0.13±0.02^{a} bc	0.02±0.002^{ab}
	Ch	3.29±0.36 abc	0.72±0.08^{a}	0.11±0.02 ab	0.62±0.07 bcd	0.05±0.006 g	0.08±0.008 bc	0.05±0.005^{d}	0.11±0.01^{a} bc	0.03±0.002^{ab}
ML*									0.05	0.02

*According to Commission Regulation (EU) 2023/915

Table 5. BAF of heavy metals in monitored samples

Locality	Cultivar	Fe	Mn	Zn	Cu	Co	Ni	Cr	Pb	Cd
1	R	0.001^{a}	0.008^{abcd}	0.03^{abc}	0.09^{a}	0.04^{abc}	0.013^{ab}	0.009^{abcd}	0.024^{abcd}	0.29^{b}
	T	0.001^{ab}	0.008^{bcd}	0.04^{abc}	0.15^{abc}	0.01^{a}	0.005^{a}	0.008^{abcd}	0.007^{abcd}	0.29^{b}
	Ch	0.002^{abcd}	0.010^{cde}	0.04^{abc}	0.14^{abc}	0.02^{abc}	0.010^{ab}	0.009^{abcd}	0.028^{abcd}	0.21^{ab}
2	R	0.002^{cd}	0.009^{bcd}	0.26^{bc}	0.26^{bc}	0.01^{ab}	0.011^{ab}	0.010^{abcd}	0.040^{bcd}	0.20^{ab}
	T	0.002^{d}	0.013^{ef}	0.28^{bc}	0.32^{c}	0.02^{abc}	0.013^{ab}	0.009^{abcd}	0.043^{d}	0.21^{ab}
	Ch	0.001^{abc}	0.012^{ef}	0.25^{bc}	0.20^{abc}	0.02^{abc}	0.006^{a}	0.010^{abcd}	0.041^{cd}	0.16^{ab}
3	R	0.003^{d}	0.007^{abc}	0.02^{ab}	0.18^{abc}	0.05^{bc}	0.011^{ab}	0.004^{ab}	0.006^{abc}	0.10^{ab}
	T	0.002^{bcd}	0.006^{a}	0.02^{a}	0.16^{abc}	0.04^{abc}	0.009^{ab}	0.004^{a}	0.004^{a}	0.04^{a}
	Ch	0.002^{bcd}	0.006^{ab}	0.02^{a}	0.11^{abc}	0.03^{abc}	0.047^{b}	0.004^{abc}	0.004^{ab}	0.02^{a}
4	R	0.002^{bcd}	0.014^{f}	0.10^{abc}	0.10^{ab}	0.05^{bc}	0.017^{ab}	0.013^{bcd}	0.010^{abcd}	0.04^{a}
	T	0.002^{d}	0.012^{ef}	0.11^{abc}	0.11^{abc}	0.04^{abc}	0.033^{b}	0.017^{cd}	0.024^{abcd}	0.05^{ab}
	Ch	0.002^{bcd}	0.010^{de}	0.09^{abc}	0.10^{a}	0.06^{c}	0.034^{b}	0.020^{d}	0.021^{abcd}	0.08^{ab}

The factors of accumulation of the determined heavy metals (BAF) are shown in Table 5. Based on the BAF, we can state that monitored cultivars show none to low accumulation of Fe (0.001-0.003), Mn (0.006-0.014), Co (0.01-0.05), Ni (0.005-0.047), Cr (0.004-0.02), and Pb (0.004-0.043) and low to medium accumulation of Zn (0.02-0.28), Cu (0.09-0.32), and Cd (0.02-0.29). No differences were observed in the accumulation of heavy metals by individual cultivars, but differences were observed between localities, which suggests that different agro-environmental factors influence the rate of accumulation. The low accumulation of heavy metals in tomato fruits was also reported by other authors (Arslan Topal et al., 2022; Musilová et al., 2022; Bounar et al., 2020). According to Murtić et al. (2018), the accumulation of heavy metals in tomato fruits is low because of the combined effects of several plant defense mechanisms. According to Taghipour and Jalali (2020), the accumulation of heavy metals in tomatoes is lower when organic wastes, especially plant wastes such as rice husk, are applied to the soil.

Table 6. Relationships between contents of monitored heavy metals

Variables	Fe	Mn	Zn	Cu	Co	Ni	Cr	Pb	Cd
Fe	**1**	**0.726**	**0.789**	**0.788**	-0.227	0.070	-0.201	0.430	0.282
Mn	**0.726**	**1**	0.539	0.410	-0.287	-0.052	-0.209	0.511	0.477
Zn	**0.789**	0.539	**1**	**0.880**	0.002	-0.094	-0.202	0.272	-0.024
Cu	**0.788**	0.410	**0.880**	**1**	0.008	-0.072	-0.003	0.389	-0.069
Co	-0.227	-0.287	0.002	0.008	**1**	0.173	0.083	-0.145	-0.492
Ni	0.070	-0.052	-0.094	-0.072	0.173	**1**	-0.065	-0.115	-0.400
Cr	-0.201	-0.209	-0.202	-0.003	0.083	-0.065	**1**	**0.624**	0.082
Pb	0.430	0.511	0.272	0.389	-0.145	-0.115	**0.624**	**1**	0.440
Cd	0.282	0.477	-0.024	-0.069	-0.492	-0.400	0.082	0.440	**1**

Values in bold are significant ($p<0.05$)

The relationships between the contents of determine heavy metals in monitored samples are shown in Table 6. The content of Fe positively correlated with the content of Mn, Zn, and Cu, and the content of Zn positively correlated with the content of Cu. Also, a positive correlation was observed between the content of Cr and Pb. Suárez et al. (2007) also observed positive correlations between Fe and Zn, and Fe and Mn, between Cu and Zn, and Cu and Mn, and between Mn and Zn.

The content of individual elements varied depending on both cultivar and locality. While we could not characterize individual cultivars by the content of heavy metals, samples from Žilina could be characterized by their Fe, Mn, and Zn content, and by accumulation of Co and Pb. Samples from Hliník nad Hronom could be characterized by the accumulation of Mn. These results suggest that locality could have a greater impact on the accumulation and content of elements than cultivars.

Health risk assessment

The percentages of tolerable intake of the determined heavy metals by consumption of monitored tomatoes are shown in Table 7. While the Fe, Mn, Zn, Cu, Co Ni, and Cr are essential elements, and their content in tomatoes does not pose a threat to human health, Pb and Cd are highly toxic (Musilová et al., 2022). According to EFSA (2012a; 2012b), food is the major source of human exposure to Pb and Cd. Based on the daily intake of tomatoes, we can state that tolerable intakes of heavy metals would not be exceeded, however, it is important to take other dietary sources of risk elements into account since monitored tomatoes alone could contribute up to 5.7% of the provisional monthly intake of Cd.

Table 7. The percentages of tolerable intake of the elements by consumption of tomatoes (%)

Locality	Cultivar	Fe	Mn	Zn	Cu	Co	Ni	Cr	Cd
	R	0.19	0.44	0.07-0.25	0.03	0.30	0.25	0.002	5.6
1	T	0.24	0.48	0.10-0.33	0.06	0.08	0.10	0.002	5.6
	Ch	0.31	0.64	0.09-0.30	0.06	0.16	0.22	0.003	4.5
	R	0.36	0.54	0.11-0.38	0.08	0.12	0.24	0.002	4.9
2	T	0.43	0.83	0.13-0.45	0.11	0.18	0.30	0.002	5.7
	Ch	0.32	0.83	0.12-0.41	0.07	0.22	0.14	0.003	4.5
	R	0.38	0.62	0.13-0.42	0.09	0.31	0.18	0.001	3.5
3	T	0.28	0.48	0.12-0.41	0.08	0.28	0.16	0.001	1.4
	Ch	0.29	0.56	0.10-0.33	0.06	0.23	0.82	0.001	0.9
	R	0.20	0.42	0.09-0.30	0.07	0.23	0.15	0.002	0.7
4	T	0.31	0.44	0.12-0.41	0.09	0.26	0.35	0.003	1.3
	Ch	0.25	0.39	0.10-0.34	0.08	0.36	0.36	0.004	1.9

CONCLUSIONS

Monitoring of heavy metals in soil, and their accumulation in tomato fruits is important to assess the contamination, and the associated health risks. While monitored cultivars are not accumulators of heavy metals, the content of Pb and Cd exceeded the maximum levels. The results of this study suggest that the accumulation and content of heavy metals in tomatoes depend mainly on agro-environmental factors. However, it is necessary to conduct further studies dealing with the heavy metal contamination of tomatoes.

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TESTING THE FERMENTATIVE POTENTIAL OF SOME LOCAL *Saccharomyces* AND non-*Saccharomyces* YEASTS

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Abstract

Considering that Starmerella bacillaris and Metschnikowia pulcherrima have repeatedly proved their desirable oenological properties, we have chosen to test the fermentative potential of local strains from our collection, in simple and sequential cultures with a local Saccharomyces cerevisiae. In order to prove their winemaking potential, the fermentative kinetics were observed, along with ethanol and sugar content, pH, total dry matter and wines colors. Local yeast S. cerevisiae BB06 finalized the fermentation with 10.5-11% ethanol and 9.5-9.3°Bx. The two sequential fermentations with S. bacillaris MI115 and M. pulcherrima MI109 led to an average ethanol content of 7.8%, respectively 7.8% and around 13.9°Bx, respectively 16.6°Bx. The pH levels were maintained between 3.0 and 4.0 throughout the experiments. The three tested strains confirmed they are suitable for winemaking in sequential steps, but several further investigations should be performed on biochemical and organoleptic level.

Key words*: autochthonous yeasts, Metschnikowia pulcherrima, oenological potential, Starmerella bacillaris.*

INTRODUCTION

Non-*Saccharomyces* (NS) yeasts show low fermentation performance, which rules out their exclusive use in the fermentation of grape must. They are not able to numerically dominate the entire fermentation process, nor can they bring the alcoholic fermentation to the end, due to their low tolerance to ethanol (Binati et al., 2020).

However, NS yeasts can be used in co-inoculations and in sequential inoculations with yeasts belonging to the *Saccharomyces* genus, because the metabolic impact of NS yeasts in the early stages of fermentation is sufficient to trigger significant changes in the wines' volatile profile (Carrau et al., 2020).

Considering that many of these non-conventional yeasts possess real winemaking properties, such as the production of higher alcohols, volatile esters, monoterpenes (Belda et al., 2017), wines can be obtained by inoculating non-*Saccharomyces* yeasts at the beginning, and by adding a *Saccharomyces* yeast after the fermented must reaches about 10% ethanol (Carrau et al., 2020).

Metschnikowia pulcherrima is a yeast which ferments glucose, but its fermentative power is low. It shows a low tolerance to ethanol, hardly withstanding concentrations that exceed 4-5% ethanol (*v/v*) (Vicente et al., 2020). *M. pulcherrima* can positively influence the content of esters, thiols and terpenes in wine and thus contribute to the aroma of the obtained wines (Benito et al., 2019).

Regarding the enzymatic activity of *M. pulcherrima*, it is known that it is represented by proteases, glucanases, pectinases, lipases (Canonico et al., 2023). In *S. cerevisiae* co-fermentation with *M. pulcherrima*, the obtained wine contains a volume of ethanol compared to simple fermentations with *S. cerevisiae*, a lower amount of malic acid and reduced total and volatile acidity (Canonico et al., 2023).

Starmerella bacillaris (also known as *Candida zemplinina*) is a non-*Saccharomyces* yeast with huge potential in winemaking, due to its strong fructophilic character and due to its low yield in the production of ethanol from consumed sugars (Magyar & Toth, 2011). Even if it shows a very close genetic relationship to *Candida stellata*, with which it was, until recently, confused,

C. zemplinina is a separate species, described as such in 2004, by Sipiczki.

According to studies carried out by several researchers, *S. bacillaris* presents the following desirable characteristics in winemaking: increased production of glycerol (Englezos et al., 2018 & 2019; Binati et al., 2020; Russo et al., 2020), low production of ethanol (Binati et al., 2019; Russo et al., 2020), tolerance to osmotic pressure (Vilela, 2019; Shen et al., 2022), pronounced fructophilic character (Wang et al., 2016), produces different compounds of aroma - linalool, geraniol, citronellal (Sadoudi et al., 2012). Due to the fact that *S. bacillaris* does not have the ability to complete the fermentation by itself, most of the characteristics described above are valid especially in co-cultures with *S. cerevisiae*, or in sequential cultures with the same wine yeast.

In order to develop an industrial winemaking process, it is necessary to go through several stages, as follows: the laboratory stage, the pilot station stage, the industrial production stage and the separation of the finished product. In the laboratory, the cultivation of yeasts is carried out on liquid media, in Erlenmeyer flasks, in static conditions (in the case of wine) or in continuous agitation, in small volumes (Lazăr et al., 2016). The next step is the cultivation in small volume bioreactors (up to 20L), where pH, temperature, aeration and agitation can be easily adjusted.

In the case of the present experiments, the volume was limited to 200 mL of grape must, to be able to carefully monitor the environmental conditions during the fermentative process using non-*Saccharomyces* yeasts, co-inoculated with *S. cerevisiae*.

MATERIALS AND METHODS

Yeast strains

The present study was conducted with the use of three yeast strains belonging to Faculty of Biotechnology Collection, two of them being non-*Saccharomyces* (*Starmerella bacillaris* and *Metschnikowia pulcherrima*) and one *Saccharomyces cerevisiae* strain (Table 1). All three yeast strains were isolated from Pietroasa vineyard, Buzău county and cryopreserved in Yeast Extract Peptone Dextrose (YEPD - yeast extract 10 g/L, peptone 20 g/L, dextrose 20 g/L, pH 6.5) containing 40% glycerol. Before initiating the experiments, the yeast strains were sub-cultured on YEPD and incubated at 20°C for 48 h. Subsequently, the strains were inoculated and maintained at 4°C on YEPD agar plates. For the analysis of the fermentation process at laboratory level, the experimental variants (Table 2) were inoculated on fresh white grape must from Pietroasa vineyard. All fermentations were conducted in triplicate.

Table 1. Tested yeast strains

No.	Species	Strain
1.	*Saccharomyces cerevisiae*	BB06
2.	*Starmerella bacillaris*	MI115
3.	*Metschnikowia pulcherrima*	MI109

Experimental variants were codified as stated in Table 2, depending on the inoculation modality (simple culture or co-inoculation). All of them were inoculated on sulfated must and sulfur-free must.

Table 2. Experimental variants

Sample	*S. cerevisiae* BB06	*S. bacillaris* MI115	*M. pulcherrima* MI109
V_1	100%	-	-
V_2	-	100%	-
V_3	-	-	100%
V_{12}	50%	50%	-
V_{13}	50%	-	50%

Grape must preparation

The must obtained from the fresh white grapes was divided in two sections, one without sulfur dioxide and the other with the addition of sulfur dioxide, according to the method used by Capece et al. (2020). The must (100 mL) was thus distributed into 250 mL Erlenmeyer flasks and sterilized by tyndallization.

For the preparation of the inoculum, a diluted must solution was obtained (50% must and 50% water) and then sterilized by autoclaving.

Primary and secondary fermentation

After cooling, the Erlenmeyer flasks containing the diluted must were inoculated with the yeast strains, according to the experimental variants presented previously (Table 2), adapting the protocol used by Dutraive et al. (2019). The 10 Erlenmeyer flasks with grape must (5 with sulfur dioxide and 5 without sulfur dioxide) were inoculated with a corresponding volume of

the pre-inoculum, to reach a level of 10^8 CFU/mL. V_{12} and V_{13} were inoculated at T_0 with the non-*Saccharomyces* strains and after 24 h with *S. cerevisiae* (protocol adapted after Englezos et al., 2019).

Primary fermentations were carried out in 250 mL Erlenmeyer flasks with silicone caps, in which a polyethylene tube was inserted, to allow the release of CO_2 and to prevent contamination. The flasks were kept at 20°C for 14 days. Throughout the two weeks, the wines were analyzed daily for weight loss, pH changes and sugar content (°Brix).

Secondary fermentations. Primary fermentations were evaluated as complete when there were no weight losses for three consecutive days. At the end of the fermentation process, the experimental wines were transferred into sterile corked bottles and left to decant for 24 h, at 4°C. The operation was repeated after another 24 h and the wines were stored at the same temperature. After this maturing period, the following analyzes were performed: weight loss, color, pH, residual sugars, total dry matter and ethanol content.

CO_2 losses

The fermentation kinetics was monitored daily by measuring the weight loss of the samples, due to CO_2 release, following a method adapted after Dutraive et al. (2019). The weight of each sample was checked regularly to track the fermentation progress. Loss was calculated by subtracting each day's weight from the initial weight (from T_0) and applying the following formula:

$$\text{Weight loss (\%)} = \frac{\text{initial weight} - \text{final weight}}{\text{initial weight}} \times 100$$

Sugar content determination

The amount of dissolved sugar in the grape must can be measured in °Brix or Brix units, which can show the potential ethanol content of the resulting wine. 1°Brix is equal to 1 g sucrose in 100 g solution, so 1°Brix is equal to 1% sucrose in must (Jaywant et al., 2022). At the laboratory level, Brix determinations were made with a Milwaukee MA-871 INR 9,800 digital refractometer. The sugar content value is obtained after converting the refractive index of the sample to % Brix.

pH determination

A Crison Basic 20+ pH meter (Barcelona, Spain) was used for these measurements, after calibration with standard solutions (pH 4.01, 7.00, 10.01) recommended by the manufacturer.

Ethanol content determination

A classic density meter was used to determine the ethanol content of the final wines. The working method involves inserting the hydrometer into a container with wine; when released, it will float freely in the liquid and allow the reading on the scale of the rod (to which the surface of the liquid will reach). The measurement is performed at 20°C, for accuracy. The result obtained represents the percentage volume of ethanol in the wine.

Total dry matter

Determination of dry matter using a thermo-balance is a fast and reliable method for determining dry matter content using the thermo-gravimetric principle. Thermo-gravimetry consists of weighing the sample before and after heating, to determine the moisture content by difference. 5 g of sample were taken, distributed homogeneously and in a thin layer on the weighing plate, in order to obtain correct results. After 105°C thermal balance treatment, total dry matter percentage was noted according to the thermo-balance display.

Color determination

A HunterLab MiniScan XE spectrocolorimeter was used to measure the color of the samples, with the following working conditions: Device geometry 45°/0°; LAV viewing area; Illuminant D65; Observatory 10°; The CIELAB 76 color system. CIELAB color system uses a rectangular three-dimensional color space that correlates its values with lightness, chroma and hue (Fairchild, 2018). The "L" axis is represented by luminance, with 0 being black and 100 being white. The "a" axis represents red-green values, with positive values being red, negative values being green, and 0 being neutral. The "b" axis represents blue-yellow values, with positive values being yellow, negative values blue, and 0 neutral.

RESULTS AND DISCUSSIONS

The three yeast strains *Saccharomyces cerevisiae* BB06, *Starmerella bacillaris* MI115 and *Metschnikowia pulcherrima* MI109 were tested for their oenological potential as follows. Ten fermentations (6 single cultures and 4 sequential cultures) were performed with and without sulfur dioxide (SO_2). All fermentations were successfully completed after 9 days, but were monitored until day 14.

Primary fermentation

Regarding the fermentation vigor displayed by the 10 cultures monitored (with and without SO_2), they all showed similar dynamics (Figures 1 and 2).

Figure 1. Primary fermentation of yeasts in must without SO_2 (left - simple cultures V_1, V_2 and V_3; right - co-fermentations V_{12} and V_{13})

Figure 2. Primary fermentation of yeasts in must with SO_2

Secondary fermentation

The secondary fermentation (Figure 3) took place after the wines obtained from the primary fermentation were transferred into sterile 250 mL bottles.

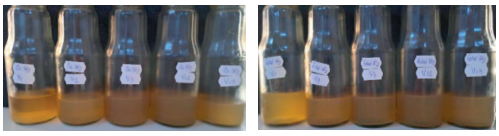

Figure 3. Obtained wines after secondary fermentation (left - with SO_2; right - without SO_2)

CO_2 losses

During the weight loss monitoring of the ten fermentations, significant differences were observed in the 4 sequentially inoculated fermentations, namely the experimental variants V_{12} and V_{13} (with and without SO_2). V_1, represented by *S. cerevisiae* BB06 followed a similar direction to V_2 (*S. bacillaris* MI115) in terms of weight loss, but V_2 maintained the same weight from day seven. V_3 had a constant weight loss during the 9 days of fermentation. V_{12} and V_{13}, on the other hand, showed significant day-to-day differences. It can be noticed in the Figures 4 and 5) that the presence of SO_2 significantly changes the CO_2 losses from the wines and, in the case of V_{13}, they stop on the seventh day (phenomenon observed in the wine without sulfur only on the ninth day.). In the case of V_{12}, the wine without SO_2 had constant and relatively low CO_2 losses from day to day, but the wine with added SO_2 followed a different trend.

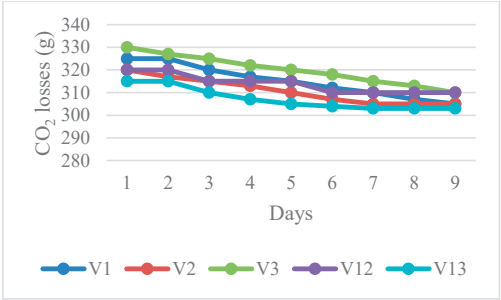

Figure 4. CO_2 release in sulfated wines

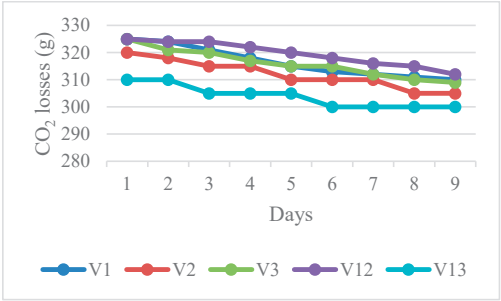

Figure 5. CO_2 release in sulfate-free wines

Sugar content

The °Brix levels were evaluated throughout the fermentation processes, in order to be able to compare the decrease in the amount of sugars in the fermented must.

On the first day of fermentation, all experimental variants had a similar sugar content, correlated with 22.6°Brix (approx. 216 g/L) in the must with SO_2, respectively with

22.3 °Brix (approx. 213 g/L) in the must without SO_2. Brix values on the ninth day of fermentation reached a minimum of 9.5°Brix and 9.3°Brix, respectively.

As presented in Figures 6 and 7) it can be seen that *S. cerevisiae* in monoculture has reached the lowest Brix level, with the previously mentioned values, and the graphs also show that the addition of SO_2 does not influence the Brix values from these experimental variants.

The other two monocultures also followed similar trends in must with SO_2 and in must without added SO_2. Also, the V_{12} and V_{13} variants had the same trend, without demonstrating any significant change in the progress of sugar consumption depending on the addition of SO_2. It should be noted that the experimental variants represented by the two sequential inoculations did not show any significant differences compared to the monocultures of each non-*Saccharomyces* strain tested in this study.

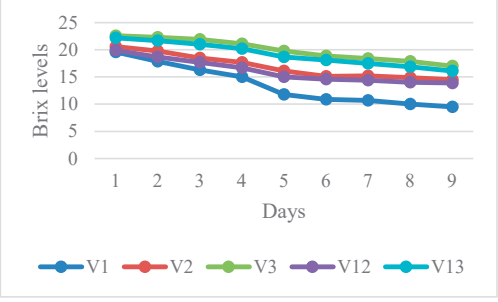

Figure 6. Brix levels of sulfated wines

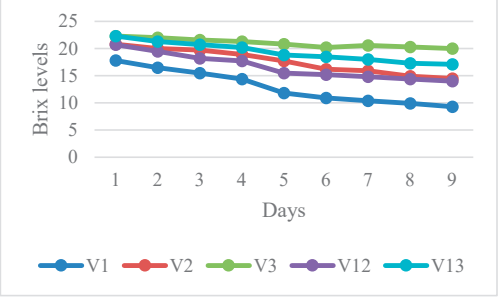

Figure 7. Brix levels of sulfate-free wines

pH values

Grape must usually has a total acidity of 6-6.5 g/L organic acids and a pH of 3.0-4.0, largely due to the malic and tartaric acids in its composition, but citric acid can also contribute to these values (Okafor, 2007). The pH is important in winemaking, because its' involvement in the microbiological stability of wine, in the start of malolactic fermentation and in the natural selection of fermentation microorganisms (Pastore et al., 2024). From the pH monitoring during 9 fermentation days, it was observed that the values of this parameter did not vary significantly neither from day 1 to day 9, nor from sulfated must to sulfate-free must. Thus, throughout the experiment and in all the experimental variants employed, the pH values were maintained between 3.0 and 4.0. Similar values were obtained by du Plessis et al. (2017), by testing simple fermentations of *S. cerevisiae* (pH 3.66) and sequential inoculations of *S. cerevisiae* with *S. bacillaris* (pH 3.70), respectively *S. cerevisiae* with *M. pulcherrima* (pH 3.77).

Ethanol content

Regarding the ethanol content of the experimental wines, the lowest value was recorded at V_3 in the wine without SO_2 (3.5%) and in the wine with SO_2 (5%). Obviously, the highest values were recorded in wines obtained only with *S. cerevisiae* (Figure 8).

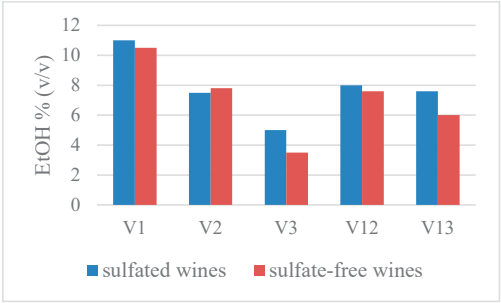

Figure 8. Ethanol content of final wines

From the values obtained, it can also be noted that the wines obtained from co-fermentations have a lower ethanol content compared to those obtained with *S. cerevisiae* in monoculture, which supports the possibility of using these experimental variants in future studies (for obtaining wines with lower percentage of alcohol).

Total dry matter

From the point of view of dry matter content (Table 3), sample V_1 presented the lowest

content, 4.97% in wine with SO_2 and 5.23% in wine without SO_2. At V_2, the same values of the dry matter and, implicitly, of the moisture were obtained, respectively 12.96% dry matter and 87.04% moisture (both in the wine with SO_2 and in the one without SO_2 addition). Sample V_{12} had a dry matter percentage similar to V_2, with 12.66% in the wine with SO_2 and 11.99% in the wine without SO_2.

V_3 showed the highest dry matter value, namely 16.96% in the wine sample with SO_2 and 20.37% in the wine sample without SO_2. Finally, V_{13} presented 14.84% dry matter (with added SO_2), respectively 16.70% dry matter (without SO_2).

Table 3. Total dry matter and moisture results

Sample	Yeast strains	Wine with SO_2 (%)		Wine without SO_2 (%)	
		Moisture (%)	Dry matter (%)	Moisture (%)	Dry matter (%)
V_1	*S. cerevisiae* BB06	95.03	4.97	94.77	5.23
V_2	*S. bacillaris* MI115	87.04	12.96	87.04	12.96
V_3	*M. pulcherrima* MI109	83.04	16.96	79.63	20.37
V_{12}	*S. cerevisiae* BB06 + *S. bacillaris* MI115	87.34	12.66	88.01	11.99
V_{13}	*S. cerevisiae* BB06 + *M. pulcherrima* MI109	85.16	14.84	83.30	16.70

Color determination

From the data presented in Tables 4 and 5, it can be seen that the luminance (L*) of the analyzed wine samples took values in the range of 8.61-17.27.

Table 4. Results of the colorimetric analyzes of the sulfated wines

Wine samples	Color indicators		
	L*	a*	b*
V_1 with SO_2	8.61	-0.07	2.31
V_2 with SO_2	16.63	-1.36	-0.24
V_3 with SO_2	16.19	-0.71	0.39
V_{12} with SO_2	16.80	-1.10	-1.43
V_{13} with SO_2	17.40	-1.13	-0.05

Table 5. Results of the colorimetric analyzes of the non-sulfated wines

Wine samples	Color indicators		
	L*	a*	b*
V_1 without SO_2	12.49	-1.06	-0.10
V_2 without SO_2	16.03	-1.51	0.60
V_3 without SO_2	17.27	-1.40	1.06
V_{12} without SO_2	16.96	-1.49	-0.43
V_{13} without SO_2	17.25	-1.49	-0.34

The color index a* (green-red) recorded negative values for all analyzed wine samples, they were in the range between -0.07 and -1.51, which places all the experimental variants in the green color zone (Figure 9).

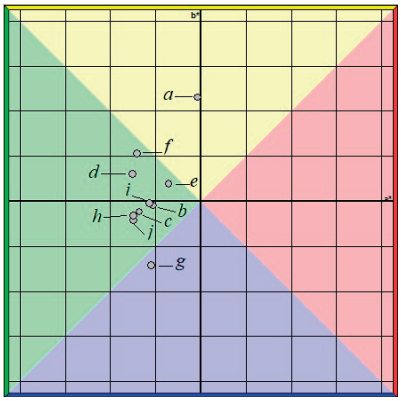

Figure 9. Graphic representation of colorimetric analyzes of the wines: (a) V_1 with SO_2; (b) V_1 without SO_2; (c) V_2 with SO_2; (d) V_2 without SO_2; (e) V_3 with SO_2; (f) V_3 without SO_2; (g) V_{12} with SO_2; (h) V_{12} without SO_2; (i) V_{13} with SO_2; (j) V_{14} without SO_2.

Regarding the b* index (blue-yellow), it can be seen that negative values predominate in the range between -0.05 and -1.43, but there are also four positive values.

The results listed below (luminance indicator column) show that the sulfated wines V_1 and V_{13} have a lighter shade than the wines obtained with the same yeasts, but without the addition of SO_2. In contrast, the sulfated wines V_2, V_3 and V_{12} showed a darker shade than the wines obtained with the same yeasts but without the addition of SO_2.

Also, sulfated V_1 (inoculated only with *S. cerevisiae*) showed a significantly lower luminosity value than the other sulfated wines (almost half less), and sulfate-free V_1 also showed a lower luminosity value, but only with few points. These values are correlated with the wines lightness as can be seen in Figure 3, where the wines obtained by *S. cerevisiae* simple culture are lighter and clearer than the rest of the wines.

It can be seen that most of the experimental variants are located on the graph in close color areas, but V_1 and V_{12} are divided into two color areas. V_1 with SO_2 is thus found on the yellow color and V_1 without SO_2 is found on the green color. V_{12} with SO_2 is found in blue and V_{12} without SO_2 is found in green.

CONCLUSIONS

In small-scale laboratory fermentations, no significant differences in fermentation dynamics were observed while using co-inoculation of *Saccharomyces* and non-conventional yeast like *Starmerella bacillaris* and *Metschnikowia pulcherrima*. The monitoring of CO_2 losses provided information on the speed of the fermentation activity, which was slower in the analyzed non-*Saccharomyces* strains compared to the *S. cerevisiae* strain, without major differences between sulfated and non-sulfated wines.

Sugar consumption followed similar curves for all experimental variants, with the mention that in simple fermentations with *S. cerevisiae* BB06 the lowest values of °Brix were reached, of 9.3 (wine without SO_2) and 9.5 (wine with SO_2). The ethanol content of the wines obtained with only *S. cerevisiae* was 11% (*v/v*) in the sulfated wine and 10.5% (*v/v*) in the non-sulfated wine. The other experimental variants contained an average volume of approx. 7.5%, with the exception of wines fermented by *M. pulcherrima*, in which 5% ethanol was produced in the sulfated wine, respectively 3.5% ethanol in the non-sulfated wine. The pH values were maintained throughout the experiment between 3.0 and 4.0. Among the results of the dry substance, a value of 4.97% (sulfated wine) and 5.23% (no sulfites wine) can be noted in the fermentations conducted only by *S. cerevisiae* BB06. The highest content of dry matter was recorded in the wine obtained from the fermentations conducted by *M. pulcherrima*, namely 16.96% in the sulfated wine, respectively 20.37% in the non-sulfated wine.

Regarding the results of the colorimetric analyses, the experimental variants represented by V_1 and V_{13} with SO_2 show a lighter shade than the wines obtained with the same yeasts, but without sulfur dioxide. V_2, V_3 and V_{12} showed a darker shade than the wines obtained with the same yeasts, but with the addition of sulfur dioxide.

This preliminary study indicates that the local non-*Saccharomyces* yeast strains isolated from Pietroasa vineyard, belonging to *Starmerella bacillaris* and *Metschnikowia pulcherrima* have fermentative potential to be used for the production of low alcohol wines, as requested nowadays on the market. Further research should be performed on biochemical and organoleptic levels to prove the oenological potential of their co-inoculation.

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LIPOLYTIC AND CUTINOLYTIC ACTIVITY OF MICROORGANISMS ISOLATED FROM POLYETHYLENE SURFACE

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Abstract

Among the most known enzymes used for plastic degradation are esterase, cutinase, and lipase. These enzymes are synthesized by a wide range of microorganisms from different species. The research aimed to estimate lipolytic and cutinolytic activity of microorganisms isolated from the surface of low-density polyethylene (LDPE) films, using three rapid screening methods. The rapid tests used in this research are based on the addition to the culture medium of tributyrin, triacetin and flaxseed oil as inducers, and dyes as indicators of enzymes synthesis. The microorganisms included filamentous fungi and yeasts, pseudomonads and actinobacteria. Among 21 isolated strains of fungi, 90% strains possessed lipase activity, 29% exhibited high lipolytic activity. Only 57% of fungal isolates showed cutinase activity. Both extracellular enzymes were recorded for 43% of yeasts isolates. Lipolytic activity exhibited 83% of the tested strains of Pseudomonas spp., and 50% of Streptomyces spp., while cutinolytic activity only 33% of bacterial isolates. Both lipase and cutinase were produced by 41% of the total number of tested microorganisms. The ability to produce these extracellular hydrolytic enzymes indicates the possibility to metabolize polymers, such as polyethylene.

Key words*: fungi, yeasts, bacteria, lipase activity, cutinase activity, polyethylene, LDPE.*

INTRODUCTION

Plastic pollution is a worldwide issue that has an impact on the environment and demands an eco-friendly solution. The use of microorganisms for bioremediation has proved to be the safest method for the environment over time, as it does not involve the use of hazardous chemicals or expensive machinery, and is more energy-efficient than methods of mechanical and chemical degradation (Soong et al., 2022; Raoufi et al., 2023).

The enzymes secreted by microorganisms can reduce the number of carbonyl groups, break them into carboxylic acids, and hydrolyze the polymer carbon chains into fragments, or bio-fragmentation, including long-chain aliphatic compounds such as alkanes and alkenes (Zhang et al., 2022; Raoufi et al., 2023). Among the most known enzymes used for plastic degradation are cutinase, lipase, polyethylene terephthalate hydrolase (PETase) and esterase. These enzymes are synthesized by a wide range of microorganisms from different species, used in the degradation of PE (polyethylene) and PET (polyethylene terephthalate) polymers in different natural environments (Monahan et al., 2020; Kaushal et al., 2021; Andler et al., 2022).

Lipases (triacylglycerol acylhydrolase, EC 3.1.1.3) are members of the α/β hydrolase fold superfamily. They are a biotechnologically important group of enzymes that act on the carboxyl ester bonds present in triacylglycerols and liberate fatty acids and glycerol. Lipases are reported to be produced by many species of animals, plants, bacteria, yeasts, and fungi (Bornscheuer, 2002; Sreelatha et al., 2017). Lipases of microbial origin are widely used in various industries due to the rapid growth of microorganisms and the possibility of obtaining high yields (Rigoldi et al., 2018). For example, representatives of the genus *Streptomyces* and *Pseudomonas* are recognized for their high exogenous lipolytic activity (Madan & Mishra, 2010; Aly et al., 2012). In addition, microbial lipases are characterized by broad substrate specificity, versatility of molecular structure and stability in organic solvents (Aly et al., 2012).

Cutinases (E.C. 3.1.1.74) are esterases belonging to the *α/β* hydrolase superfamily, usually quite stable, do not require cofactors, are active in organic solvents and can be used both in hydrolysis reactions and in synthesis reactions. Cutinases are mostly produced by phytopathogenic microorganisms, which use

these extracellular enzymes to break down cutin which is the major component of the cell wall in plants. Cutinases of fungal origin are capable of breaking down not only cutin, but also soluble esters and insoluble triglycerides (Macedo & Pio, 2005; Furukawa et al., 2019; Ueda et al., 2021). Many polyester hydrolases related to cutin-hydrolyzing esterases are active against PET (Wei & Zimmermann, 2017; Zhu et al., 2022). Cutinases of bacterial origin, according to the literature, are most frequently synthesized by the genera *Thermobifida*, *Streptomyces and Pseudomonas* (Furukawa et al., 2019; Xu et al., 2020).

Fungi are a preferable source of lipase and cutinase, because in most cases these are extracellular enzymes, which facilitate their extraction from the culture liquid (Gopinath et al., 2005; Rai et al., 2014; Raoufi et al., 2023). Apart from this, due to the fact that these enzymes are extracellular, various rapid screening tests of microorganisms with the capacity to synthesize lipase and cutinase have been developed. However, they differ in sensitivity, cost and ease of preparation (Ramnath et al., 2017).

Three primary screening methods of the ability to synthesize lipase and cutinase enzymes by fungi and bacteria were tested and evaluated in this study. The premise was our previous research, which revealed microbial consortia of mixed composition, isolated from the surface of low-density polyethylene (LDPE). These consortia included filamentous fungi, yeast, and bacteria (Rastimesina et al., 2022). Research has shown that the LDPE films, populated by microorganisms, have undergone processes of mechanical degradation and chemical changes (Rastimesina et al., 2023).

Thus, the research aimed to estimate the lipolytic and cutinolytic activity of microorganisms isolated from the surface of low-density polyethylene films.

MATERIALS AND METHODS

Biological material

The soil was collected from the landfill, mostly of plastic waste, located near the village of Slobozia-Duşca, the Criuleni district, the Republic of Moldova. The sampling of the complex soil sample collected from 20-30 subsamples from determined sampling unit (area of the sampling with relative similar conditions) was performed according to the protocol (GOST 17.4.4.02-2017). The total soil sample volume was near 10 kg. The complex soil sample was taken from the depth 0-10 cm. LDPE films (35 μm) used in this work were produced by Kraus Folie Sp.J. (Poland). After six months of incubation in the soil LDPE films were extracted and placed in the flasks with liquid mineral salt media MSM 2 (g/L: K_2HPO_4 - 1.0, KH_2PO_4 - 1.0, NH_4NO_3 - 1.0, $(NH_4)_2HPO_4$ - 1.0, $MgSO_4 \cdot 7H_2O$ - 0.2, $FeCl_3$ - 0.05, $CaCl_2$ - 0.02) and MSM 4 (g/L: $FeCl_3$ - 10.0, $NaNO_3$ - 2.0, $MgSO_4 \cdot 7H_2O$ - 0.5, $CaCl_2$ - 0.5, KCl - 0.5, $BaCl_2$ - 0.2) (Postolachi et al., 2021) and cultivated under continuous stirring conditions at 28°C. All mineral salts were purchased from Sigma-Aldrich, Millipore, Germany.

Through enrichment techniques cultivation was continued for 270 days for microbial consortia formation. Microorganisms were isolated by serial dilution technique, purified, and identified to the genus. All bacterial isolates were studied for their colony morphology, cell morphology (Gram reaction), pigmentation and spore production as per Bergey's Manual of Determinative Bacteriology (Holt et al., 1994). Differentiation of pseudomonads was performed by culturing on King B Agar medium (Millipore, Germany) (King et al., 1954). The identification of fungi isolates was performed according to the colonial aspects and microscopic morphology (Garybova & Lekomtseva, 2005). Morphological peculiarities of the microorganisms were studied under the optical microscope Optica® Microscopes B-510 PH, Italy. The isolated fungi were maintained on slants of potato dextrose agar medium (Millipore, Germany), bacteria and yeasts on nutrient agar medium (Oxoid, United Kingdom).

Screening for lipase production by the isolated microorganisms

All the previous isolated microscopic fungi and bacteria were inoculated on solid tributyrin agar medium (NutriSelect® Basic, Switzerland) containing (g/L), special peptone 2.5; yeast extract 3.0; agar-agar 12.0 and 10.0 mL tributyrin (glycerol tributyrate) (Sigma-Aldrich,

Switzerland). Final pH was 7.5. The plates were inoculated at 28°C for a period of 10 days. The presence of clear zone around the colonies indicated lipase production. The diameter of the clear zone was measured at regular intervals of 24-h incubation, started from the 3rd day (Gopinath et al., 2005; Aly et al., 2012). Statistical analysis was performed using MS Excel. The results were expressed as mean of two experiments in three individual replicates ± CI (confidence intervals). All differences were considered significant at $P<0.05$. Lipase activity was qualitative evaluated based on the diameter of the clear zone: slight activity - zones 1-2 mm, medium activity - zones 2-5 mm, large activity - zones > 5 mm, absent activity - no zones (Ramnath et al., 2017).

Screening for cutinase production by the isolated microorganisms

Basic indicator method

The screening of the cutinase activity of the microorganisms was performed on modified Czapeck-Dox medium (3 g $NaNO_3$, 1 g K_2HPO_4, 0.5 g KCl, 0.01 g $FeSO_4 \cdot 7H_2O$ and 17 g agar-agar in 1 L of distilled water) with the addition of flaxseed oil (10 g/L), as the sole source of carbon, and phenol red (Sigma-Aldrich, India) (5 g/L) as an indicator at pH 9, was used. All mineral salts were purchased from Sigma-Aldrich, Millipore, Germany. Cutinase activity was qualitative evaluated based on the color change of the medium from red to yellow: ++ = growth of microorganisms and detectable color change on selective medium, + = growth of microorganisms but no detectable color change on selective medium, − = no growth (Dickman & Patil, 1986; Rueda-Rueda et al., 2005).

Rhodamine B method

The method involves the cultivation of fungal and bacterial strains on triacetin (TRI) agar medium (NutriSelect® Basic, Switzerland) (g/L: special peptone 2.5; yeast extract 3.0; agar-agar 12.0) with the addition of triacetin (Sigma-Aldrich, United Kingdom) and the colorant Rhodamine B (Sigma-Aldrich, India). The production of cutinase enzyme is determined by the formation of a fluorescent halo around the colony during growth. Cutinase activity was qualitative evaluated based on the appearance of a fluorescent halo: ++ = growth of microorganisms and detectable fluorescent halo on selective medium, + = growth of microorganisms but no detectable fluorescent halo on selective medium, − = no growth of microorganisms.

The cutinase activity of the strains, inoculated on modified Czapek and TRI media with Rhodamine B, was evaluated starting with the 6th day of growth, until the 14th day (Bornscheuer, 2002; Macedo & Pio, 2005). The results represent the data from two experiments in three repetitions.

RESULTS AND DISCUSSIONS

The use of microbial enzymes in the biodegradation of plastic has been demonstrated in a lot of researches. Thus, the cutinase produced by *Thermomonospora* spp. (Raoufi et al., 2023), yeasts *Cryptococcus magnus* (Ueda et al., 2021), filamentous fungi *Fusarium solani pisi*, *Fusarium oxysporum*, *Humicola insolens*, *Aspergillus fumigatus* (Zhu et al., 2022; Raoufi et al., 2023), was effective in degradation of PE, PET, polybutylene succinate (PBS), and polybutylene terephthalate (PBT). Some species of fungi, known as lipase producers, also are involved in the degradation of LDPE (*Phanerochaete chrysosporium*) (Janapaty, 2021), PBS (*Cryptococcus* sp., *Rhizopus delemer, Candida antarctica, Thermomyces lanuginosus, Candida rugosa*) (Srikanth et al., 2022), PET and PE (*Aspergillus, Acremonium, Alternaria, Beauveria, Candida, Eremothecium, Fusarium, Geotrichum, Humicola, Mucor, Ophiostoma, Penicillium, Rhizomucor, Rhizopus, Trichoderma*) (Raoufi et al., 2023).

Although there is more information about cutinases of fungal origin, a number of bacteria capable of actively producing cutinase have been identified. The most studied source of bacterial cutinase is the actinobacteria *Thermobifida fusca*, followed by other representatives of this genus (*T. vulgaris, T. cellulosilytica, T. alba,* and *T. halotolerans*) (Furukawa et al., 2019; Xu, 2020; Amobonye, et al., 2023), as well as representatives of the genus *Streptomyces* (*Streptomyces scabies*)

(Jabloune et al., 2020), which have demonstrated PET-degrading potentials. It was reported about PET degrading ability of the marine bacterium *Pseudomonas aestusnigri* (Bollinger et al., 2020).

In our research from representatives of the order Hypocreales, five strains of the genus *Trichoderma* and one strain of the genus *Fusarium* were isolated according to the colonial aspects and microscopic morphology (Garybova & Lekomtseva, 2005). The representatives of this order mostly showed a weak lipolytic activity, the exceptions being strains *Trichoderma* sp. PE1 and *Trichoderma* sp. PE4, which had a medium tributyrin lysis activity (Table 1). It should be noted that lipolytic activity was not observed in all the strains from the 3rd day of growth and lasted throughout the testing period. Thus, zones of tributyrin lysis appeared from the 4th day of growth of the *Trichoderma* sp. PE4, and the strain *Trichoderma* sp. PE3 on the 10th day of growth no longer presented lipolytic activity. Tests for the determination of cutinolytic activity revealed only *Trichoderma* sp. PE2 as a cutinase-producing strain.

Table 1. Lipolytic and cutinolytic enzymes in representatives of the order Hypocreales

Strains	Lypolitic activity, diameter of the lysis zone (mm)	Rating Phenol red	Rating Rhodamine B
Trichoderma sp. PE1	2.88 ± 0.67	+	-
Trichoderma sp. PE2	1.08 ± 0.25	++	+
Trichoderma sp. PE3	1.33 ± 0.51	+	-
Trichoderma sp. PE4	4.83 ± 0.32	-	-
Trichoderma sp. PE5	1.96 ± 0.13	+	+
Fusarium sp. PE1	1.42 ± 0.25	-	-

Penicillium strains were found to be more active producers of extracellular cutinolytic and lipolytic enzymes. Thus, out of 13 representatives of the genus *Penicillium*, 4 had increased activity of lipase production, 6 strains - average activity, 1 strain - reduced synthesis, and 2 strains were lipase-negative, but produced cutinase (Table 2). In some strains, the synthesis of the lipase enzyme started later, for example at *Penicillium* sp. PE3 from day 4, at *Penicillium* sp. PE5 from day 5, and *Penicillium* sp. PE11 from day 6. There were strains at which lipase synthesis was not fixed throughout the experiment. So, at *Penicillium* sp. PE1 the tributyrin lysis activity disappeared in the 7th day, and at the strains *Penicillium* sp. PE6 and *Penicillium* sp. PE10 - on the 10th day.

Tests to determine the synthesis of cutinase enzyme by *Penicillium* strains revealed the discoloration of phenol red or the appearance of fluorescent halos in 10 isolates.

It was established that 8 from 13 strains of genus *Penicillium* show both lipolytic and cutinolytic activity. *Penicillium* sp. PE8, *Penicillium* sp. PE11 and *Penicillium* sp. PE13 showed only lipolytic activity, and *Penicillium* sp. PE1 and *Penicillium* sp. PE4 showed only cutinolytic activity.

The strain *Aspergillus* sp. PE1 proved to be an active producer of the lipase enzyme, but does not produce cutinase, while the representative of the genus *Alternaria* sp. PE1 active produces both enzymes.

Table 2. Lipolytic and cutinolytic enzymes in representatives of the order Eurotiales and Pleosporales

Strains	Lypolitic activity, diameter of the lysis zone (mm)	Rating Phenol red	Rating Rhodamine B
Penicillium sp. PE1	0.17 ± 0.32	++	-
Penicillium sp. PE2	3.08 ± 0.91	++	-
Penicillium sp. PE3	1.54 ± 0.31	++	-
Penicillium sp. PE4	0.00	++	++
Penicillium sp. PE5	3.17 ± 0.51	++	+
Penicillium sp. PE6	3.25 ± 0.34	++	++
Penicillium sp. PE7	5.79 ± 0.31	++	++
Penicillium sp. PE8	17.00 ± 1.76	+	+
Penicillium sp. PE9	2.92 ± 0.25	++	-
Penicillium sp. PE10	6.83 ± 0.51	-	-
Penicillium sp. PE11	2.75 ± 0.34	++	++
Penicillium sp. PE12	6.67 ± 0.75	++	++
Penicillium sp. PE13	4.00 ± 0.56	+	-
Aspergillus sp. PE1	8.25 ± 0.86	+	+
Alternaria sp. PE1	10.88 ± 0.26	+	++

Figure 1. Phenol red method: orange plate - *Ascomycota* sp. PE1, without cutinolytic activity, yellow plate - *Penicillium* sp. PE6, demonstrates cutinolytic activity

Among the representatives of Ascomycota filum were three strains of yeasts (PE3, PE4, PE5), which had the property of synthesis of both enzymes, lipase and cutinase, and the strain *Ascomycota* sp. PE1, which actively produces lipase (Table 3). It should be mentioned that the activity of tributyrin lysis in the Yeast PE5 strain was reported starting with the 5th day of growth.

Table 3. Lipolytic and cutinolytic enzymes in representatives of the phylum Ascomycota

Strains	Lypolitic activity, diameter of the lysis zone (mm)	Rating Phenol red	Rating Rhodamine B
Yeast PE1	0.00	+	+
Yeast PE2	0.00	+	+
Yeast PE3	3.00 ± 0.39	+	++
Yeast PE4	5.00 ± 0.79	+	++
Yeast PE5	6.83 ± 0.32	+	++
Yeast PE6	0.00	+	+
Yeast PE7	0.00	+	+
Ascomycota sp. PE1	7.17 ± 0.51	+	+

Representatives of the genus *Pseudomonas*, for the most part, possess lipolytic activity except for the strain *Pseudomonas* sp. PE1 (Table 4). Testing the cutinolytic activity showed that all strains of pseudomonads grew on the media with the addition of Phenol red and Rhodamine B, but only 2 strains, Pseudomonas sp. PE2 and Pseudomonas sp. PE5, reacted positively by forming fluorescent halos. It should be noted that these two strains also have good lipolytic activity.

Table 4. Lipolytic and cutinolytic enzymes in representatives of the genus *Pseudomonas*

Strains	Lypolitic activity, diameter of the lysis zone (mm)	Rating Phenol red	Rating Rhodamine B
Pseudomonas sp. PE1	0.00	+	-
Pseudomonas sp. PE2	3.00 ± 0.56	+	++
Pseudomonas sp. PE3	1.67 ± 0.51	+	+
Pseudomonas sp. PE4	2.67 ± 0.32	+	+
Pseudomonas sp. PE5	3.29 ± 0.41	+	++
Pseudomonas sp. PE6	3.25 ± 0.34	+	+

Among the representatives of actinobacteria, the genus *Streptomyces*, of 6 strains, 3 formed zones of tributyrin lysis, denoting good (*Streptomyces* sp. PE3 and *Streptomyces* sp. PE6) and very good (*Streptomyces* sp. PE5) lipolytic activity (Table 5). Cutinolytic activity was observed only in two strains, *Streptomyces* sp. PE3 and *Streptomyces* sp. PE6.

The research has revealed that 19 out of the 21 isolated filamentous fungi strains produce exocellular enzymes with lipolytic activity (90%), including 6 isolates (29%) showed high lipolytic activity. The number of strains exhibiting cutinase activity was significantly smaller - 12, which is 57% of the total micromycetes. Both extracellular enzymes were recorded for 43% of yeasts isolates (three of 7 cultures).

Table 5. Lipolytic and cutinolytic enzymes in representatives of the genus *Streptomyces*

Strains	Lypolitic activity, diameter of the lysis zone (mm)	Rating Phenol red	Rating Rhodamine B
Streptomyces sp. PE1	0.00	+	-
Streptomyces sp. PE2	0.00	-	-
Streptomyces sp. PE3	3.17 ± 0.32	+	++
Streptomyces sp. PE4	0.00	-	-
Streptomyces sp. PE5	5.58 ± 0.47	+	-
Streptomyces sp. PE6	4.17 ± 0.75	+	++

Of the 6 tested strains of pseudomonads, 83% (5 strains) exhibited lipolytic activity, 2 of which also had cutinolytic activity (33%). Of the tested streptomycetes, 2 strains produce

lipase and cutinase (33%), and one strain has high lipolytic activity.

The data obtained in our research are in accordance with the multiple researches presented in the specialized literature (Dickman & Patil, 1986; Rueda-Rueda et al., 2005; Gopinath et al., 2005; Aly et al., 2012; Ramnath et al., 2017).

CONCLUSIONS

The screening study of the production of extracellular enzymes by the microorganisms populating the LDPE surface demonstrates the effectiveness of rapid testing. Namely, it has been shown that not all strains of microorganisms produce lipases or cutinases, that the production of extracellular enzymes is an individual physiological process, which in different strains begins at different growth phases.

Of the total number of tested microorganisms, including fungal and bacterial strains, 75% possess lipolytic activity and 46% cutinolitic activity, 41% can produce both lipase and cutinase. It can be highlighted, that from 17 isolates which possesses both activities, 59% are mycelial fungi, including representatives of the genus *Penicillium*, making up 47% of active strains.

The ability to produce these extracellular hydrolytic enzymes indicates the possibility to metabolize polymers, such as polyethylene.

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BIODEGRADATION OF LIGNOCELLULOSIC SUBSTRATES WITH IMPROVED FUNGAL STRAINS

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Abstract

Lignocellulosic biomass is considered to be the most abundant renewable biomass in the world. However, since only 5% of it is valorised, research is still focused on a better degradability of this waste due to its potential in various applications. Given the structural complexity, recalcitrance and variety depending on the source, several enzymes are involved in a better degradation of lignocellulose. This research study is focused on testing two fungal isolates on their ability to depolymerise two lignocellulosic substrates with a different chemical composition: beech sawdust and wheat bran. The fungal isolates were obtained through random UV mutagenesis from Aspergillus brasiliensis ATCC 16404. The fungal strains were compared based on their enzymatic activities regarding the production of endoxylanase, acetyl xylan esterase, feruloyl esterase and laccase. The highest specific enzymatic activities for all the enzymes analysed were registered for Aspergillus brasiliensis UV_{14}, a strain obtained after exposure to UV irradiation for 50 minutes.

Key words*: acetyl xylan esterase, Aspergillus brasiliensis, endoxylanase, feruloyl esterase, laccase.*

INTRODUCTION

Lignocellulosic biomass is one of the most abundant renewable resources, with an amount of approximately 181.5 billion tons every year. Unfortunately, only 5% of this biomass is currently utilised (Singh et al., 2022), the rest being wasted or burned in land fields which has a major impact on the environment (Liang et al., 2020).

Lignocellulosic waste has a tremendous potential as a substrate for producing various products used in a multitude of applications such as: food, beverages and feed industries, bleaching of pulp for the paper industry, obtaining antioxidants, enzymes and low-cost chemicals etc. However, the valorisation of this biomass is not fully achievable due to its problematics with biodegradability. Therefore, several studies are still focused on complete degradation of lignocellulose by targeting the main components: cellulose, hemicellulose and lignin and their structural units (Monica et al., 2024; Rusu et al., 2022). The composition of these three polymers varies with the source of the waste, usually being 25-65% cellulose (higher contents found in sugarcane bagasse, softwood or hardwood stalks, grains straw etc.), 11-50% hemicellulose (higher contents evaluated in *Gramineae*, wheat or barley straw, softwood or hardwood stalks etc.) and 5-40% lignin (higher contents detected in nut shells or wood stalks etc.) (Harmsen et al., 2010; Palonen, 2004; Pandey, 2015).

Due to the structural variability of lignocellulosic waste, several enzymes are required for best yield regarding lignocellulose degradation (Dincă et al., 2024; Giurescu et al., 2023), such as: cellulases (β-glucosidase, β-1,4-endoglucanase, β-1,4-exoglucanase), hemicellulases (β-1,4-endoxylanase, β-xylosidase, α-glucuronidase, α-L-arabinofuranosidase, acetyl xylan esterase, feruloyl esterase) and ligninases (laccase, heme peroxidase, versatile peroxidase) (Li et al., 2022).

In view of the fact that cellulases are widely studied since their isolation from *Trichoderma reesei* in 1940 (Liu et al., 2021), this research was focused more on analysing the biodegradation of lignocellulosic substrates (wheat bran and beech sawdust) with hemicellulases (endoxylanase, acetyl xylan esterase, feruloyl esterase) and ligninases (laccase).

MATERIALS AND METHODS

Microorganisms

Two fungal strains were tested in this study: *Aspergillus brasiliensis* UV_7 and *Aspergillus brasiliensis* UV_{14}, both obtained through random UV mutagenesis from a parental strain *Aspergillus brasiliensis* ATCC 16404, after exposure to a UV lamp for 20 min (UV_7) and 50 min (UV_{14}), as described in previous research (Burlacu et al., 2017).

Growth medium

Two growth mediums were used for testing the ability of the fungal strains to degrade lignocellulosic substrates:

Medium 1 (g/L): 8 g beech sawdust, 5 g Tween-80, 0.005 g $NaNO_3$, 0.05 g $MgSO_4 \cdot 7H_2O$, 0.005 g $CaCl_2$, 0.012 g $MnSO_4$, 0.009 g $FeSO_4 \cdot 7H_2O$, 0.002 g $ZnSO_4$, 0.23 g KCl, 0.23 g KH_2PO_4, 2 g peptone;

Medium 2 (g/L): 8 g pre-treated wheat bran, 5 g Tween-80, 0.005 g $NaNO_3$, 0.05 g $MgSO_4 \cdot 7H_2O$, 0.005 g $CaCl_2$, 0,012 g $MnSO_4$, 0.009 g $FeSO_4 \cdot 7H_2O$, 0.23 g KCl, 0.002 g $ZnSO_4$, 0.23 g KH_2PO_4, 2 g peptone.

The wheat bran was pre-treated according to the protocol described by Sarangi et al. (2009). Thus, they were repeatedly washed with distilled water, then filtered and washed again with an aqueous solution of 1.5% Tween-80. The wheat bran was then dried and incubated for 20 minutes at 121ºC and stored at -20ºC.

The Erlenmeyer flasks with the inoculated liquid mediums were incubated at 30±2ºC, 140 rpm agitation for 7 days. At every 24 h samples were collected, centrifuged, filtrated and subjected to analysis for different enzymatic activities: xylanase, acetyl xylan esterase, feruloyl esterase and laccase.

Endoxylanase assay

The method used for evaluating endoxylanases activity was based on the quantification of reducing sugars using DNS reagent, endoxylanases catalysing the hydrolysis of xylan, and the reducing carbohydrates formed (xylose) are determined spectrophotometrically at 540 nm. A modified assay described by Chidi et al. (2008) was followed. Briefly, 0.5 ml diluted filtrate (collected at every 24 h) was mixed with 0.5 ml xylan (0.6% in sodium acetate buffer 0.05M, pH 5.3) and the mixtures were incubated at 40ºC for 10 minutes. After adding 1 ml DNS reagent, the samples were boiled for 5 minutes and left to cool down before adding 3 ml distilled water. After 30 minutes, the absorbance was read at 540 nm. A standard calibration curve was created with known concentration of D-xylose. One unit of endoxylanase activity was defined as the amount of the enzyme that released one µmol of xylose per minute under these experimental conditions.

Acetyl xylan esterase assay

The activity of acetyl xylan esterase was evaluated based on the quantification of p-nitrophenol (pNP) released following the hydrolysis of p-nitrophenyl acetate (pNPA), according to the method proposed by Atta et al. (2011). The assay mixture contained 0.1 ml diluted filtrate, 0.9 ml pNPA (10 mM in DMSO) and 1 ml sodium phosphate buffer (0.1M, pH 7.00). After incubation at 37ºC for 10 minutes, the absorbance was read at 410 nm. A standard calibration curve was created with known concentration of p-nitrophenol (pNP). One unit of acetyl xylan esterase activity was defined as the amount of enzyme able to release one µmol of pNP per minute under these experimental conditions.

Feruloyl esterase assay

The method for measuring the enzymatic activity of feruloyl esterase is based on the release of ferulic acid following the hydrolysis of the substrate (Mastihuba et al., 2002). Therefore, 0.2 ml diluted filtrate was mixed with 15 µL ethyl ferulate (10 mg/mL in dimethylformamide) and 0.8 ml sodium phosphate buffer (100 mM, pH = 6.00). After incubation at 37°C for 2 h, the samples were boiled at 100°C for 3 minutes and the absorbance was read at 338 nm. A standard calibration curve was created with known concentration of ferulic acid. One unit of feruloyl esterase activity was defined as the amount of enzyme able to release one µmol of ferulic acid per minute under these experimental conditions.

Laccase assay

Laccase activity is measured based upon the degradation of the substrate (guaiacol) in one minute based on a continuous spectrophotometric method, according to the protocol described by Desai et al. (2011). Briefly, 1 ml diluted filtrate was mixed with 1 ml guaiacol (2 mM) and 3 ml sodium acetate buffer (10 mM pH = 5.00). After incubation at 30ºC for 15 minutes the absorbance was read at 450 nm.

The enzyme activity is calculated based on the formula:

$$\text{A.E.} = \frac{A*V}{e*t*v}$$

where:

A = absorbance value at λ = 450nm;

V = Total volume of the reaction mixture;

e = extinction coefficient of guaiacol at 450 nm (12,100 $M^{-1}cm^{-1}$);

t = incubation time (min);

v = volume of the filtrate (that contains the enzyme).

One enzyme unit was defined as the amount of enzyme required to oxidize one µmol of guaiacol per minute, under the described experimental conditions.

Total soluble protein assay

Lowry's method (Lowry et al., 1951) was used for the evaluation of protein concentration in the collected filtrates at every 24 h. For this, 1 mL of properly diluted sample was mixed with 1 mL reagent A+B (A: NaOH, Na_2CO_3, $KNaC_4H_4O_6 \cdot 4H_2O$ and B: $CuSO_4$ 0.5%). After 10 minutes, 10 ml of dilluted Folin-Ciocâlteu reagent was added and the mixtures were incubated for 10 minutes at 50°C. The extinctions were read at 660 nm. A standard calibration curve was created with known concentration of bovine seric albumin (BSA).

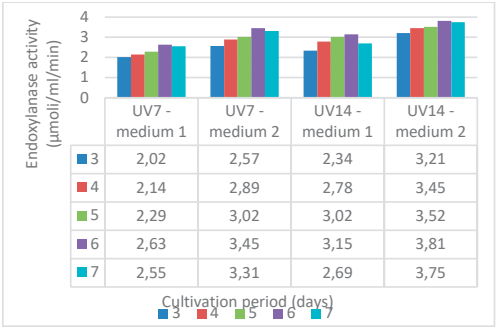
RESULTS AND DISCUSSIONS

The importance of fungal hemicellulases resides from their range of optimal parameters such as efficiency at temperatures below 50ºC and especially acidic pH range of 4-6, since bacterial hemicellulases are active mainly in alkaline conditions (Beg et al., 2001).

The substrates were selected for this study based upon their different composition in major lignocellulosic components. Therefore, beech sawdust contains: 38-50% cellulose, 23-32% hemicellulose and 15-26% lignin (Liu et al., 2023; Świątek et al., 2020). Wheat bran has a relatively different composition: 11-19% cellulose, 11-35% hemicellulose, 5-6% lignin and other components such as 14-25% starch, 13-18% protein etc. (Glaser et al., 2023; Merali et al., 2015). The pretreatment applied to wheat bran was carried out for the removal of starchy residues, which tend to affect the activity of lignocellulases.

Following the cultivation of the two fungal strains on the medium containing beech sawdust, it was found that better results were obtained with the mutant strain *Aspergillus brasiliensis* UV_{14}, compared to *Aspergillus brasiliensis* UV_7 (Figure 1). *A. brasiliensis* UV_{14} had the maximum endoxylanase activity of 3.81 µmoles/mL/min, when it was cultivated on wheat bran medium.

Analysing the results with ANOVA with a single variable, significant differences in the endoxylanase activity are determined ($p<0.05$) between the two fungal strains and also between the same fungal strain cultivated on different substrate.

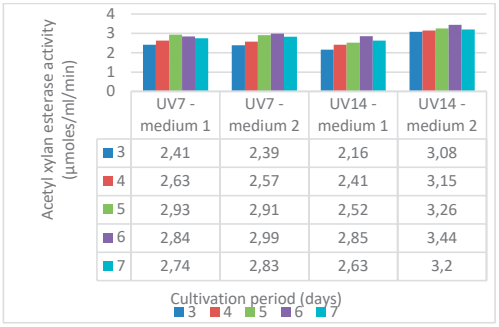


	UV7 - medium 1	UV7 - medium 2	UV14 - medium 1	UV14 - medium 2
3	2,02	2,57	2,34	3,21
4	2,14	2,89	2,78	3,45
5	2,29	3,02	3,02	3,52
6	2,63	3,45	3,15	3,81
7	2,55	3,31	2,69	3,75

Figure 1. Endoxylanase activity of mutant *A. brasiliensis* strains cultivated on beech sawdust (medium 1) and wheat bran (medium 2)

Comparing the endoxylanase activity of each microbial strain cultivated on both mediums, it is noticeable that the medium containing wheat bran determined a higher enzymatic activity, mainly due to the higher hemicellulose content of this substrate.

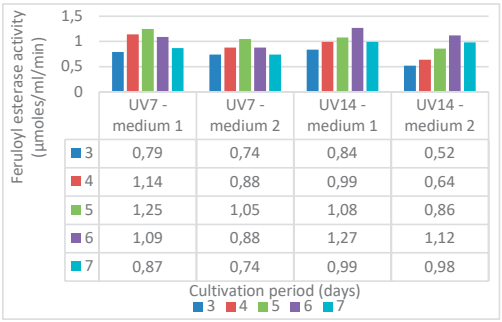
In addition, it is noticeable that the highest endoxylanase activities were registered on the 6th day of cultivation for all the strains cultivated on both mediums (Figure 1).
Regarding acetyl xylan esterase activity, better results were obtained with the mutant strain *A. brasiliensis* UV_{14} compared to *A. brasiliensis* UV_7, on both cultivation media, but more pronounced on the medium containing wheat bran (Figure 2).



	UV7 - medium 1	UV7 - medium 2	UV14 - medium 1	UV14 - medium 2
3	2,41	2,39	2,16	3,08
4	2,63	2,57	2,41	3,15
5	2,93	2,91	2,52	3,26
6	2,84	2,99	2,85	3,44
7	2,74	2,83	2,63	3,2

Figure 2. Acetyl xylan esterase activity of mutant *A. brasiliensis* strains cultivated on beech sawdust (medium 1) and wheat bran (medium 2)

The maximum acetyl xylan esterase activity was 3.44 µmoles/ml/min for the mutant strain grown on the medium containing 0.8% wheat bran as the sole carbon source. In addition, by using medium 1, which had beech sawdust as a carbon source, it was observed that the differences in acetyl xylan esterase activity were insignificant ($p>0.05$), following statistical processing with a single variable ANOVA. Moreover, by using medium 2 which had pre-treated wheat bran as a carbon source, it was observed that the differences in acetyl xylan esterase activity were significant ($p<0.05$), following statistical processing with one-variable ANOVA.
In addition, it is noticeable that the highest acetyl xylan esterase activities were registered on the 6th day of cultivation for all the strains cultivated on both mediums (Figure 2), except for *A. brasiliensis* UV_7 when cultivated on beech sawdust (5th day).
Analysing the results regarding feruloyl esterase activity, it was found that by cultivating the fungal strains on medium with beech sawdust, better results were obtained compared to their cultivation on medium with pre-treated wheat bran (Figure 3). This is explained by the high lignin content of beech sawdust, compared to that of wheat bran, thus there are more ester bonds between lignin and hemicellulose.



	UV7 - medium 1	UV7 - medium 2	UV14 - medium 1	UV14 - medium 2
3	0,79	0,74	0,84	0,52
4	1,14	0,88	0,99	0,64
5	1,25	1,05	1,08	0,86
6	1,09	0,88	1,27	1,12
7	0,87	0,74	0,99	0,98

Figure 3. Feruloyl esterase activity of mutant *A. brasiliensis* strains cultivated on beech sawdust (medium 1) and wheat bran (medium 2)

The differences in feruloyl esterase activity of the fungal strains compared between cultivation on the medium with beech sawdust and that with pre-treated wheat bran were insignificant by statistical processing with one-variable ANOVA ($p>0.05$). The highest feruloyl esterase activity (1.27 µmoles/ml/min) was observed in the case of the mutant strain of *A. brasiliensis* UV_{14}, cultivated on medium containing beech sawdust as the only carbon source (Figure 3). Regardless of the cultivation mediums used, the highest feruloyl esterase activities were registered on the 6th day of cultivation for *A. brasiliensis* UV_{14} and 5th day for *A. brasiliensis* UV_7 (Figure 3).
When measuring laccase activities, it was determined that *A. brasiliensis* UV_{14} clearly has a higher enzymatic activity, unrelatedly to the medium in which it was cultivated (Figure 4). Moreover, the differences between the laccase activities of the strains regarding their activity compared between the two-cultivation media are ensured by statistical processing with one-variable ANOVA ($p>0.05$).

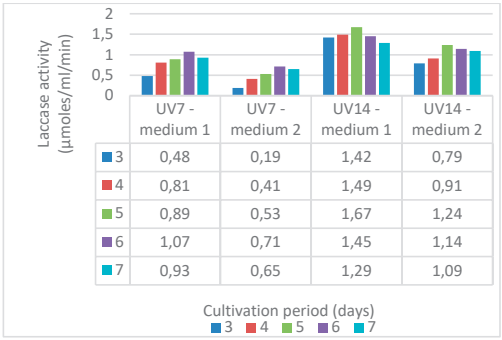


Figure 4. Laccase activity of mutant *A. brasiliensis* strains cultivated on beech sawdust (medium 1) and wheat bran (medium 2)

The maximum laccase activity for both strains was determined when the fungal strains were grown on medium 1 containing beech sawdust as a carbon source (Figure 4), which could be correlated with the higher lignin content of this substrate, the phenolic compounds being more easily hydrolysable by laccases.

Measuring the amount of soluble protein synthesized by the tested strains allowed the calculation of specific enzymatic activities for endoxylanases, acetyl xylan esterases, feruloyl esterases and laccases (Table 1). The specific enzymatic activity allows a relevant comparison of the action of the enzymes synthesized by the tested strains.

Table 1. Specific enzymatic activities of mutant *A. brasiliensis* strains cultivated on beech sawdust (medium 1) and wheat bran (medium 2)

Fungal strain	Specific endoxylanase activity (µmol/mg protein)	Specific acetyl xylan esterase activity (µmol/mg protein)	Specific feruloyl esterase activity (µmol/mg protein)	Specific laccase activity (µmol/mg protein)
UV_7 medium 1	1.15	1.26	0.49	0.47
UV_7 medium 2	1.35	1.34	0.44	0.33
UV_{14} medium 1	1.21	1.15	0.52	0.68
UV_{14} medium 2	2.05	1.61	0.61	0.59

In the case of the specific activity of endoxylanases, the maximum value was obtained with the strain *A. brasiliensis* UV_{14} cultivated on medium with pre-treated wheat bran (Table 1).

Regarding the specific activity of acetyl xylan esterases, the maximum value was recorded with the strain *A. brasiliensis* UV_{14} cultivated on medium with pre-treated wheat bran. When cultivated on medium with beech sawdust, the *A. brasiliensis* UV_7 strain registered a higher acetyl xylan esterase activity than the activity of the mutant strain *A. brasiliensis* UV_{14} (Table 1).

The isolate *A. brasiliensis* UV_7 had a lower specific feruloyl esterase activity than the enzymatic activity of the mutant strain *A. brasiliensis* UV_{14}. However, *A. brasiliensis* UV_7 had a higher specific enzymatic activity when cultivated on beech sawdust, compared with *A. brasiliensis* UV_{14} who had a higher activity when cultivated on wheat bran (Table 1).

In regards to specific laccase activity, the highest values were registered for *A. brasiliensis* UV_{14} regardless of the cultivation media. Also, both isolates had a higher specific enzymatic activity when cultivated on beech sawdust than on wheat bran (Table 1).

CONCLUSIONS

In this experimental study, two fungal strains were tested: *Aspergillus brasiliensis* UV_7 and *Aspergillus brasiliensis* UV_{14} - mutant strains obtained by random mutagenesis with UV radiation, after exposure for 20 and 50 minutes. These strains were cultivated on two lignocellulosic substrates that had different compositions: medium with 0.8% wheat bran and medium with 0.8% beech sawdust.

The results of this study led to the conclusion that the synthesis of the components of the xylanolytic system and laccase is regulated by the substrate used, obtaining maximum enzyme activities of endoxylanase and acetyl xylan esterase following cultivation on medium with pre-treated wheat bran, and higher feruloyl esterase and laccase activities when they were grown on medium with beech sawdust. The different proportions of the majority components (cellulose, hemicellulose and lignin) of the substrates used explain these differences.

The results obtained can be important contributions to the development of biodegradation processes of lignocellulosic waste and, in addition, to the valorization of the

compounds obtained in biotechnological applications, in areas of acidic pH, given the fact that most applications are based on bacterial enzymes that act mainly in the alkaline range.

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ASSESSMENT OF GRAPES INDIGENOUS MICROBIOME FROM "ȘTEFAN VODĂ" PROTECTED GEOGRAPHICAL INDICATION

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Abstract

The diversity of the yeast microflora significantly contributes to the chemical and sensorial characteristics of wine. Lately, the significance of microbiome of grape berries of different geographical origin and varieties, winemaking practices and climatic conditions has led to the suggestion of the microbial terroir. Under micro winery conditions were studied indigenous flora of Cabernet Sauvignon and Merlot grapes from "Ștefan Vodă" PGI. This study is reflecting that the dynamics of the alcoholic fermentation process presented several peculiarities: the spontaneous fermentation had a latency period of 4 days until the beginning of the active phase, and then presented difficulties in completion, especially at the end of the fermentation. Due to possible early and significant development of Kloeckera apiculata, spontaneous fermentation was accompanied by an increase in volatile acidity. The microscopy of studied samples allowed their visual evaluation and the preventive determination of some morphological characters of the microbiome. An efficient use of the grape microbiome would be the selection and subsequent multiplication to be used for fermentation (starter cultures).

Key words*: authenticity, grape microbiome, indigenous flora, wine.*

INTRODUCTION

Winemaking is characterized by a constant decrease of microbial diversity, both bacteria and yeasts. In the vineyard, yeast populations are low and compete with moulds. Most species are oxidative, so they do not, or very little, transform grape sugars into alcohol. This is why it can be found very few fermentation yeasts of the *Saccharomyces cerevisiae* type on grapes (Delteil, 2000; Pretorius, 2020). But in the fermenting must, we do not systematically find yeasts from the grapes, both in terms of present species and of strains deriving from them. At the same time, the cellar is characterized by an atmosphere loaded with both fermentative and oxidative yeasts (Poulard, 2008).

When the use of SO_2 in the must is low or zero, and the fermentation temperatures are in the range from 10 to 25˚C, the fermentation is generally carried out by several species of yeasts which intervene at different fermentation stages. So, apart from grapes microbiome, the cellar can also be a significant source of contamination (Beguin et al., 2003; Coarer M., 2008).

Spontaneous fermentation is ensured by indigenous flora which is not selected. According to Poulard studies (2008) several peculiarities are revealed. Immediately after settling, different species of apiculate and oxidative yeasts develop, producing very low amount of alcohol: *Kloeckera, Candida, Metschnikowia,* etc. Afterwards, a large number of different strains of *Saccharomyces cerevisiae* (between 10 and 20) take over, including only 1 or 2 are dominant either by their numerical importance (50%), or by their presence throughout the fermentation process (Poulard, 2008). Other strains of *Saccharomyces cerevisiae* may appear exceptionally depending on environmental conditions (particularly alcohol content), and always in insignificant proportions. Oxidative species can, however, intervene occasionally during various winemaking operations, notably involving a supply of oxygen in the musts: pumping over, aeration, etc. (Ribereau-Gayon et al., 2004).

The evolution of dominant strains has a direct impact on fermentation. These strains can sometimes persist for several years, but it is more common to record an annual renewal (Poulard, 2008).

Torulaspora delbrueckii yeasts produce specific aromatic profiles during fermentation, improve the organoleptic properties of wines

and it is recommended to use them together with *Saccharomyces strains*. The use of *Torulaspora delbrueckii* yeasts in winemaking is possible in combination with *Saccharomyces cerevisiae* strains, yeasts that ensure the completion of alcoholic fermentation in a reasonable time. Studies on the synergism of the interaction between these two species are relatively few, especially from the point of view of quantifying the effect of this interaction (Lonvaud, 2004; Beguin et al., 2008; Poulard, 2008; Liu et al., 2019). Another factor that is important to be taken in view is the influence of the nutritional environment on the development of these two different strains (Poulard, 2008).

Regarding the terroir, it is difficult to associate active microflora on the must in winemaking because of great diversity: geographical (no individual or population strictly associated to a region), temporal (no rigorous rule, except variability), taxonomic (no single species or genus), genetic (no single-strain fermentation) (Liu et al., 2019; Kazou M., 2023). On the other hand, the terroir, just like the cultivation practices and the climatic conditions of the vintage, undoubtedly has a more or less direct impact on the overall structure of the yeast microflora (Renouf et al., 2006; Liu et al., 2019; Oyuela Aguilar et al., 2020).

If spontaneous fermentations can often take place without any problem, in around 30% of cases we see difficult and sluggish fermentations. Unlike fermentation accidents, these situations are difficult to correct and have consequences on aroma profile involving more or less significant depreciation. The presence of lactic bacteria during these sluggish fermentations can also lead to undesirable effects. Maintaining significant populations of acetic bacteria until the start of alcoholic fermentation increases the phenomena of SO_2 recombination (Beguin et al., 2008).

Certainly, spontaneous fermentation is not responsible for all the quality problems that may arise, but it is carried out by a microbial consortium that is difficult to control. Poorly controlled, it leaves the way open to multiple complications. Spontaneous fermentation therefore constitutes, in all cases, a risk that must be carefully calculated and taken with full knowledge of the facts (Beguin et al., 2003).

It can be mentioned that yeast diversity does not rhyme with complexity, nor with diversity. On the other hand, a large number of fermentative strains can create problems in terms of fermentation. The struggle for nutrients between different strains may be the cause of sluggish fermentations (Coarer M., 2008).

Winemakers could not rely on the spontaneous development of indigenous yeasts to benefit from the positive effects because of the risk of uncompleted fermentation and inherent sensory deviations. In this context, an important task in the field of wine microbiology is the identification and selection of indigenous yeasts with valuable technological properties and easily adaptable to the environment, which ferment completely the carbohydrates from the must in order to obtain natural wines with high organoleptic qualities and to guarantee wines authenticity (Koralewski, 2010; Pretorius, 2020).

Therefore, there is a need to know the biochemical and physico-chemical characteristics of the wines produced in each winemaking center based on the selection of certain strains of local yeasts, the results of which could be used to guarantee the authenticity of wines with protected designations of origin or protected geographical indications.

MATERIALS AND METHODS

Researches were carried out within the Oenological Research Center of the Technical University of Moldova. The grape samples were harvested in October 2023 in Javgur village from Cimișlia district, which is part of a natural amphitheatre located on the ancient "Valul lui Traian de Sus", belonging to PGI "Ștefan Vodă".

Grapes of Cabernet Sauvignon and Merlot varieties were randomly collected from vineyards and transported immediately (4 hours on average) for microbiological and physico-chemical analyses. Subsequently, the harvested grapes were crushed and destemmed. Afterwards, the must was submitted to the maceration-fermentation process in micro winery conditions in 5.0 L glass container on indigenous microflora at a constant temperature

of 28 ± 1°C. The spontaneous fermentation of the must occurred starting with the 4th day after the grapes processing and lasted for 31 days until the complete fermentation of sugars. After the completion of the fermentation-maceration process, the must was pressed, the young red wine was directed to post fermentation and subjected to physico-chemical and microbiological analyses.

The physico-chemical analyses were performed in order to establish the basic composition (alcoholic strength, total acidity, volatile acidity, reducing sugar, pH), the polyphenolic composition (Folin-Ciocalteu index) and the chromatic characteristics (Color intensity, shade) in accordance with official International Organization of Vine and Wine (OIV) practices (OIV-MA-INT-00-2021, Compendium of International Methods of Wine and Must Analysis) and national Technical Regulation "Methods of analysis in the field of winemaking" (HG RM no. 708 of 20.09.2011).

The content of total phenolic substances was determined by the UV–VIS spectrophotometry method with the Folin-Ciocalteu reagent, gallic acid (Sigma-Aldrich) being used as a calibration substance. Prior to determination of phenolic compounds, the red wines were centrifuged at 8000 rpm for 15 minutes.

Chromatic indices were measured spectrophotometrically using quartz cuvettes with 1 mm optical path. The UV-VIS spectrophotometer was used to measure the absorption and transmission of light in the UV spectrum and the visibility of samples. The chromatic indices were calculated according to the formulas (OIV-MA-AS2-07B-2021, Compendium of International Methods of Wine and Must Analysis):

Colour intensity: $Ic = (A_{420} + A_{520} + A_{620})$;

Colour shade: $Nc = A_{420} / A_{520}$.

A_{420} – the absorbance value at 420 nm, characterizes the yellow component of the color; A_{520} – the absorbance value at 520 nm, characterizes the red component of the colour;

A_{620} – the absorbance value at 620 nm, characterizes the purple component of the colour.

The sensory analysis of the was carried out by a group of 12 tasters, which provided the description of the sensorial profile. Each descriptor was scored by points between 1 (least felt) and 10 (most felt) and then recorded in a special descriptive evaluation sheet (Regulation on the Evaluation Method of the Organoleptic Characteristics of Wine Products through Sensory Analysis).

The microbiological study (microscopy, yeast cells counting, seeding on different nutrient media, etc.) was carried out according to the national instructions of the microbiological control of wine production (IC MD 67-42582515-01-2010) and the OIV resolution (OENO-MICRO 08-370, version 2012). The results of the studied samples microbial load were compared with the limit values described in the normative documents (OIV-MA-INT-00-2021, OENO-MICRO 08-370).

In order to assess the microbiota of Merlot and Cabernet-Sauvignon grapes was used the method of washing water microscopy, which consisted in washing for 3 minutes 300 grames of grapes (the samples must contain both berries and portions of stems) in 500 mL distilled water so the microorganisms from the grape elements would pass into the water. Afterwards, the washing water was used for direct microscopy and seeding on media: Sabouraud 4% Dextrose Agar (SDA) medium (agar 18 g/L, dextrose 40 g/L, peptone 10 g/L) (Sigma-Aldrich); MRS Agar Vegitone medium (yeast extract 5 g/L, proteose peptone (vegetable) 10 g/L, dextrose 20 g/L and agar 15 g/L) (Sigma-Aldrich); Brettanomyces Agar Base (yeast extract 3 g/L, malt extract 3 g/L, peptone 5 g/L, dextrose 10 g/L and agar 20 g/L) (HiMedia); Lauril Triptose Broth (tryptose 20 g/L, lactose 5 g/L, sodium chloride 5 g/L, potassium hydrogen phosphate 5.5 g/L) (HiMedia).

The inoculation was carried out on the surface of the sterilized media, melted and cooled by scarification or streaking, after which the media were placed in a thermostat at 30ºC. The samples are thermostated for 7 days, but periodically visual analysis of the media was performed to monitor the development and growth of microorganisms on the media.

In order to establish the purity of the isolated yeasts, microscopy of the grown cultures was performed. For this, the yeast strains were pre-incubated for 5 days on a nutrient medium at a temperature of 30°C. Samples were seeded on the following media: Sabouraud Dextrose Agar

(for the detection of pathogenic and non-pathogenic fungi), MRS Agar (for the detection of *Lactobacillus* lactic bacteria), Broth (non-selective detection of microorganisms), Brettanomyces Agar (for the detection of *Brettanomyces*).

After the completion of the spontaneous fermentation, a new seeding was performed on the MRS Agar, Brettanomyces Agar and Sabouraud Dextrose Agar medium and after a 5-day thermosetting, the visual analysis and microscopy of the cultures was performed. Depending on the results of the samples microscopy the microbiological state of the wine samples was established.

RESULTS AND DISCUSSIONS

The geographical location of the "Ștefan Vodă" PGI area is characterised by insufficient humidity and specific climatic conditions due to nearness of Black Sea. According to statistical data, the amount of precipitation varies from 450 mm to 550 mm. The presence of tertiary red minerals, rich in iron and micro-elements of the iron group explains the production of superior quality wines in this region.

Red grapes of Cabernet Sauvignon and Merlot varieties were taken from the vineyards of Javgur village, physical-chemical indices were recorded in Table 1.

Table 1. Physical-chemical indices of studied grapes

Physical-chemical indices	Cabernet Sauvignon	Merlot
Mass concentration of sugars, g/L	220 ± 1	234 ± 1
Mass concentration of titratable acids, g/L	6.5 ± 0,1	6.2 ± 0.1
Phenolic compounds technological potential, mg/L	3700 ± 15	3380 ± 15

The values of the content of phenolic compounds for Cabernet Sauvignon and Merlot varieties are characteristic for wines from the Southern region of Moldova, which stand out for a sum of active temperatures reaching an annual rate of 3200 to 3400°C, which ensures a full ripening of the grapes and highlight the wine-growing value of the region. Especially that the 2023 year in Republic of Moldova was characterized by an extremely high thermal regime and a significant deficit of precipitation in the July-October period (State Hydrometeorological Service from Republic of Moldova, 2023).

Nevertheless, from Table 1 it can be noticed that the content of phenolic compounds of the Cabernet Sauvignon variety is higher compared to that of Merlot, an aspect that is fully reflected in the specialized researches (Țardea C., 2007; Musteață et al., 2012; Tudose-Sandu-Ville et al., 2012) and foremost due to an increased technological reserve of phenolic compounds of Cabernet Sauvignon grapes.The dynamics of the alcoholic fermentation process of the studied musts presented several peculiarities: the spontaneous fermentation had a latency period of 4 days until the beginning of the active phase, and then presented difficulties in completion, especially at the end of the fermentation (Figure 1).

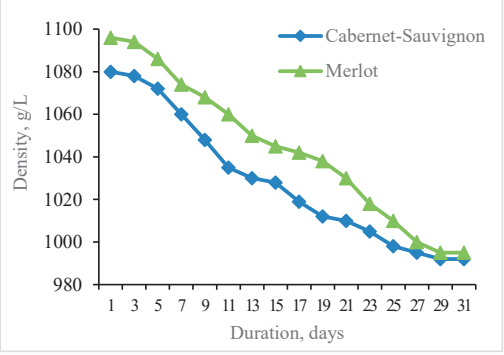


Figure 1. Kinetics of spontaneous fermentation of musts

The increasing concentration of alcohol, which accumulated in the must fermentation process, inhibited the activity of some yeast strains and slowed down the alcoholic fermentation process. Thus, spontaneous fermentation turned out to be less efficient: latency time - 4 days, total duration of alcoholic fermentation - 31 days, which once again proves the advantage of using starter cultures. After the completion of the alcoholic fermentation, the obtained wines were subjected to physical-chemical analysis, the results being presented in Table 2.

Table 2. Physical-chemical indices of studied wines

No	Physical-chemical indices	Cabernet Sauvignon	Merlot
1.	Concentration of alcohol, % vol	12.63 ± 0.1	13.45 ± 0.1
2.	Mass concentration of sugars, g/L	3.4 ± 0.1	4.5 ± 0.1
3.	Mass concentration of titratable acids, g/L	7.7 ± 0.1	7.6 ± 0.1
4.	Mass concentration of volatile acids, g/L	0.75 ± 0.05	0.68 ± 0.05
5.	pH	3.65 ± 0.01	3.37 ± 0.01
6.	Ic	18.6 ± 0.3	17.4 ± 0.3
7.	Nc	0.63 ± 0.02	0.653 ±0.02
8.	Organoleptic quality, points	7.80 ± 0.05	7.85 ± 0.05

According to the results presented in Table 2, it can be mentioned that the dry red wines obtained by spontaneous fermentation are characterized by a high concentration of alcohol.

The mass concentration of titratable acids in the wines obtained under micro winery conditions changed insignificantly, and the variation of the pH index values in the samples of dry wines obtained by spontaneous fermentation is within a limited range and constitutes 3.65 for Cabernet Sauvignon and 3.37 for Merlot.

The mass concentration of volatile acidity varies in both obtained wines (0.68-0.75 g/L), which can be explained by the development of different enzymatic reactions and latent fermentation conditions. These results could be explained by the early development of *Kloeckera apiculata* during spontaneous fermentation, which is accompanied by an increase in volatile acidity and ethyl acetate content (Poulard, 2008). Thus, especially due to the early and significant development of *Kloeckera apiculata*, spontaneous fermentation is accompanied by an increase in volatile acidity compared to induced fermentation.

Regarding the chromatic indices, it can be observed that Cabernet Sauvignon wine has a colour intensity with about 6.5% higher than in case of Merlot wine, which is specific for these varieties (Musteață et al., 2012; Tudose-Sandu-Ville et al., 2012).

On the other hand, the colour hue is only 1% bigger in the case of Merlot wine compared to Cabernet Sauvignon. The color hue, defined as ratio (A420/A520) showing the red color shift to orange hues is correlated with aging of the wine (Ribéreau-Gayon et al., 2006). Thus, the obtained results (Nc<1) are completely representative for young red wines, which are defined by the red colour of anthocyanidins (Musteață et al., 2012; Tudose-Sandu-Ville et al., 2012). This specificity grants them a better resistance and a higher color stability during the maturation and aging processes. Most often, spontaneous fermentations can take place without problems, but in about 30% of cases, difficult and prolonged finishing can take place (Coarer, 2008). The research carried out did not highlighted the diversity of the wines obtained by spontaneous fermentation complexity, the only measurable impact being the extension of the complete fermentation duration compared to selected yeasts. The microscopy of yeast preparations allowed their visual evaluation and the preventive determination of some morphological characters, such as: size, shape, grouping, as well as cell homogeneity. Following the microscopy of the washing water of Cabernet-Sauvignon and Merlot grapes, the presence of yeasts of the genus *Saccharomyces* and *Kloeckera apiculata*, of acetic bacteria in the form of bacillus, were detected in both samples.

Within three days, after further microscopy, it was found that the cells of the studied strains differed in shape and size, it was also established that the cells of the studied strains were eukaryotic and in budding state (Figure 2).

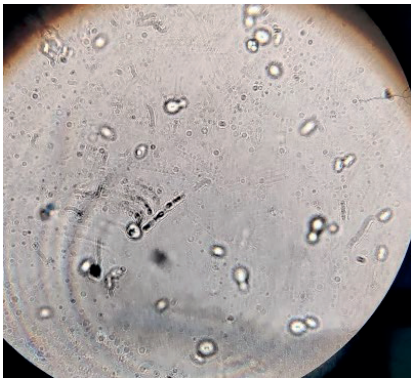
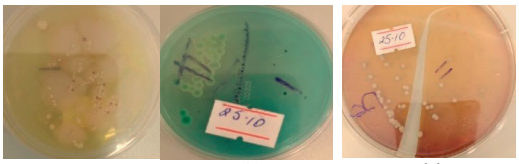


Figure 2. Microscopy of the washing water after 3 days

Seeding was performed on several types of media, and growth of different types of cultures was observed after visual analysis. In Figure 3 are represented the final results of the thermosetting of the media (Sabouraud SDA, Brettanomyces, Broth) and their visual analysis.



(a) (b) (c)

Figure 3. Results of seeding on different media:
(a) - Sabouraud SDA; (b) – Brettanomyces; (c) - Broth

Thus, it was found that on the Sabouraud SDA medium - from sample I (Cabernet Sauvignon) nothing developed and from sample II (Merlot) 3 different kinds of colonies have been evidenced:

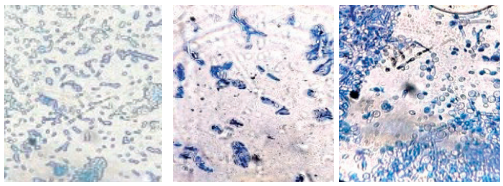
- colony 1 - red-brown, small, round, slightly convex, smooth, glossy;
- colony 2 - white, round, medium-sized, slightly convex, glossy;
- colony 3 - matte white color, with a large surface, irregular shape, flat.

On the Brettanomyces medium from both samples developed white-gray colonies of small size, convex and glossy. From the first sample, 4 colonies develop, and from the 2nd a much larger amount.

On the Broth medium from both samples developed a large number of medium-sized, round, glossy, slightly convex, grayish-white colonies.

Following the microscopy of the cultures developed on the media, the presence of the following microorganisms was found:

- on the Sabouraud SDA medium - the presence of yeasts from the genus *Saccharomyces cerevisiae*, *Kloeckera apiculata* and *Torulopsis* was detected (Figure 4 a).
- on the Bretanomyces agar medium - yeasts from the genus *Brettanomyces* were detected (Figure 4 b).
- on the Broth medium – presence of yeasts from *Saccharomyces* and *Brettanomyces* genus was found (Figure 4 c).



(a) (b) (c)

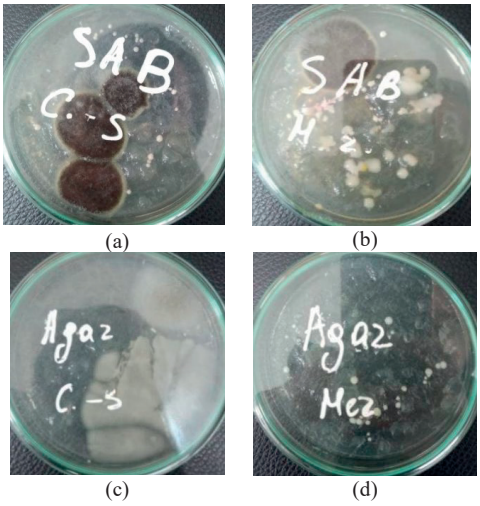
Figure 4. Results of seeding on different media:
(a) - Sabouraud SDA; (b) – Brettanomyces; (c) - Broth

After finalizing of the spontaneous alcoholic fermentation and settling of the wine, a new seeding was performed on the Agar and Sabouraud medium and after a 5-day thermosetting, the visual analysis and microscopy of the colonies was performed (Figure 5).

Following the visual analysis of both media, the development of two types of colonies was observed from both samples:

- the first type - grayish-white colonies, glossy, medium and small in size, round, with continuous, regular, slightly convex edges.
- the second type - white, matte, large, irregular, flat colonies.

In specialized literature (Kurtzman & Fell, 2000) is revealed that yeasts of the genus *Saccharomyces* have a round or ellipsoidal cell shape, the cells of the yeasts of the genus *Torulopsis* are spherical, and the lemon or cylindrical shape is characteristic of the cells of the genera *Hanseniaspora*, *Kloeckera*.



(a) (b) (c) (d)

Figure 5. Results of wine samples seeding
on different media: (a), (b) - Sabouraud SDA;
(c), (d) - Brettanomyces agar

Therefore, it was established that the studied yeasts do not form real mycelia, they all multiply vegetatively by multilateral budding and sexually by spores, which confirms that these strains belong to the genus *Saccharomyces*.

Thus, following the evaluation of the morphological, physiological and reproductive properties, it was found that the new yeast cultures isolated from the indigenous microflora are characterized by uniform yeast cells and are microbiologically viable.

CONCLUSIONS

The presence of a diverse microbiome, both beneficial and pathogenic, was detected on grapes in Javgur of the Cimișlia district. Thus, it was concluded that the grapes from the mentioned region have yeasts from the *Saccharomyces* genus that allow spontaneous fermentation to take place on indigenous yeasts. This fact would ensure a decrease of expenses, but beside fermentation yeasts, other types of yeasts (*Brettanomyces*) and even bacteria (*Acetobacter*) that could danger the fermentation process were detected in the analysed samples.

An efficient use of the grape microbiome would be the selection of specific microorganisms and their subsequent multiplication to be used for fermentation (starter cultures), thus, this could possibly limit problems regarding the infection of the wine with other types of microorganisms which are contained on the grapes, and more the authenticity of the wine from the specific geographical area is preserved.

However, if spontaneous fermentation on native yeasts is an option, it is necessary to carry out systematic monitoring of the fermentation process, in order not to admit the triggering of unwanted processes, because the use of indigenous yeasts does not allow obtaining good reproducibility of the must microbiome and, therefore, of consistent wine quality, leaving much room to competition between strains and random contamination.

Thus, the spontaneous fermentation of the grape must could be followed by a number of disadvantages, such as: obtaining wines with an incomplete fermentation; the danger of wine contamination with pathogenic flora; low alcohol content; high content of volatile acids; slower wine clarification comparing to wines produced by inoculation of selected yeast cultures.

Therefore, it is important to mention that the inoculation of a starter culture could reduce the latency time before starting the must fermentation, as well as other advantages listed above, especially compared to spontaneous fermentation.

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SCREENING OF MICROBIAL STRAINS ABLE TO PRODUCE EXTRACELLULAR LIPASES

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Abstract

Lipases have gained attention worldwide, due to their potential for diverse applications and their stability and selectivity. The study's aim was to identify microorganisms with the ability to synthesize extracellular lipase. In order to achieve this, 20 microbial strains were cultivated on selective solid agar media and lipase producing microorganisms were selected based upon their ratio of the diameter of the halo (if present) and the colony's diameter. The bacterial strains were cultivated on three different selective media, the results indicating that the best screening medium was TBA and the strain that was estimated to have the highest lipase activity was Bacillus subtilis ICCF 20. Yeast strains were cultivated on two selective media and M4D was selected as the best screening medium. The highest producer of lipases was considered to be Yarrowia lipolytica ATCC 16618 ICCF 214. The fungal strains were cultivated on two selective media and the best screening medium was determined to be YS. Two fungal strains were selected as having the highest lipase activity: Aspergillus niger (P4 C36) ICCF 24 and Aspergillus awamory (P2 C114) ICCF 259.

Key words*: lipase, screening, bacteria, yeast, fungi.*

INTRODUCTION

Due to their usefullness in various versatile industrial applications, lipases are ranked as the third most used enzymes after proteases and amylases (Javed et al., 2017).

Depending upon the source, lipases can be grouped into: plant lipases, animal lipases (milk, pancreatic lipases, lipoprotein lipases, hormone-sensitive lipases) and microbial lipases (Jurcoane et al., 2009). Among all of the types of lipases, microbial ones are preferable because they have high specificities for their substrates, higher stability, and lower production costs compared to lipases synthesized from plants and animals (Lee et al., 2015).

Various industries require enzymes that can be used as organic catalysts in various commercial-scale processes. The specialized literature consider that microbial enzymes are of particular interest, including lipases and amylases (Tomulescu et al., 2015).

Lipases are glycerol-ester hydrolases and therefore are present in the form of aqueous emulsions, in a heterogeneous system they are carboxyl-esterases, which hydrolyze glycerides (Gerhartz, 1990; Pascoal et al., 2018). Lipase is responsible for the catalysis of the hydrolysis of triglycerides at the oil-water interface with the formation of glycerol and fatty acids, with a high rate of cleavage (Javed et al., 2017; Gopinath et al., 2013; Guldhe et al., 2015). Also, they have the ability to catalyze transesterification and interesterification reactions into organic solvents (Singh and Mukhopadhyay, 2012; Villeneuve et al., 2000). Due to these special properties, lipases are widely used in the detergent industry, food production and processing, pharmaceuticals, paper, cosmetics, and chemical synthesis industry (Pascoal et al., 2018; Guldhe et al., 2015; Jaeger and Reetz, 1998).

According to the data from the specialized literature, some confusions were noted regarding the exact meaning of the terms lipase and esterase, since both hydrolyze the ester bonds of carboxylic acids. Some researchers believe that the essential difference between lipases and esterases lies in the physical state of the substrate on which they act. Thus, esterases

can hydrolyze soluble or totally dispersed substrates, and lipases cannot. That is why it was proposed by Wills and Jensen that lipases are enzymes that hydrolyze esters at an oil-water interface, in a heterogeneous environment (Jurcoane et al., 2009).

Due to their wide applications, low production costs and higher stability, a lot of research work was done in order to find new microbial strains capable of lipase synthesis at superior level. Yeats and fungi belonging to *Candida*, *Yarrowia*, *Aspergillus, Penicillium* and *Trichoderma* genera were investigated for their ability of extracellular lipase production. Also, a lot of strains of *Bacillus, Pseudomonas, Staphylococcus, Serratia* and *Streptomyces* species were used for obtaining this type of hydrolases with thermostability and higher enzymatic activity at neutral and alkaline pH (Chandra et al., 2020).

The aim of the present study was to investigate 6 bacterial strains, 7 yeast strains and 7 fungi strains for their ability to produce high amount of lipase. The microorganisms were cultivated on specific culture media (3 for bacteria, 2 for yeasts and 2 for fungi) and the lipolytic activity was determined according to the area of opacity around the microbial colonies.

MATERIALS AND METHODS

The microbial strains tested in this study are from the Collection of Industrially Important Microorganisms (IIMC) belonging to the National Institute for Chemical Pharmaceutical (Table 1).

Table 1. Potential lipase-producing microbial strains that were studied from the ICCF collection

Microbial strains analysed		
Bacteria	**Yeast**	**Fungi**
Bacillus megaterium CA1 ICCF 280	*Candida albicans ATCC 10231 ICCF 91*	*Aspergillus awamory (P2 C114) ICCF 259*
Bacillus subtilis ICCF 20	*Candida famata ICCF 181*	*Aspergillus awamory (P2 C287) ICCF 245*
Bacillus subtilis ICCF 77	*Candida paraffinica ICCF 184*	*Aspergillus niger (P4 C36) ICCF 249*
Bacillus subtilis ICCF 84	*Candida tropicalis CMGB 114*	*Aspergillus niger (P6 C317) ICCF 244*
Bacillus subtilis NCIB 8646 ICCF 19	*Rhodotorula glutinis CMGB 169*	*Aspergillus niger ICCF 170*
Bacillus subtilis USAMV 4 ICCF 294	*Yarrowia lipolytica ATCC 16618 ICCF 214*	*Aspergillus niger IP 106 ICCF 222*
-	*Yarrowia lipolytica ICCF 215*	*Beauveria bassiana CA 6 ICCF 335*

ATCC - American Type Cultures Collectionst; Center for Research, CMGB - Forming and Consultancy in Microbiology, Genetics and Biotechnology - MICROGEN, University of Bucharest; NCIB - National Collection of Industrial Bacteria, Aberdeen, Scotland; USAMV -University of Agronomic Sciences and Veterinary Medicine of Bucharest; CA - Antibiotics Research Center Iasi.

Different specific culture media were used for the selection of microbial strains with lipase-producing potential. The three culture media tested for the selection of lipase-producing bacteria are: M4B, TBA and T80.

M4B: tryptone 1% (w/V), calcium chloride 0.1% (w/V), Tween 80.1% (w/V), yeast extract 0.5% (w/V), agar 2% (w/V).

TBA: bacto-peptone 0.5% (w/V), tributyrin 1% (w/V), meat extract 0.3% (w/V), agar 2% (w/V).

T80: bacto-peptone 1% (w/V), Tween 80.1% (w/V), sodium chloride 0.5% (w/V), calcium chloride 0.01% (w/V), agar 2% (g /V).

Two specific culture media were used for screening lipase-producing yeasts: M4D and YS.

M4D: tryptone 1% (w/V), glucose 2% (w/V), calcium chloride 0.1% (w/V), Tween 80.1% (w/V), yeast extract 0.5% (w/V), agar 2% (w/v).

YS: bacto-peptone 0.5% (w/V), glucose 2% (w/V), Tween 80.1% (w/V), calcium chloride 0.1% (w/V), yeast extract 0.5% (w/V), agar 2% (w/V).

All the five culture media described above were sterilized at 121°C for 20 minutes and the pH was adjusted to 6.5.

Two specific culture media were tested for screening of lipase-producing fungi: CDA si YS.

CDA: sodium nitrate ($NaNO_3$) 0.2% (w/V), magnesium sulfate ($MgSO_4*7H_2O$) 0.05% (w/V), potassium chloride (KCl) 0.05% (w/V), iron sulfate ($FeSO_4*7H_2O$) 0.001% (w/V), dipotassium phosphate (K_2HPO_4) 0.01% (w/V), starch 1% (w/V), agar 2% (w/V).

These culture media were sterilized at 115^0C for 30 minutes, adjusting the pH to 6.5.

All microorganisms were cultivated in Petri dishes, with a diameter of 10 cm, for 24-72 hours at 28-30^0C. The method for determining lipase activity for the strains grown on inducer agar

media consists of the presence or absence of areas of opacity (halo) around the colonies.
The lipolytic activity of microorganisms was expressed by the ratio of the diameter of the opacity zone added to the colony diameter and the colony diameter (Ionita et al., 1997), as follows:

$$Vr = (R+r)/r$$

Where:
R = diameter of the opacity zone;
r = colony diameter;
Vr = size of the opacity area.
Strains for which Vr > 2 are considered to be good lipase producers.

RESULTS AND DISCUSSIONS

The six bacterial strains mentioned above were tested on the three specific culture media, the lipase activity being assessed according to the Vr value (Table 2).
After the end of the incubation period, several aspects were analysed: the presence or absence of the opacity area around the colonies and the halo aspect, thus determining the lipolytic activity for the bacterial strains.

Table 2. Evaluation of lipase activity synthesized by bacterial strains according to the calculated Vr values

	TBA		**M4B**	
Bacillus subtilis NCIB 8646 ICCF 19	Compact, matte, clear halo	2.83	Semi-compact, matte halo	2.66
Bacillus subtilis ICCF 20	Compact, clear, transparent halo	5.33	It developed on this medium, but the halo is missing	-
Bacillus subtilis ICCF 77	It did not develop on this medium	-	Compact, matte halo	2.66
Bacillus subtilis USAMV 4 ICCF294	Compact, transparent, well-defined halo	2.88	It developed on this medium, but the halo is missing	-
Bacillus subtilis ICCF 84	Compact, matte halo	2.33	It developed on this medium, but the halo is missing	-
Bacillus megaterium CA1 ICCF 280	Compact, transparent halo	2.5	It developed on this medium, but the halo is missing	-

On the specific T80 culture medium, in the conditions of the experiment, only the strain *Bacillus subtilis ICCF 84* developed, but it didn't show any halo, while the other tested strains of bacteria did not develop on this medium.
The best lipolytic activity was recorded for the *Bacillus subtilis ICCF 20* strain, with a well-formed and clear halo of 5.33, on TBA medium (Figure 1). In fact, on this culture medium the tested strains of bacteria showed lipolytic activity with halos over 2 cm, except for *Bacillus subtilis ICCF 77* that did not grow on TBA medium.
On M4B specific medium, all the bacterial strains were developed, but only two of them synthesized lipases: *Bacillus subtilis NCIB 8646 ICCF 19* and *Bacillus subtilis ICCF 77* (Figure 2).

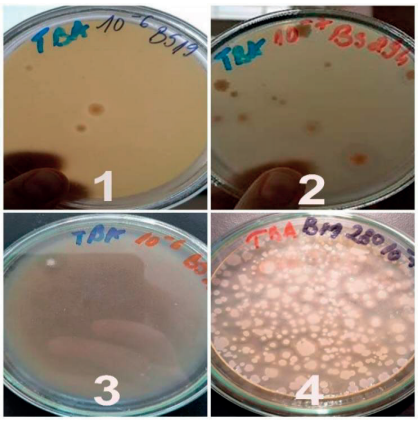

Figure 1. Screening of bacterial strains grown on TBA medium:
1- *Bacillus subtilis NCIB 8646 ICCF 19;*
2- *Bacillus subtilis USAMV 4 ICCF294;*
3- *Bacillus subtilis ICCF 20;*
4- *Bacillus megaterium CA1 ICCF 280*

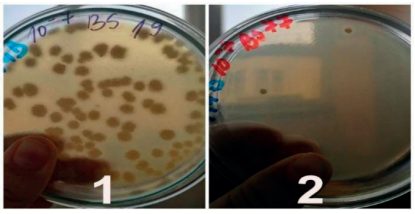

Figure 2. Screening of bacterial strains grown on M4B medium:
1- *Bacillus subtilis NCIB 8646 ICCF 19;*
2- *Bacillus subtilis ICCF 77*

For estimating the lipolytic activity, several aspects were monitored for the yeasts cultivated on the two specific media: the type and the diameter of the obtained halo and the area of opacity (Table 3).

The enzyme activity with the highest value was recorded for the strain *Yarrowia lipolytica ATCC 16618 ICCF 214*, on the M4D medium (Vr = 4.33), and the strains *Candida albicans ATCC 10231 ICCF 91* and *Candida tropicalis CMGB 114 USAMV* presented well-defined halos with a Vr equal to 3, grown on M4D medium (Figure 3).

Table 3. Evaluation of lipase activity synthesized by yeast strains according to the calculated Vr values

	YS		M4D	
Candida albicans ATCC 10231 ICCF 91	It has developed on this medium, but it does not show a halo	-	Compact, transparent halo	3
Candida famata ICCF 181	It has developed on this medium, but it does not show a halo.	-	It has developed on this medium, but it does not show a halo.	-
Candida paraffinica ICCF 184	It has developed on this medium, but it does not show a halo.	-	It has developed on this medium, but it does not show a halo.	-
Yarrowia lipolytica ATCC 16618 ICCF 214	It has developed on this medium, but it does not show a halo.	-	Compact, matte and well-defined halo	4.33
Yarrowia lipolytica ICCF 215	It has developed on this medium, but it does not show a halo.	-	It has developed on this medium, but it does not show a halo.	-
Rhodotorula glutinis CMGB 169 USAMV	Compact, transparent halo	2.5	It did not develop on this medium.	-
Candida tropicalis CMGB 114 USAMV	It has developed on this medium, but it does not show a halo.	-	Compact, transparent halo	3

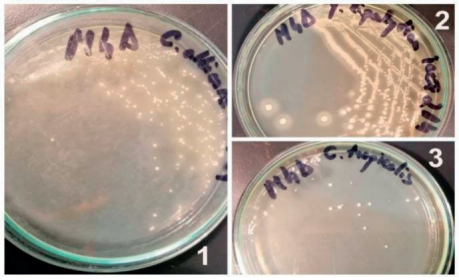

Figure 3. Screening of yeast strains grown on M4D medium:
1- *Candida albicans ATCC 10231* ICCF 91;
2- *Yarrowia lipolytica ATCC 16618 ICCF 214;*
3- *Candida tropicalis CMGB 114* USAMV

On the specific YS medium, all seven yeast strains used in this screening experiment developed, but the strain *Rhodotorula glutinis CMGB 169 USAMV* was the only one that showed enzymatic activity and a well-defined halo (Figure 4).

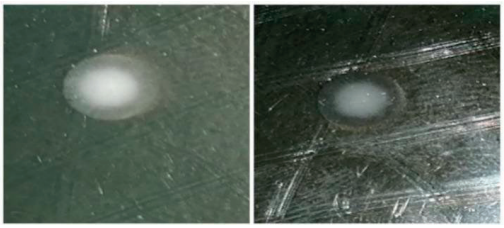

Figure 4. Screening of the yeast strain *Rhodotorula glutinis CMGB 169 USAMV* grown on YS medium

The 7 strains of fungi, analysed in this study, were cultivated on the two specific culture media YS and CDA, and the lipase activity was evaluated according to the diameter of the halo (Table 4).

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Table 4. Evaluation of lipase activity synthesized by fungal strains according to the calculated Vr values

	YS		CDA	
Aspergillus niger ICCF 170	It has developed on this medium, but it does not show a halo.	-	Compact and transparent halo	2.4
Aspergillus niger (P6 C317) ICCF 244	It has developed on this medium, but it does not show a halo.	-	It has developed on this medium, but it does not show a halo.	-
Aspergillus niger (P4 C36) ICCF 249	Compact and transparent halo	3.75	It did not develop on this medium.	-
Aspergillus niger IP 106 ICCF 222	Compact halo, matte	3	It has developed on this medium, but it does not show a halo.	-
Aspergillus awamory (P2 C287) ICCF 245	It did not develop on this medium.	-	It did not develop on this medium.	-
Aspergillus awamory (P2 C114) ICCF 259	Semi-compact and matte halo	4	It did not develop on this medium.	-
Beauveria bassiana CA 6 ICCF 335	Compact halo, matte	3	It has developed on this medium, but it does not show a halo.	-

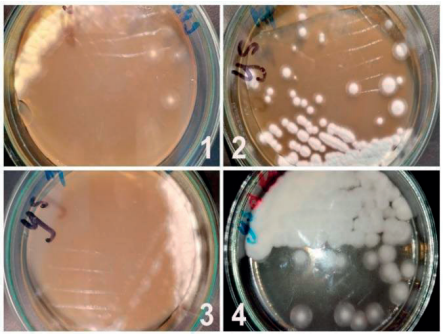

Figure 5. Screening of fungal strains grown on YS medium:
1- *Aspergillus niger (P4 C36)* ICCF 249;
2- *Aspergillus niger IP 106* ICCF 222;
3- *Aspergillus awamory(P2 C114)*ICCF 259;
4- *Beauveria bassiana CA 6* ICCF 335

The strain *Aspergillus awamory (P2 C114) ICCF 259* recorded the highest lipolytic activity when cultivated on the specific culture medium YS, with a Vr value of 4. The strain *Aspergillus niger IP 106 ICCF 222* and *Beauveria bassiana CA 6 ICCF 335* registered an equal lipase activity, Vr = 3, on the same culture medium, YS.

On the specific culture medium for fungi, CDA, enzymatic activity was observed only for the strain *Aspergillus niger ICCF 170*, with a compact and transparent halo. The other six strains of fungi tested in this experiment did not show any lipolytic activity.

CONCLUSIONS

The purpose of this research was to identify the microorganisms able to produce lipases, testing bacterial, yeasts and fungi strains from the Culture Collection Of Industrial Importance Microorganisms (CMII), from the National Institute of Chemical and Pharmaceutical Research and Development in Bucharest (ICCF) and two yeast strains (*Rhodotorula glutinis CMGB 169 USAMV* and *Candida tropicalis CMGB 114 USAMV*) from the Faculty of Biotechnology of the USAMV of Bucharest.

The best culture medium for testing bacteria was TBA, and the strain that showed high lipolytic activity was *Bacillus subtilis ICCF 20*.

The best screening medium for testing yeast strains was M4D, and the strain that synthesized lipase with high activity was *Yarrowia lipolytica ATCC 16618 ICCF 214*.

The fungi synthesized lipases especially when cultivated on the specific culture medium, YS, and the strains that were selected as best lipase producers were *Aspergillus niger (P4 C36) ICCF 249* and *Aspergillus awamory (P2 C114) ICCF 259.*

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INFLUENCE OF MEMBRANE SEPARATION TECHNIQUE UPON THE PHENOLIC CONTENT OF RED CORN BRAN EXTRACT

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Abstract

Different extracts from coloured corn varieties are well known for their profile of high-value phenolic compounds. In this context, the current study proposes an evaluation of the phenolic content for the hydroalcoholic extract obtained from bran of red corn, a variety grown in Romania. Therefore, the native extract, obtained by ultrasounds treatment, was separated using different membranes (regenerated cellulose and polyethersulphone), with nominal molecular weight cut-off of 5000 and 10000 KDa, by employing different working parameters. Several extract fractions were obtained and characterized in terms of their total phenolic content and total flavonoid content by means of UV-Vis spectrophotometry and phenolic acids content also, by HPLC. Total antioxidant capacity was also measured by means of UV-Vis spectrophotometry. Membrane separation has been observed to lead to extract fractions rich in phenolic compounds, especially with flavonic structures, such as rutin and quercetin, with great potential for today's modern industries (pharmaceutical, cosmetic or food industry).

Key words: *antioxidants, membrane separation, phenolics, red corn, ultrasound extraction.*

INTRODUCTION

In a world with increasing environmental issue, the plant resources, man-made or spontaneous, are some of the most valuable sources of various raw materials for bioactive molecules and for sustainable technologies also (Cantão Freitas et al., 2021; Zahmanova et al., 2023; Chaachouay & Zidane, 2024). In the last years, agricultural resources, grains and their derived byproduct became more and more explored for their potential as natural sources for bioactive molecules (Priya et al., 2023; Hazem et al., 2023). Many scientific works in this field have focused their attention on various pigmented corn species (white, yellow, red, purple, black), due to the recognized properties of their great polyphenols and anthocyanins content (Serna-Saldivar & Perez Carrillo, 2019; Colomco et al., 2021; Colombo et al., 2022; Özdemir et al., 2023; Sánchez-Nuño et al., 2024). The corn colour is given by the anthocyanin molecules (Colomco et al., 2021; Özdemir et al., 2023). Also, is well known that corn with a darker colour (black, purple, red) has a generous profile of anthocyanins, phenolic compounds and carotenoids also (Lopez-Martinez et al., 2009), thus behaving higher antioxidant activity. The oxidative stress is very important being a problematic aspect, on one hand associated to many human/animal diseases and on the other hand, for the resistance of a final product prone to oxidation (Chen et al., 2017; Herrera-Sotero et al., 2020; Hao et al., 2022). For this, phenolic compounds derived from vegetable resources have attracted their attention as very useful molecules to prevent many oxidative processes (Simukova et al., 2021; Hao et al., 2022; Jamova et al., 2022). Apart from corn kernel, high polyphenols, anthocyanins and pigments content may be found in the non-edible parts, classified as byproducts or waste, like bran, husk, cob or silk (Cristianini & Guillén Sánchez, 2020; Hao et al., 2022).

Even if there is a lot of research exploring the coloured corn species, there are also many recent papers reporting improvements in the extraction processes (Jayaprakash et al., 2023; García-Ortíz et al., 2023; Duah Boateng et al., 2023). This fact highlights that corn still represents a source to be explored and led towards various modern applications. Water, ethanol, methanol and hydroalcoholic mixtures are the common solvents involved in the extractive processes for the polyphenols and anthocyanins; additionally, hydrochloric and formic acids are used as acidifying reagents (Cevallos-Casals & Cisneros-Zevallos, 2004; Li et al., 2013; Abdel-Aal et al., 2014; Mazewski et al., 2017; Hao et al., 2022). Also, several studies focused on the extraction methods, conventional (as maceration, percolation, Soxhlet extraction and so on) or modern ones (ultrasounds treatment, microwave or enzymatic assisted extraction, supercritical fluid extraction etc.) being reported (Deepika & Gagandeep, 2014; Aourabi et al., 2020; Elsayed et al., 2022; Frosi et al., 2024). So that, using this combined research strategies, there was possible to be established the efficiency of solvents/acidifying agents or the optimal extraction methods able to conduct to some extracts derived from coloured corn rich in bioactive molecules (phenolics/anthocyanins) and great antioxidant activity.

A recent report of our team (Arlet et al., 2023) established the optimal experimental conditions to obtain a polyphenol-rich extracts from bran of red corn variety (Bloody Butcher red corn, cultivated in Romania). It was established that the mixture of equal-parts ethanol-water, under ultrasonic-assisted extraction (20 min) led to a corn extract with great biological activity as reflected on total polyphenolic content (TPC) and total antioxidant activity (AA).

Therefore, the current work is focused on the optimal red corn bran extract and its exploration for the separation of a polyphenols-rich fraction through membrane-processing as a green technology, due to the main advantages such as mild operating conditions with low energy consumption (Baptista et al., 2015). Different membranes were explored within this research and different separation/work parameters also. The obtained fractions [named concentrate (C)/permeate fraction (P)] were characterized in terms of their total phenolic content, flavonoid content, phenolic acids and antioxidant capacity. The polyphenols-rich fractions are great candidates to be tested as antioxidant ingredient for different products.

MATERIALS AND METHODS

Raw material

Dried corn grains of red corn, the raw material, represents a Bloody Butcher variety, cultivated in Romania (Brăila region), harvested in 2022. Red corn bran (RCB), obtained as a by-product from the grinding of the raw material, was used for the extraction of polyphenolic compounds.

Materials

Ethanol (Sigma Aldrich, 96% purity), purified water (Milli-Q), gallic acid (GA; Sigma Aldrich), Folin-Ciocâlteu reagent (Merck), 2,2′-Azino-bis (ABTS; 3-ethylbenzo-thiazoline-6-sulphonic acid; Sigma Aldrich), 6-hydroxy-2,5,7,8-tetramethylchroman-2 carboxylic acid (Trolox; Sigma Aldrich) and aluminium chloride (Sigma Aldrich) were used as reagents for the extraction and extract characterization. Other substances (sodium carbonate, sodium acetate, potassium persulfate, from Sigma-Aldrich) were used as received.

Acetonitrile (Sigma Aldrich, HPLC purity), ultrapure water, gallic acid (Sigma Aldrich, analytical standard), syringic acid (Sigma Aldrich, analytical standard), chlorogenic acid, (Sigma Aldrich, (≥95%), caffeic acid (Sigma Aldrich, ≥98%), p-coumaric acid (Sigma Aldrich, ≥98%), rutin (Sigma Aldrich, reference substance), quercetin (Sigma Aldrich, ≥95%) were used for HPLC determination.

Corn extract preparation

Based on previous results (Arlet et al., 2023), RCB raw material was subjected to the extraction under ultrasound treatment (30W power) for 20 min, using hydro-alcoholic solvent (1:1 (v/v) mixture of purified water and ethanol), maintaining the ratio RCB/solvent of 1/10. Several extraction phases were performed, hydro-alcoholic product being filtered (Grade 1 filter paper, Whatman), collecting ~1 L red corn extract. Considered as

reference samples, further named **P0**, the initial extract sample was stored at approx. -18°C (freezer), in brown containers, hermetically closed.

Extract processing through membrane processes. Experimental set-up

Separation experiments were performed by using a KMS Laboratory Cell CF 2 (Koch Membranen GmbH, Germany) equipment. The main characteristics of the membrane module (tangential flow) are, as follows: membrane diameter = 75 mm; effective membrane surface = 28 cm^2; maximum hold up volume = 600 mL; maximum operating pressure = 6 bars without nitrogen gas bottle; maximum operating temperature = 70°C; maximum pump capacity = 1.8 L/min at 6 bar; material = stainless steel. For the determination of ultrapure water and separation flows the following formula was used:

$$J = \frac{V}{S \times t}$$

where: J = ultrapure water flow or separation flow ($L/m^2/h$); V = permeate collected volume (L); S = effective membrane surface (m^2, in this particular case = 28 cm^2); t = time (h)

Three types of membranes were used for separation tests: 5 KDa Ultracel regenerated cellulose (Millipore, USA), Polyether sulfone 5 KDa (Sartorius, Germany) and 10 KDa K131 Polyether sulfone (Sartorius, Germany).
Ultrapure water flow as well as separation flows varied based on the membrane used due to different base polymer, cut-off values and preparation methods.

Table 1. Separation tests carried out for the corn extract

Concentration test		Working conditions	
		membrane type	pressure
1		5 KDa regenerated cellulose	5 bar
2		5 KDa polyether sulfone	5 bar
3	3.1.	10 KDa polyether sulfone	2 bar (first pass)
	3.2.		3 bar (second pass)

After each membrane separation/concentration process, resulted a concentrate fraction (referred to as concentrate, not. **C**) and a permeate fraction (referred to as permeate, not. **P**). All samples were stored in brown and hermetically close vials, at approx. -18°C.

Table 2 below summarizes the membrane concentration experiments and the sample code that were given to each obtained fraction.

Table 2. The membrane concentration experiments and the samples code

Experiment No.	Sample code	Sample description
1.	P0	Initial extract
2.	P1-C	Concentrate fraction (RC[a], 5 KDa, 5 bar)
3.	P1-P	Permeate fraction (RC[a], 5 KDa, 5 bar)
4.	P2-C	Concentrate fraction (PES[b], 5 KDa, 5 bar)
5.	P2-P	Permeate fraction (PES[b], 5 KDa, 5 bar)
6.	P3-C1	Concentrate fraction (PES[b], 10 KDa, 2 bar, first pass)
7.	P3-P1	Permeate fraction (PES[b], 10 KDa, first pass)
8.	P3-C2	Concentrate fraction (PES[b], 10 KDa, 3 bar, second pass)
9.	P3-P2	Permeate fraction (PES[b], 10 KDa, 3 bar, second pass)

[a]*RC - regenerated cellulose membrane*
[b]*PES - polyethersulfone membrane*

Processing of the resulted fractions

Each fraction (P1-P3, concentrate or permeate fraction), as well as the initial extract (P0) were conditioned in two steps: first, ethanol was eliminated by reduced pressure evaporation. Then, they were frozen (-55°C) and lyophilized (Freeze Dryer, D-37520; Osterode am Harz, Germany) for 24h, to remove also the water (and traces of ethanol). The dry extracts obtained were stored in hermetically closed vials, protected from light at approx. -18 °C.
For their evaluation, the lyophilized samples were resolubilized in the extraction solvent (mixture ethanol:water 1:1, v/v), at a final concentration of 10 mg DM/mL.

Determination of total phenolic content

Total phenolic content (TPC) was determined using the Folin-Ciocalteu method (ISO 14502-1:2005(E); Munteanu & Apetri, 2021). 0.01 mL corn extract (concentration of 10 mg/mL) were diluted with purified water (0.99 mL), then being added 5 mL of Folin-Ciocâlteu reagent (sol., 10%). After 4 min, 4 mL Na_2CO_3 solution (7.5%) and resulting mixtures were incubated in dark place for 1h (at room temperature).
Absorbance was registered at 750 nm using an ultraviolet-visible spectrophotometer (Helios

Beta UV-Vis, Thermo Electron Corporation, Waltham, MA, SUA; Thermo Scientific™ VISION pro™ software).
The TPC was expressed as mg gallic acid equivalent per gram dry weight (mg GAE/g DM), based on the standard curve of gallic acid (0.5 to 50 mg/mL).

Determination of total flavonoid content
Total flavonoid content (TFC) was determined using an aluminium chloride complexation assay (Giurescu et al., 2023). The protocol proposed by Pękal & Pyrzynska (2014) was modified as follows: dilluted samples were mixed with sodium acetate (1:1) and after filtration, 2.5 ml sample was mixed with 1 ml aluminium chloride solution (2.5%) and 1.5 ml distilled water. After 45 minutes incubation period, the optical densities of the samples were measured at 420 nm. A calibration curve was obtained with rutin solution with various concentrations (10-160 µg/ml). Total flavonoid content was expressed as mg of rutin equivalent per gram dry weight (mg RE/g).

Determination of phenolic compounds by HPLC method
HPLC analysis was performed using a Waters 2695 Alliance system equipped with a quaternary pump, autosampler and UV/Vis detector. Separation was achieved by reversed-phase with 5 µm SunFire Column (3.9 x 150 mm). A binary elution gradient consisting in 0.5% orthophosphoric acid in water (A) and acetonitrile (B) was used according to the following gradient: 90/10 as initial condition, changed to 75/35 at 25 min, changed to 10/90 at 40 min, held 10/90 for 5 min, changed to 90/10 at 45.10 min, held 90/10 for 10 min. The column temperature was controlled at 40°C and the samples temperature at 20°C. Chromatograms were acquired at 280 and 300 nm wavelength.
For separation the samples were appropriate diluted, filtered by 0.22 nylon syringe filters and injected (5 µL).
The identification of phenolic compounds was done according to their retention times compared to those obtained by injecting of the standard solutions (4.35 min for gallic acid, 9.85 min for chlorogenic acid, 12.15 min for caffeic acid, 12.90 min for syringic acid, 17.30 min for 4-coumaric acid, 19.40 min for rutin and 24.58 min for quercetin). For analytes quantification sample peak area was processed using the calibration curves obtained for the corresponding standards.

Determination of total antioxidant capacity
Total antioxidant activity (AA) of the colored corn extracts was evaluated using **TEAC method** (Trolox equivalent antioxidant capacity (Kim et al., 2023; Tociu et al., 2023). ABTS salt (7.0 mM) and potassium persulfate solution (2.45 mM) were mixed and maintain in dark place at room temperature for 16 h. Then, the $ABTS^{\bullet+}$ stock solution was normalized (by ethanol dilution) to a final absorbance of 0.68 ± 0.02 at 734 nm.
The calibration curve was done by using 0.25-1.25 mM Trolox-ethanol solutions. For this, 990 µL $ABTS^{\bullet+}$ solution and 10 µL Trolox standard solution were well mixed; after exactly 1 min, the absorbance was registered at 734 nm, against a suitable blank solution, the percentage of inhibition being calculated for each Trolox solution, then plotted as function of concentration. The same procedure was performed for the test samples (the different fractions of coloured corn extract), the percentage of inhibition of absorbance being calculated for each sample based on the calibration curve.
AA of the corn extracts (of 10 mg/mL concentration) were expressed as mg Trolox equivalents/100 g dry matter (mg TE/g DM).

RESULTS AND DISCUSSIONS

In attempt to fractionate the complex mixture of polyphenolic components contained by the RCB hydroalcoholic extract, ultrafiltration experiments were considered. The impact of membrane separation process upon the phenolic composition of the resulted fraction was investigated.

Concentration by membrane processes
Comparison of ultrapure water and separation flows obtained when using the same working pressure (5 bar) proved that much higher values are obtained when using regenerated cellulose as base polymer. On the other hand, different cut-off values for the same base polymer

(polyether sulfone) led as expected to higher ultrapure water and separation flows for higher cut-off values. The experimental results are presented in Table 3 and Figure 1.

Table 3. Experimental results for the flow rate of membrane separation process

Results	Concentration test			
	Test 1	Test 2	Test 3	
			First pass	Second pass
Jw initial[a] ($L/m^2/h$)	75.140	53.493	221.785	81.225
Js[b], (mL/min)	1.101	0.125	1.898	1.586
Jw final[c] ($L/m^2/h$)	67.798	47.408	71.410	71.666

a - initial ultrapure water flow ($L/m^2/h$);
b - separation flow (mL/min);
c - ultrapure water flow after separation ($L/m^2/h$).

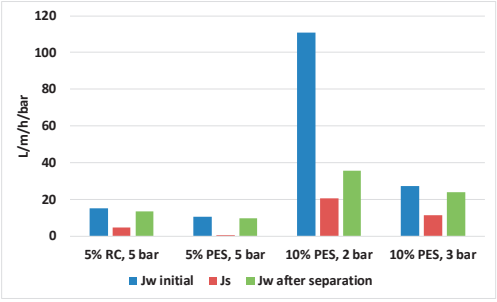

Figure 1. Representation of the separation flows for the different membrane separation tests

TPC assays

The products resulted following each separation experiment (C and P fractions) and the initial corn extract (P0), adjusted to a concentration of 10 mg DM/mL, were explored in terms of their TPC by means of Folin-Ciocalteu determination.

Based on the obtained results, as shown in Figure 2, the higher TPC was registered for the P1-C extract fraction, obtained by using RC membrane, greater also than other studied corn species (Zhang et al., 2017).

Also, it may be observed that the different membrane separation procedure (membrane type, RC/PES; membrane cut-off, 5/10 KDa; the working pressure, 2/3/5 bar) led to different concentrate/permeate fractions in terms of their phenolic content. The observed differences are related to the cut-off of each used membrane, which will separate the polyphenols present in the P0 extract comparatively to their average molecular weight.

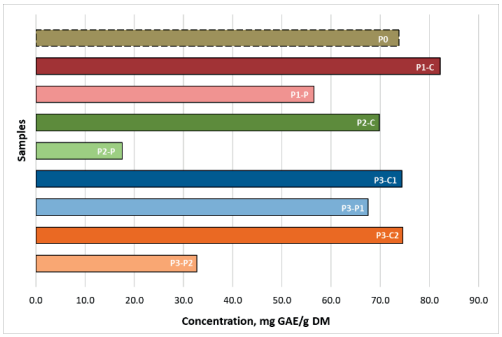

Figure 2. TPC of the initial corn extracts (P0) and resulted concentrate/permeate fractions resulted by membrane separation experiments

The polyphenols content is clearly higher for all the concentrate fractions (P1-C, P2-C, P3-C1, P3-C2) when compared the TPC values with those obtained for the permeate fractions (P1-P, P2-P, P3-P1, P3-P2), as observed from the graphical representation depicted in Figure 2.

Of the two membranes tested in this study, the RC type (cut-off 5 KDa) leads to a concentrate fraction richer in polyphenols, with a TPC value around 82 mg GAE/g DM (P1-C sample). At same working pressure (5 bar) but using PES membrane (cut-off 5 KDa), the obtained concentrate fraction P2-C registered a TPC around 70 mg GAE/g DM. The same way, permeate fractions resulted by these two experiments indicate very different TPC, near 57 mg GAE/g DM for P1-P and 18 mg GAE/g DM for P2-P.

Therefore, a higher retention of polyphenols may be reached by using RC membrane, that will promote the enrichment of the extract in valuable phenolic molecules.

Testing another type of PES membrane (cut-off 10 KDa), looks like the higher cut-off promotes the improvement of polyphenol content for the concentrate fraction, probably allowing the suspension in hydroalcoholic media of those compounds with higher molecular weight, avoiding their deposition on the membrane surface (losses) or their entrapment in the membrane pores (and, again, their loss). A possible conjugation of small phenolic species with higher glycoside fragments, poly- and

monosaccharides may be considered, being known the tendency of these entities to join in different molecular arrangements (Balasundram et al., 2006; Alara et al., 2012).

Total flavonoid content (TFC)

Natural flavonoids from different sources have been widely used due to their biological activity, such as antioxidant, antidiabetic, and antihyperlipidemic (Li et al., 2023). However, a great correlation of the corn extracts with potential biological activities was not established yet.

For the corn extract and derived C or P fractions, TFC was determined, being established the influence of the membrane separation process and membrane type upon the flavonoid bioactive components within each collected fraction.

According to Figure 3, the resulted values of TFC for P1-C, P3-C1 and P3-C2 fractions, were higher than the one obtained for P0 extract. The results showed that regenerated cellulose membrane separation (5 KDa, 3 bar working pressure) lead to the flavonoid-richest fraction (P1-C, 37.411 RE/g DM). However, the most efficient separation of the flavonoids was recorded when using PES membrane at the second pass, because less active compounds remained in the permeate fraction whose volume was also very small (20 ml). A strong correlation of TPC and TFC was registered (r = 0.928).

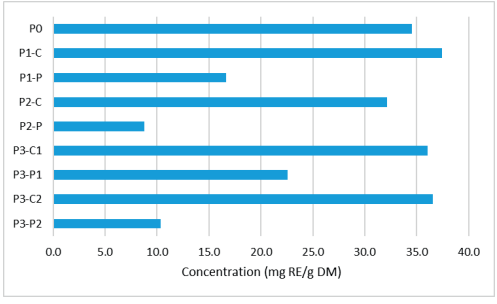

Figure 3. Total flavonoids content (TFC) of the initial corn extracts (P0) and different C/P fractions resulted by membrane separation

Quantification of individual phenolic acids, rutin and quercetin

Dominant group of cereals phenolic species, phenolic acids (PAs) are very important for their biologic activity (Kasprzak et al., 2018; Horvat et al., 2020). Thus, corn extracts, due to the PAs content became a valuable source of natural antioxidants.

Separation by HPLC of the obtained fractionated corn extract, resulted in a great content of syringic acid, as shown in Figure 4. The results are consistent with those reported by Kapcum et al. (2016) who found 31-200 mg/100 g syringic acid in red corn cob.

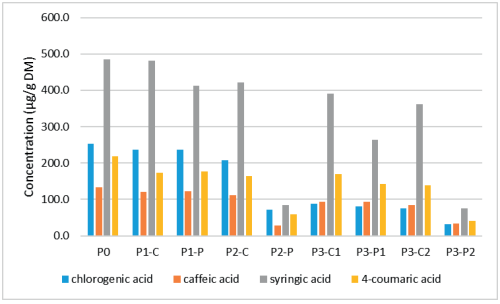

Figure 4. Phenolic acids quantification of the initial corn extracts (P0) and the resulted separation fractions

Initial P0 extract indicates the greatest content and concentrated fractions indicate significant syringic acid contents, a promising phenolic derivative with a wide range of pharmacological properties, including antioxidant, hepato-, cardio-, neuro-protective, antidiabetic or antimicrobial properties (Srinivasulu et al., 2018; Mirza et al., 2022; Sahari et al., 2024).

No less significant is the content of chlorogenic and 4-coumaric acids, well known for their therapeutic effects (Kaur, J., Kaur, R., 2022; Tehami et al., 2023; Huang et al., 2023; Nguyen et al., 2024).

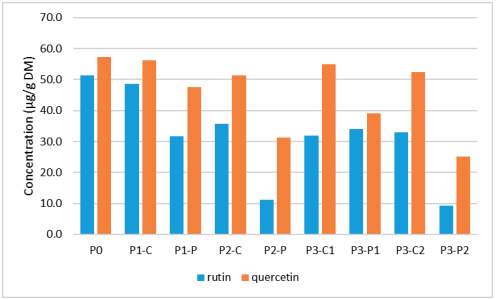

Figure 5. Rutin and quercetin quantification of the initial corn extracts (P0) and the resulted separation fractions

Rutin and quercetin are two important flavonols with potential applications in the pharmaceutical industry as well as that of food supplements. The values determined for these compounds in the analysed samples (Figure 5) were rather low, but together with the other polyphenols may contribute to the whole biological activity of the obtained extract.

Antioxidant activity

The total antioxidant activity was evaluated for C and P extract fractions and initial extract, P0, the obtained results being graphically represented in Figure 6. They are in good agreement with the TPC results, also to other report (Bani et al., 2023).

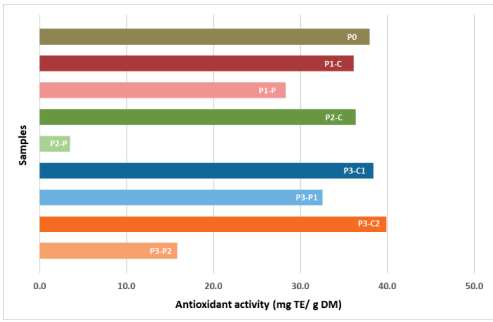

Figure 6. Graphic representation of the antioxidant activity for the initial corn extracts (P0) and different C/P fractions resulted by membrane separation

Higher antioxidant activity values are well correlated with the TPC values. When fractionate, P0 extract conduce to concentrated fractions with AA ranging from 36 near to 40 mg TE/ g DM.

As Figure 6 presents, the higher antioxidant activity is exerted by P3-C2 extract fraction, resulted when PES membrane was used for the separation, on the working pressure of 3 bar (P2-C3, AA of almost 40 mg TE/ g DM), well correlated to other studied corn species (Zhang et al., 2017).

Also, permeate fractions registered lowest AA values, P2-P barely reaching a value of 3.5 mg TE/ g DM. Conversely, the concentrate fraction resulted from this test indicates great antioxidant capacity (P2-C, 36.330 mg TE/g DM). Corroborating these experimental data, it can be concluded that the PES 5 KDa membrane is efficient for the concentration of corn extract in biological compounds with notable antioxidant activity.

CONCLUSIONS

A byproduct derived from coloured corn (husk of a Bloody Butcher red corn variety) was used to obtain a polyphenol-rich extract using mild extraction condition, with ethanol-water (1/1) by ultrasound treatment for 20 minutes.

By means of membrane separation several extract fractions were obtained with different concentration of phenolic compounds, starting from the investigated initial corn extracts.

According to obtained results, both regenerated cellulose membrane and polyethersulfone membrane can lead to efficient separation process. Higher cut-off of the used polyethersulfone membrane leads to higher ultrapure water and separation flows.

All the obtained fractions after separation process were characterized in terms of total phenolic content and antioxidant activity but also related to their flavonoid content and phenolic acids also. Total phenolic content and antioxidant capacity of the studied extract fractions were found to be comparative with other reports (around 80 mg GAE/g DM and respectively 40 mg TE/ g DM).

Important results were obtained for the concentrated fractions, well reflected both in the total content of phenols and antioxidant activity.

Also, significant flavonoid content and phenolic acids were identified also for the concentrated fractions derived by the corn extract.

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PERFORMANCES OF TWO EXTRACTION KITS OF AFRICAN SWINE VIRUS GENOME

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Abstract

Currently, the ASF (African Swine Fever) diagnosis is carried out by the detection of viral DNA. The successful amplification of the targeted DNA fragment needs a proper quantity of the genetic material. The aim of this study was to compare the yield and quality of DNA extracted using two dedicated commercial kits. It was compared the product obtained using the Pure Link Genomic DNA Mini Kit (Invitrogen) and QIAamp Cador Pathogen Mini Kit (Qiagen). The DNA has been quantified using Qubit DNA HS Assay Kit (Qubit 3.0 Fluorometer, ThermoFisher Scientific), a highly selective over RNA and accurate tool for DNA at levels between 10 pg/µL and 100 ng/µL.
The standard DNA of the ASF virus has been diluted 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5} and 10^{-6} as both extracts obtained with Pure Link Genomic DNA Mini Kit (Invitrogen) and the QIAamp Cador Pathogen Mini Kit (Qiagen). According to the dilutions (10^{-2} to 10^{-6}), the DNA yield with the QIAamp Cador Pathogen Mini Kit was 132 ng/µl, 71 ng/µl, 45 ng/µl, 21.2 ng/µl and 10.2 ng/µl, respectively.
The yield obtained with the Pure Link Genomic DNA Mini Kit was 126 ng/µl, 65 ng/µl, 31 ng/µl, 15.2 ng/µl and 4.2 ng/µl, respectively.
The highest concentration was obtained using the QIAamp Cador Pathogen Mini Kit (Qiagen). In conclusion, the sensitivity of the Qiagen Kit is more suitable to be used for further investigation on the ASF genome.

Key words*: ASFV, DNA extraction, fluorometer.*

INTRODUCTION

ASF (Africane Swine Fever) is caused by African swine fever virus (ASFV) (DNA genome), belonging to *Asfivirus* genus, *Asfarviridae* family (Dixon et al., 2005). The transmission is made by direct contact with the infected animals (Sánchez-Vizcaíno J.M., 2016) or indirect contact by secondary source of infection like: feed, water, other materials, means of transport contaminated with secretions and excretions from contaminated animals or through contaminated clothing of pig farmers and veterinarians (Răpuntean & Răpuntean, 2014).
African swine fever (ASF) is a significant and intricate notifiable disease affecting both domestic and wild pigs. It is caused by the African swine fever virus, classified under the genus *Asfivirus* within the Asfarviridae family Uniquely, ASFV is the only known DNA virus transmitted by an arthropod vector (Pietschmann et al., 2016).
ASFV and Classical Swine Fever virus (CSFV) are unrelated pathogens. Pigs immunized against CSFV do not gain protection against ASFV, and the same is true the other way around. This lack of antigenic relationship necessitates separate and specific laboratory diagnostic methods for detecting ASF (Mânzat, 2005).
There is a continuing risk of the spread of ASF from these areas due to the uncontrolled movement of infected individuals, pork products, fomites and wild boar. Pig industry of each country is at risk. The low biosecurity of small and medium farms is particularly vulnerable. In the absence of a vaccine or effective treatment, the best strategy against ASF is to put in place an early detection strategy, together with an early response mechanism in case of outbreaks. In this context, awareness

raising and training of veterinary professionals and other front-line staff will be essential (Costard et al., 2009).

The pig food industry and their relationship with wild pigs has an important role preserving the virus in local and regional systems. By looking the pig market in the European countries, it can be noted the necessity to see this structure in the surveillance systems of pig healthy and in the outbreaks diseases (Guinat et al., 2016).

The potential for live pig trade networks between EU member countries to face and spread ASF, is increasing particularly when there is a long period between infection appearance and reporting of disease, has also been noticed.

The first outbreak of ASF in Romania has been confirmed by the Institute of Diagnosis and Animal Health in a farm located on the outskirts of Satu Mare in 2017. This outbreak was related to the large scale spread of the virus in the neighboring countries: Ukraine and Republic of Moldova (Ladoși et al., 2023). The losses suffered by Romanian's economy was made by a massive depopulation strategy.

ASFV is a large, enveloped virus with icosahedral morphology and a double-stranded DNA genome of 170 to 190 kbp (Fauquet & Fargette, 2005), containing heads with terminal inverted repeats and closed by hairpin loops (Salas & Andrés, 1999). The ASFV replication cycle is mainly cytoplasmic, but an early stage of replication in the nucleus has been described (Garcia-Beato et al., 1992; Rojo et al., 1999).

Accurate diagnosis is essential for the rapid control of ASF, particularly due to the rising prevalence and the ongoing emergence of variant strains. Commonly used methods for detecting ASFV include the hemadsorption test (HAD), conventional PCR, real-time PCR, antigen detection via the fluorescent antibody test, and enzyme-linked immunosorbent assay (ELISA) (Muzykina et al., 2024).

The disease confirmation is done in accordance with the procedures, sampling methods and criteria for the evaluation of laboratory test results described in the Operational Manual for Intervention in ASF Outbreaks emitted by Romania's National Sanitary and Food Veterinary and Food Safety Authority (NSVFSA) World Organization for Animal Health (2019). The diagnosis was based on epizootiology, clinical, pathological data, laboratory tests and bioassays. Currently, the confirmation of ASF is carried out by PCR. The viral DNA purification is done by lysing cells and solubilizing DNA, followed by removal of contaminated substances: proteins, RNA and other macromolecules (Agüero et al., 2004).

The field of molecular biology has developed very rapidly, since `80, when the polymerase chain reaction (PCR) was discovered in 1987 by biochemist Karry Mullis (Agüero et al., 2004). The PCR method can detect any pathogen that has nucleic acid in its structure: bacteria, viruses, protozoa, fungi, parasites because through this method a fragment of a DNA molecule is multiplied/amplified in numerous copies. The main advantages of this technique are the speed, the sensitivity and the undoubtful specificity (O'Donnell et al., 2017).

Using the PCR method, molecular biology changed the course once the enzymatic synthesis of target DNA was achieved *in vitro*. The PCR method is based on two fundamental properties of nucleic acids:

a) reversible thermal denaturation at 90°C of the two chains of the DNA molecule;

b) renaturation based on the complementarity of the nitrogenous bases: adenine - thymine in the case of DNA and adenine - uracil in the case of RNA, and guanine - cytosine.

The primary structure of nucleic acids is always oriented from the 5′end of the molecule towards the 3′ end so that a DNA double helix has two complementary anti-parallel mono-strands, one "sense" (5′→3′) and the other "antisense" (3′→5′) (Minchin & Lodge, 2019).

Nucleic acid extraction is a non-specific step, the whole nucleic acids being extracted from the biological sample. Extraction is done by cell lysis, inactivation of nucleases and removal of cell debris. The technique includes mechanical destruction (shredding, homogenization), chemical treatment or enzymatic digestion with proteinases.

Purification of nucleic acids differs depending on the nucleic acid extracted, DNA or RNA.

DNA extraction from the biological sample is carried out according to the type of the biological sample (serum, blood, animal tissue, virus, bacteria, etc.) and according to the desired DNA quality (genomic DNA, highly pure

DNA), without PCR - inhibitors (hemoglobin, plant, or synthetic phenols).
DNA can be collected from a range of biological samples by isolating it from the cellular mixture. This purified DNA can then be used in a variety of molecular biology analyses. DNA isolation can be performed by manual and automated methods using standardized commercial kits or the classical phenol-chloroform-isoamyl alcohol method can be used.
The successful amplification of the targeted DNA fragment needs a proper quantity of the genetic material. The aim of this study was to compare the yield and quality of DNA extracted using two dedicated commercial kits. It was compared the product obtained using the Pure LinkGenomicDNA Mini Kit (ThermoScientific) and QIAamp Cador Pathogen Mini Kit (Qiagen). The DNA has been quantified using Qubit DNA HS Assay Kit (Qubit 3.0 Fluorometer, Thermo Fisher Scientific), a highly selective over RNA and accurate tool for DNA at levels between 10 pg/µL and 100 ng/µL.

MATERIALS AND METHODS

We used in the present study molecular biology methods for the detection of ASF nucleic acid, standard ASF (synthetic standard) from the diagnostic test ASFV Monodose dtec-qPCR (GPS genetic PCR solution, Orihuela, Spain) to optimize the molecular biology methods. We made serial dilution, quantified. For 2 µl synthetic standard we add 8 µl ultrapure water for 10^{-2} and so on.
Most of the time extraction is done by using dedicated extraction kits from different manufacturers. The quality of the extracted nucleic acids is important in order to avoid PCR inhibitors and to have the best-preserved sequences.
DNA purification is done by lysing cells and solubilizing DNA, followed by the removal of contaminated substances: proteins, RNA and other macromolecules (Agüero et al., 2004).
The reference materials were used and protocols were followed. One kit extracts DNA and RNA and the other extracts only DNA, this comparison aimed to evaluate the quantity of extracted material from each kit.
The QIAamp Cador Pathogen Mini Kit is designed for the isolation of all types of nucleic acids out of many biological samples like blood, swabs, and organ tissues. The principle used is a rapid spin-column technique, where the contaminants and inhibitors are eliminated in order to extract the nucleic acids. The nucleic acid extract can be used in classical PCR or real-time PCR. The benefits of the QIAamp Cador Pathogen Mini Kit are: unique protocol for all types of nucleic acids (RNA, DNA), the isolation out of many biological samples - blood, swabs etc., the extract of isolated nucleic acids ready for analysis by real-time PCR or classical PCR.
QIAamp Cador Pathogen Mini Kit uses silica column principle in order to extract RNA and DNA from different type of samples. There are used optimized buffers and different enzymes in order to lyse the samples. The nucleic acid is bound to the silica membrane while the contaminants will pass through the membrane into the column. Then are used washing buffers in order to eliminate all kinds of PCR inhibitors like bivalent cations and proteins. Nucleic acids are then eluted with AVE buffer (according the manual of the kit: Samples are lysed under highly denaturing conditions at room temperature (15-25°C) with proteinase K and Buffer VXL to inactivate nucleases. Buffer ACB is then added to adjust the conditions for binding DNA and RNA. The lysate is transferred to a QIAamp Mini column, where nucleic acids attach to the silica membranes during centrifugation, while contaminants pass through. Two thorough wash steps remove any remaining impurities and enzyme inhibitors, and the nucleic acids are eluted in Buffer AVE). These nucleic acids can be used in all kinds of molecular biology methods. Compared to other kits that are using the silica membrane, in this kit can be used up to 200 µl of blood sample, without producing the clogging of the column filter.

Table 1. Nucleic acid extraction protocol (QIAamp Cador Pathogen Mini Kit)

Reagent	µl/sample	No. samples	Total
Proteinase K	20 µl		
Sample	200 µl		
Buffer VXL	100 µl		
Pipetting/vortex mixing			
Incubate for 15 minutes at room temperature			
Spin centrifuge for liquid collection			
Buffer ACB	350 µl		
Pipetting / vortex mixing			
Spin centrifuge for liquid collection			
Transfer of samples to purification column			
Centrifuge at 8000-10.000 rpm for 1 minute. Replacement manifold tube.			
Buffer AW 1	600 µl		
Centrifuge at 8.000 – 10.000 rpm for 1 minute.			
Eluted remove			
Buffer AW2	600 µl		
Centrifuge at 8.000 – 10.000 rpm for 1 minute. Replacement manifold tube.			
Eluted remove			
Centrifuge - maximum speed - 2 minutes. Introduction of columns into the collection tube.			
Buffer AVE	50 µl		
Incubation for 1 minute at room temperature.			
Centrifuge at maximum speed for 1 minute.			
Storage the elute at 1°C - 2°C until the amplification step.			

Table 2. Nucleic acid extraction protocol (PureLink® Genomic DNA Kit)

Reagent	µl/sample	No. samples	Total
200µl blood	20 µl		8 ml
Remove the eluate			
Resuspend in Pure Link Digestion Buffer	180 µl		1440 µl
Proteinase K	20 µl		160 µl
Incubation at 55 ºC 1h with vortex during incubation			
RNase A	20 µl		160 µl
Vortex and incubation at room temp.			
PureLink Genomic Lysis/Binding Buffer	200 µl		1600 µl
Ethanol 96º-100º	200 µl		1600 µl
Transfer the lysate 640 µl to the column - centrifugation at 10,000 x g 1 min.			
Remove the supernatant and transfer the column to a new tube			
Wash Buffer 1	500 µl		4000 µl
Centrifuge at 10,000 x g 1 min. Remove supernatant			
Wash Buffer 2	500 µl		4000 µl
Centrifuge at maximum speed 3 min. Remove supernatant			
Transfer the column to a 1.5 ml tube			
PureLink Genomic Elution Buffer 20 µl			
Centrifugation at maximum speed 1 min			
Remove the column and store the DNA at -80ºC			

The PureLink® Genomic DNA Kits (Thermo Scientific, Carlsbad CA USA) are designed for purification of all genomic DNA. The kit can be used for sample like: organ tissues, blood samples, buccal swabs, bacteria, yeast, and FFPE (formalin paraffin-embedded) tissues.
The principle of the kit is similar with other kits using silica membrane where DNA binds in the presence of chaotropic salts (guanidine-HCL).
DNA isolation was done in four steps. In the sample processing step, RNA digestion was performed to prevent contamination of the purified DNA sample with RNA. For a good lysis of tissues and cells it is used proteinase K. DNA is selectively bound to the silica membrane and subsequently washed in two steps to remove all contaminating cellular components. In the last elution step, DNA is released from the silica membrane.
The second extraction of the DNA was made with the PureLink® Genomic DNA Kit according with the manufacturing kit (Table 2).

The advantages of using the PureLink® Genomic DNA kit are: An efficient extraction process for genomic DNA from different sample types, like organ tissues, blood, and swabs, is designed to be quick and yield high-quality DNA, using of the proteinase K, without the mechanical lysis, low quantities of contaminants and performance of purifying DNA for using it in different protocols of molecular biology, not using organic solvents.
The Qubit 3.0 Fluorometer ThermoFisher Scientific system is used for quantifying the DNA concentration of the extracted samples. The Qubit 3.0 Fluorometer, is an equipment that can be used to quantify DNA, RNA, microRNA and proteins using highly sensitive and accurate fluorescence-based quantification assays.
A dedicated DNA quantification kit called Qubit DNA HS Assay Kit has been used to read DNA concentration. The assay is highly selective for DNA and will not determine free RNA, protein or nucleotides.

Common contaminants like salts, free nucleotides, solvents, detergents, or proteins are well tolerated by this kit. The test kit is designed for DNA concentrations in the sample between 250 pg/µl and 100 ng/µl. The kit contains dilution buffer and pre-diluted DNA standards. We dilute the reagent using the buffer provided, add the sample (any volume between 1 µl and 20 µl can be used) and read the concentration. The samples 5 in number, were reference material and read the DNA yield we extract it previously with the dedicated extraction kits.

The photometry reading protocol consists of:

1. We use the 0.5 ml tubes supplied with the kit in which the mixture will be prepared. The tubes will be for both the sample reading and the two standards required to read the DNA concentration.
2. The working solution is prepared in 1/200 dilution with the Qubit DNA HS reagent and the Qubit DNA HS buffer. It is necessary to use a sterile tube each time when it is made the working Qubit solution. It is forbidden to make the working solution in a glass tube. The final volume in each tube will be 200 µl. The standard needs 190 µl of the working solution and samples need any amount between 180- 199 µl. It is necessary to prepare a sufficient volume of working solution in order to read all standards and samples.
3. It is added 10 µl from each standard, mix for 2-3 seconds.
4. It is added each sample to test the concentration, then vortex for 2-3 seconds. The final volume in each tube should be 200 µl.
5. The tubes must be incubated at room temperature for 2 minutes. Then we read the tubes with the solution choosing the appropriate DNA reading program.

RESULTS AND DISCUSSIONS

After fluorometer reading of the African Swine Fever standard dilutions 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5} and 10^{-6} of both the extracts obtained with the Pure Link Genomic DNA Mini Kit (Thermo Scientific) and the QIAamp Cador Pathogen Mini Kit (Qiagen), it can be said that the results were in accordance with the dilutions. So, the highest concentrations were registered at dilution 10^{-2} and the lowest at dilution 10^{-6}. For the Pure Link Genomic DNA Mini Kit (Thermo Scientific) the following results were recorded: 125 ng/µl at dilution 10^{-2}, 62 ng/µl at dilution 10^{-3}, 31 ng/µl for dilution 10^{-4}, 15.6 ng/µl at dilution 10^{-5} and 7.8 ng/µl at dilution 10^{-6}.

For the QIAamp Cador Pathogen Mini Kit (Qiagen) the following results were obtained: 134 ng/µl for dilution 10^{-2}, 71 ng/µl for dilution 10^{-3}, 43 ng/µl for dilution 10^{-4}, 21.2 ng/µl for dilution 10^{-5}, 10.3 ng/µl for dilution 10^{-6}.

The concentration of the negative control which is ultrapure water: Ultrapure™ DNase/RNase-Free Distilled Water Invitrogen™, in the extraction was also read for each of the two kits used and the results were negative - "out of range to low" - confirming that the extraction was validated with no contamination. According to the instrument's manual the Qubit will give you an out-range error (it will tell you if too low or too high) so you can dilute your samples to be within range, and will display the message "out of range to low". Preventing and controlling ASF requires the early detection of the disease through rapid field identification and accurate laboratory diagnosis. Essential components of this strategy include robust surveillance programs, adequate facilities and resources, and the preparedness of veterinary services. Handling samples potentially infected with the ASFV necessitates laboratories with appropriate biocontainment levels, which are often limited in ASF-free countries (Kosowska et al., 2021).

The aim of this study was to highlight the effectiveness of some of the most popular extraction kits. The performance of the extraction kit is essential in the case of this virus because depending on the age of the sample and the way it is collected, for example in wild boar carcasses, partially denatured, with a difficult to specify age, which do not provide you with blood or other tissues, the amount of DNA is small. This forces you to have a more efficient extraction that will generate maximum yield.

During the early stages of an outbreak, detecting the virus genome through various PCR assays is the most sensitive and specific method. As the disease progresses, serology becomes more valuable for diagnosis because specific antibodies appear later in the course of the disease (7-10 days).

With no effective vaccine or treatment available, the optimal strategy against ASF is to implement an early detection system paired with a rapid response mechanism for outbreaks. Therefore, it

is crucial to increase awareness and provide training for veterinary professionals and other frontline personnel (Beltran-Alcrudo et al., 2017). The ongoing development of commercial kits with improved sensitivity and specificity is evident. Despite having well-established diagnostic tests for ASF, the global epidemiological situation highlights the need for improved tools to quickly identify new cases and reduce response times. Recent advances in ASF diagnostics, focusing on new sample matrices relevant to Europe's epidemiological context. Rapid and reliable diagnosis depends on selecting appropriate samples and methods (Muzykina et al., 2024).

CONCLUSIONS

DNA concentrations obtained from extraction with the QIAamp Cador Pathogen Mini Kit are higher than those obtained from extraction with the Pure Link Genomic Mini Kit. The sensitivity of the QIAamp Cador Pathogen Mini Kit is higher compared to the Pure Link Mini Kit. This is also because the QIAamp Cador Pathogen Mini Kit extracts both RNA and DNA, and also pathogenic DNA, compared to Pure Link Mini Kit, which extracts total DNA.

Based on the obtained results, we can conclude that the QIAamp Cador Pathogen Mini Kit produced the highest DNA concentration, indicating that it has higher sensitivity compared to the Pure Link Genomic DNA Mini Kit. This suggests that the QIAamp kit may be more effective for applications that require the detection of lower quantities of DNA.

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PREPARATION AND CHARACTERIZATION OF NOVEL BIOMATERIALS BASED ON MIXTURES OF FISH COLLAGEN AND PEPTIDES WITH CHITOSAN FOR BIOMEDICAL APPLICATIONS

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Abstract

In the present study, novel biomaterials based on mixtures of collagen and peptides isolated from aquaculture fish residues and marine chitosan solution at pH 5 were prepared, conditioned as 3D porous sponges and characterized to select best variant for biomedical applications. The physico-chemical properties of the biomaterials were investigated by determination of their in vitro biodegradability, swelling degree and porosity, while morphology was observed by scanning electron microscopy (SEM). In addition, the biocompatibility of the prepared biomaterials was assessed by cell viability of L929 fibroblast cultures cultivated in the presence of the prepared sponges by MTT assay. The results indicated that the biomaterials prepared from mixtures of collagen and chitosan at pH 5 and mixtures of collagen, chitosan at pH 5 and peptides had lower in vitro biodegradability in the presence of collagenase, higher degree of swelling and increased porosity, compared to a sponge of fish collagen (control). The cell viability values recorded in L929 fibroblast culture cultivated in the presence of the prepared biomaterials were high, indicating a good biocompatibility with skin specific cells. In conclusion, this study demonstrated that the biomaterials prepared from fish collagen and marine chitosan at pH 5 with or without addition of fish peptides have optimal physico-chemical and structural properties and good biocompatibility, recommending their further testing and use in skin tissue engineering.

Key words*: biomaterials, chitosan, collagen, fish, peptides.*

INTRODUCTION

The fish processing industry generates a multitude of residues, which can be further valorized as raw materials for obtaining collagen or bioactive peptides (Ideia et al., 2020). The usefulness of both collagen and peptides is huge, especially in the healing of tissue injuries, and the fish residues represent a viable alternative to bovine source (Coppola et al., 2020). Thus, in the literature, evidences were found regarding a biomaterial based on sodium alginate, calcium carbonate and fish peptides that stimulated osteogenesis, indicating the possibility to be used in the restoration of bone tissue (Zheng et al., 2024). In addition, it was demonstrated the antioxidant activity of fish collagen peptides and their property to stimulate the dermal fibroblast growth (Wang et al., 2023). Other studies have confirmed that the peptides extracted from fish residues by enzymatic treatment exerted antioxidant, antihypertensive and antiproliferative activity, and had cellular biocompatibility *in vitro* (Toma et al., 2021; Tascias-Pascacio et al., 2021). It has been observed that chitosan addition in the composition of biomaterials based on collagen improved their biological and mechanical properties (Fatemi et al., 2021). *In vivo* testing of a collagen and chitosan gel covering burns made on rat skin showed an increase of angiogenesis, proliferation of fibroblasts and, finally led to the restoration of the damaged tissue (Fatemi et al., 2021). Analysis of a mixture of gelatin and chitosan solution, pH 5, indicated the formation of stable complexes due to the electrostatic interaction between constituent polymeric chains (Chen et al., 2023).

The present study aimed to prepare novel biomaterials based on mixtures of collagen and peptides isolated from aquaculture fish and marine chitosan, conditioned as porous

sponges, and to comparatively investigate their biodegradability, swelling degree, porosity and *in vitro* biocompatibility, in view of their use in biomedical applications.

MATERIALS AND METHODS

Residues (skin, bones) of aquaculture carp were kindly provided by a local fishery after fish processing (Tulcea, Romania). Chitosan from crustaceans was acquired from Sigma-Aldrich (Germany). All chemical reagents used in this study were of analytical grade and acquired from Sigma-Aldrich (Germany), unless otherwise stated.

Isolation of fish collagen and peptides

Both collagen and peptides were obtained from carp residues. The fish collagen was extracted using an enzymatic method with pepsin in acetic acid, pH 2. The peptides were prepared from fish collagen by enzymatic hydrolysis with 4% papain solution, as previously described (Toma et al., 2022).

Preparation of 3D porous biomaterials based on fish collagen

A solution of 50 mg/ml chitosan was obtained in 1 M acetic acid by magnetic stirring over-night and then it was adjusted at pH 5 with 5 M NaOH. Two variants of 3D porous biomaterials were prepared using fish collagen solution (50 mg/ml) mixed with chitosan in different weight ratios (Table 1, variants 2 and 3).

Table 1. The variants of biomaterials prepared from mixtures of fish collagen and peptides with chitosan, conditioned as 3D sponges

Variant no.	Biomaterial composition	Mixing ratio (w/w, w/w/w)	pH
1.	Collagen	-	5
2.	Collagen-chitosan	1:1	5
3.	Collagen-chitosan	1:0.5	5
4.	Collagen-chitosan-peptides	1:1:0.1	5
5.	Collagen-chitosan-peptides	1:1:0.2	5

The mixtures were homogenized by magnetic stirring for 2 h and fish peptide solution (50 mg/ml) was added to obtain variants 4 and 5 (Table 1). All mixtures were conditioned by freeze-drying in glass Petri dishes, at -35^{0}C, for 48 h using a Martin Christ lyophilizer equipment (Germany) (Craciunescu & Moldovan, 2011).

Determination of *in vitro* biodegradation degree in the presence of collagenase

The biodegradation degree of porous biomaterials was determined in the presence of collagenase, as previously described in a study performed by Craciunescu et al. (2011). Samples of biomaterial were incubated in TES buffer pH 7.4, containing collagenase and $CaCl_2$, for 5 h, at 37ºC. At the end of incubation period, the solution was put on ice to deactivate the enzyme and centrifuged at 5000g, for 10 min. Aliquots of supernatant were analyzed for protein content using ninhydrin-based reagent (Park et al., 2002). The absorbance was read at 660 nm using a V-650 UV-VIS spectrophotometer (Jasco, Japan).

Determination of swelling degree

Samples of biomaterial were weighed (w_i) and then, they were immersed in TES buffer pH 7.4. At predetermined periods of time, the biomaterials were removed, the excess water was absorbed on a filter paper and then, the biomaterials were weighed (w_f) (Craciunescu et al., 2011). The degree of swelling was calculated according to the formula:

$$\text{Swelling degree} = \frac{wf - wi}{wf} * 100$$

Determination of porosity

Biomaterials porosity was determined by water replacement method (Craciunescu et al., 2011). Samples of biomaterial were weighed and immersed into a graded cylinder with a known volume of water (V1). The porous biomaterial was periodically pressed to release the air from the pores and fill them with water. The total volume of water and water-impregnated matrix was measured (V2). Then, the biomaterial was removed and the volume of water remaining in the cylinder was measured (V3). The porosity of samples was calculated using the formula:

$$\text{Porosity} = \frac{V1 - V3}{V2 - V3} * 100$$

SEM observations

The morphology of 3D porous samples of biomaterial was observed by scanning electron microscopy (SEM) using a Hitachi SU1510 equipment. The samples were previously coated with a gold layer.

Evaluation of *in vitro* biocompatibility

A cell culture of mouse fibroblasts from the stabilized cell line NCTC (clone L929) was used to determine *in vitro* biocompatibility of the prepared biomaterials. The viability of cells cultivated in 96-well culture plates in direct contact with samples of lyophilized biomaterials was determined after 24 and 48 h by MTT (thiazolyl tetrazolium bromide) assay. The optical density (OD) of the resulting solution was measured at 570 nm at LB 940 plate reader (Berthold Mithras, Germany). Cells cultivated in culture medium without sample were used as a control and their viability was considered 100%.

$$\text{cell viability (\%)} = OD_{sample}/OD_{control} * 100$$

RESULTS AND DISCUSSIONS

Four variants of composite biomaterials were prepared from fish collagen and marine chitosan supplemented or not with fish peptides and conditioned as 3D porous sponges (Figure 1).

Figure 1. Sponge biomaterial based on fish collagen mixed with chitosan solution, pH 5 in 1:1 weight ratio

Biodegradability of fish collagen-based biomaterials

The results of the biodegradation degree of biomaterials are presented in Figure 2.

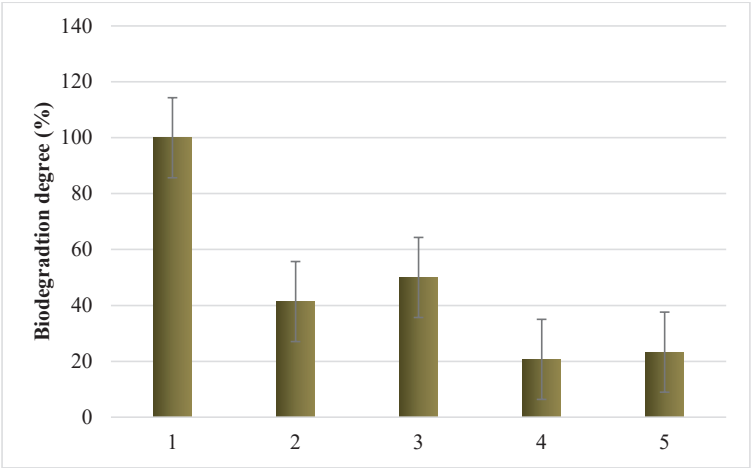

Figure 2. *In vitro* biodegradation degree of biomaterials based on fish collagen

The biodegradation degree of the collagen sponge (variant 1, control) was 100% after 5 h of incubation in the presence of collagenase. Mixing collagen with chitosan solution, pH 5 increased the resistance of the biomaterial to collagenase action *in vitro* with 58.63% in variant 2 and 50% in variant 3, compared to collagen sponge. The lowest biodegradation degree of approx. 20% was observed in the case of variants 4 and 5 obtained by mixing collagen with chitosan solution pH 5 and fish peptides. It was observed that the addition of peptides in the composition of the biomaterial based on collagen and chitosan pH 5 increased the resistance to the action of collagenase *in vitro* with 79.31%, compared to collagen sponge, and with 28.26%, compared to variant 1. The increased resistance to enzyme action of biomaterials based on collagen and chitosan compared to collagen biomaterial was also observed in Ressler's study (Ressler et al., 2023).

Swelling degree of fish collagen-based biomaterials

The results of the swelling degree of biomaterials are presented in Figure 3.

The swelling degree of the composite biomaterials was lower than that of the fish collagen sponge, except for the variant 3 consisting of a mixture of collagen and chitosan pH 5 in a ratio of 1:0.5. The lowest swelling degree was recorded for variants 4 and 5 that contained fish collagen, chitosan solution pH 5 and fish peptides. It was observed that the addition of peptides decreased the degree of swelling of the biomaterials by 50.67% in variant 4 and by 39.08% in variant 5. Still, a value of 900-1000% swelling degree registered for variants 4 and 5 is mostly due to the presence of a polysaccharidic constituent (chitosan) in the composition of the biomaterial and represent an optimal parameter, indicating the capacity to absorb the exudate at the wound situs. Yang observed that the degree of swelling is not proportional to the amount of chitosan added to the collagen-based biomaterial, a result similar to the result obtained in the study presented in this article (Figure 3) (Yang et al., 2021).

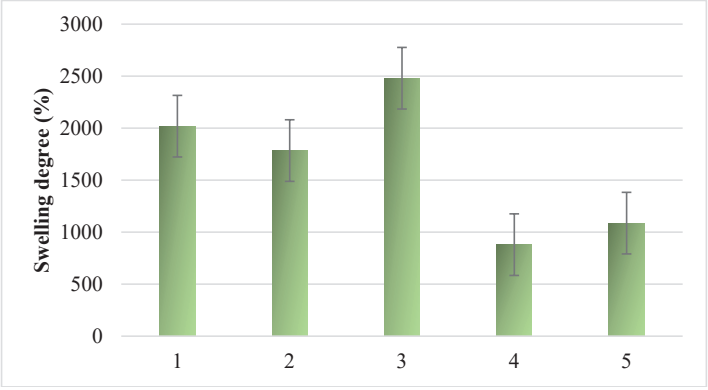

Figure 3. Swelling degree of biomaterials based on fish collagen

Porosity of fish collagen-based biomaterials

The results of biomaterials porosity are presented in Figure 4.

The porosity of composite biomaterials was higher than that of the fish collagen sponge, except for variant 3. The values varied between 54.42-61%. The highest porosity value (61%) was found in the case of variants 4 and 5 containing in addition fish peptides. Variant 2 of collagen and chitosan pH 5 in a ratio of 1:1 had a porosity of 58.8%, higher than that of variant 3 (54.4%), containing less chitosan (1:0.5).

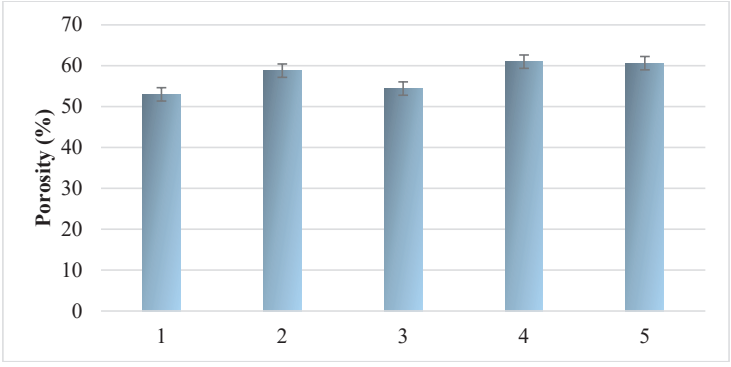

Figure 4. Porosity of biomaterials based on fish collagen

These results confirmed previously observations reported in the case of a hydrogel made of collagen and chitosan, i.e. the porosity increased for higher amount of chitosan in the composition of the biomaterial (Valentino et al., 2023).

Morphology of fish collagen-based biomaterials

Through SEM visualization, the morphology of composite biomaterials was observed (Figure 5). The presence of interconnected pores with variable sizes was apparent and characteristic to biomaterials used as scaffolds for tissue regeneration.

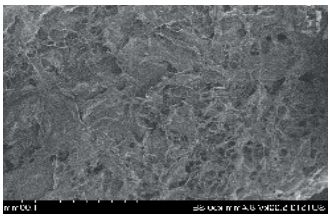

Figure 5. Representative SEM image of composite biomaterial consisting of fish collagen and chitosan at pH 5 in a ratio of 1:1

***In vitro* biocompatibility of fish collagen based biomaterial**

The results of cell viability testing are presented in Table 2.

Table 2. Cell viability of L929 cells cultivated in the presence of composite biomaterials based on fish collagen, chitosan and fish peptides, determined by MTT assay

Incubation time	Variant 1	Variant 2	Variant 3	Variant 4	Variant 5
24 h	108.77 ± 2.52	92.03 ± 2.54	93.51 ± 3.54	107.08 ± 2.71	115.44 ± 1.91
48 h	91.47 ± 2.32	80.08 ± 2.45	85.08 ± 1.79	94.84 ± 2.45	103.16 ± 2.34

The cell viability values after cultivation in the presence of composite biomaterials were high, exceeding 90% in direct contact for 24 h, and above 80% after 48 h. In the case of composite biomaterials supplemented with fish peptides, it was highlighted a stimulation of fibroblast metabolism (107-115%), similar (variant 4) or higher (variant 5) than in the case of fish collagen sponge (variant 1). Te results of cell viability are presented in Table 2. Similar studies showed that a hydrogel based on collagen and chitosan mixtures stimulated the adhesion and cell proliferation of human dermal fibroblasts *in vitro* (Valentino et al., 2023). They reported that higher cell proliferation was found in the case of samples with lower amount of chitosan. In the specialty literature, biomaterials based on collagen and chitosan did not show cytotoxicity in the case of treating cell cultures with them (Valentino et al., 2023; Yang et al., 2021; Ressler et al., 2023).

CONCLUSIONS

By mixing the collagen solution extracted from aquaculture fish with a solution of chitosan pH 5, a biomaterial with an increased resistance to collagenase action *in vitro* was obtained. Fish peptides addition to the polymeric composition increased even more the resistance to collagenase action *in vitro*. The degree of swelling was lower for composite biomaterials containing fish collagen, chitosan pH 5 and fish peptides, compared to that of fish collagen sponge. The porosity of biomaterials increased in variants containing chitosan and fish peptides. The novel biomaterials based on fish collagen did not show cytotoxicity in L929 fibroblast culture and, in addition, the biomaterials containing fish peptides stimulated cell proliferation. All these results demonstrated optimal physico-chemical and structural properties, and good biocompatibility of 3D porous biomaterials resulted from mixing collagen with chitosan at pH 5 or with chitosan and peptides extracted from fish, recommending their use in biomedical applications. Future work will investigate their interaction with skin cells in terms of mechanism of action, to better solve tissue engineering issues.

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TOXIGENIC MOLDS CONTAMINANTS IN SUDANESE POULTRY FEED AND THEIR POTENTIAL BIOCONTROL

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Abstract

This study focuses on Penicillium and similar other mold contaminants found in poultry feed sourced from Sudan. Such fungi were found in sorghum kernels, shelled peanuts, wheat bran and peanut meal from seven Sudanese regions. These fungi were isolated and characterized based on their microscopic features and colony morphology on Dicloran Rose Bengal Chloramphenicol Agar (DRBC), Potato Dextrose Agar (PDA), and Pentachloronitrobenzene - Rose Bengal - Yeast extract - Sucrose Agar (PRYES). To evaluate their mycotoxigenic potential, chromatographic and genetic approaches were used. Fungal extracts were evaluated for mycotoxins content by Thin-Layer Chromatography (TLC). Structural and regulatory genes such as nor-1, ver-1, omt-A, aflR, ota.nps and patN, involved in mycotoxins production were analyzed by classical PCR technology. The strains were also identified by sequencing the ITS1 – 5,8S – ITS2 region. To inhibit the growth of Penicillium isolate certain biocontrol agents were used, revealing good antifungal potential. These findings provide valuable insights into the nature of Penicillium contaminants in Sudanese poultry feed, providing fundamental knowledge for further research on managing mycotoxigenic contaminants and feed safety.

Key words*: Biocontrol, Mycotoxin, Penicillium, Poultry feed, Thin-Layer Chromatography (TLC), PCR, sequencing.*

INTRODUCTION

Mycotoxin contamination, in poultry feed threatens the health of animals and the safety of our food (Haque et al., 2020). Among the fungi known to produce mycotoxins, *Alternaria, Aspergillus, Fusarium,* and *Penicillium* species have been identified as contaminants in different agricultural products (Perrone & Susca, 2017).
Revealing the mycotoxigenic potential of mold contaminants is essential to understand the health impact of these substances and to ensure the safety of poultry products and protect consumer health (Hocquette et al., 2005; Bou et al., 2009).
In the current study, the prevalence of blue-like mold contaminants and their mycotoxigenic potential was investigated in poultry feed sourced from Sudan. Microbiologic, molecular and chromatographic approaches were used to examine the prevalence of blue mold contaminants and their toxigenic metabolites in four types of feed. Certain microbiologic strategies to suppress *Penicillium* spp. growth were also examined in order to find a managing solution to reduce the prevalence of mycotoxigenic contaminants to enhance feed safety strategies.

MATERIALS AND METHODS

Fungal isolation from poultry feed

Four types of Soudanese poultry feed were microbiologic analyzed to evaluate the presence of blue mold contaminants: sorghum kernels (A samples), shelled peanuts (B samples), wheat bran (C samples), and peanut meal (D samples). The samples were collected from different locations in Sudan.
The sorghum kernels and shelled peanut samples were ground as powder, in laboratory conditions, using sterile mortar and pestle.
For the microbiological analysis, 10 g of each powder sample were infused in 90 ml sterile distilled water, and vigorously shaken. Serial dilutions were prepared till 10^{-3} in aseptic conditions. To evaluate the fungal load 0.1 ml of suspension was spread (in triplicate) on

Dichloran Rose-Bengal Chloramphenicol Agar (DRBC medium; Carl Roth GmbH+Co.KG, Karlsruhe, Germany) (Beuchat, 1992; 1995). All plates were incubated at room temperature for 4 days to allow fungal growth. Visual analysis was periodically made to enumerate the fungal load. Blue-like colonies were selected and purified on Rose-Bengal Chloramphenicol Agar (RBC; Merck KCaA, Darmstadt, Germany), and subcultured on Potato Dextrose Agar (PDA; VWR Chemicals, Louvain, Belgium).

Microscopic and macroscopic analysis

Isolated fungal cultures were visually analyzed for macroscopic characterization. Slide cultures were also prepared on PDA for microscopic analysis. When needed, methylene blue staining was used to highlight the conidiophores and conidia architecture.

Molecular analysis for fungal identification

Fresh fungal biomass was used for genomic DNA extraction. Commercial ZR Fungal/ Bacterial MiniPrep™ kit (ZymoResearch, SUA) was used, according to the manufacturer instructions, for DNA purification. The resulting DNA was used as template for the amplification of the ITS1-5.8S-ITS4 region. In the PCR was performed in 50 µl reaction volume, containing 1X Green Buffer with $MgCl_2$, 0.2 mM dNTPs, 0.5 µM of each ITS1 and ITS4 primers (Table 1), 0.2 U of DreamTaq DNA Polymerase (Thermo-Scientific, Baltics, UAB, Vilnius, Lithuania), and ~10 ng of template DNA, all mixed in sterile MilliQ water. The amplification program included an initial denaturation at 94°C of 4 min, followed by 35 cycles of 1 min at 94°C for denaturation, 1 min at 45°C for primers' annealing, and 2 min at 72°C for elongation, followed by a final elongation at 72°C for 10 min. The PCR products were revealed through gel electrophoresis, using 1% agarose with ethidium bromide, in 0.5X TBE buffer. The electrophoretic profiles were analyzed under UV light, compared to a 100 bp DNA ladder (ThermoScientific, Baltics, UAB, Vilnius, Lithuania), using a BioDoc-It Imaging System (Ultra-Violet Products Ltd., Upland, CA, USA). The PCR products were purified and sequencing by Sanger dideoxy sequencing method, at CeMIA (Cellular and Molecular Immunological Applications, Greece). The BioEdit biological sequence alignment editor was used to assembly the forward and reverse sequencing results. For taxonomic identification the online NBLAST software was used and the partial sequences of the ITS1-5.8S-ITS4 region were compared to similar sequences found in the National Center for Biotechnology Information (NCBI) database.

To evaluate the mycotoxigenic potential of the isolated fungi, chromatographic and genetic approaches were used.

Molecular analysis for mycotoxin encoding genes

The fungal isolates were screened for five structural and regulatory genes involved in mycotoxins production, *aflR, omt, otanps, nor-1,* and *ver-1*. The PCR was performed in 25 µl reaction volume, according to the previously mentioned protocol. The amplification programs slightly varied, depending on the optimal annealing temperature for each tested primer set (Table 1).

The PCR was prepared in 3 steps. The first step was performed for initial denaturation of template DNA, at 94°C for 4 min. The second step had 30 cycles of 1 min at 94°C for denaturation, 1 min at proper primers' annealing temperature, and 2 min at 72°C for elongation. The third step was carried out for the final elongation at 72°C for 7 min.

The PCR products were migrated in agarose gel electrophoresis and visualized in UV light, as previously mentioned.

Thin-Layer Chromatography (TLC).

Fungal strains were grown for 6 days at 28°C, on Pentachloronitrobenzene - Rose Bengal - Yeast extract - Sucrose Agar (PRYES), containing 150 g/L sucrose, 20 g/L yeast extract, 0.1 g/L pentachloronitrobenzene, 0.025 g/L rose Bengal, 0.05 g/L chloramphenicol, 20 g/L agar, and a pH of 5.6±0.2. Four mycelial plugs, each of 6 mm diameter, were collected to perform the mycotoxin extraction. Grounded samples were immersed in 1 ml chloroform, vigorously vortexed, and kept for 2 days at 4°C to improve mycotoxins extraction. Samples liquid phase was then collected by centrifugation, and let for total evaporation. The crude extracts were stored by refrigeration until evaluation. For the TLC analysis, the crude

extracts were resuspended in 25 µl of chloroform, and 10 µl of each sample were spotted on thin-layer silica gel-coated glass plates. An ochratoxin A standard solution (10 µg/ml), provided from Trilogy Analytical Laboratory Inc., was used as 2 µl/spot.

Table 1. Primers sets and their nucleotide sequences

Target DNA region / gene	Primer code	Sequence 5' to 3'	Annealing temperature	Product size (bp)	Reference
ITS1-5.8S-ITS2	ITS1	TCCGTAGGTGAACCTGCGG	45°C	variable	White et al (1990)
	ITS4	TCCTCCGCTTATTGATATGC			
nor-1 or *aflD* gene encoding for norsolorinic acid	NorF	ACCGCTACGCCGGCACTCTCGGCAC	65°C	400	Abdel-Hadi et al. (2010)
	NorR	GTTGGCCGCCAGCTTCGACACTCCG			
ver-1 or *aflM* gene encoding for versicolorin	VerF	GCCGCAGGCCGCGGAGAAAGTGGT	65°C	537	Abdel-Hadi et al. (2010)
	VerR	GGGGATATACTCCCGCGACACAGC			
omt-A or *aflP* gene a structural aflatoxin gene	OmtF	GTGGACGGACCTAGTCCGACATCAC	65°C	797	Abdel-Hadi et al. (2010)
	OmtR	GTCGGCGCCACGCACTGGGTTGGGG			
aflR gene an aflatoxin regulatory factor	AflrF	TATCTCCCCCCGGGCATCTCCCGG	65°C	1032	Abdel-Hadi et al. (2010)
	AflrR	CCGTCAGACAGCCACTGGACACGG			
ota.nps gene encoding for ochratoxin	otanps F	AGTCTTCGCTGGGTGCTTCC	58°C	750	Rashmi et al. (2013)
	otanps R	CAGCACTTTTCCCTCCATCTATCC			
panN gene encoding for isoepoxydon dehydrogenase (IDH) enzyme	patN F	CAATGTGTCGTACTGTGCCC	52°C	600	Paterson et al. (2003)
	patN R	ACCTTCAGTCGCTGTTCCTC			

Plates were placed in the migration tank, loaded with 15 ml migration mixture, containing toluene: ethyl acetate: formic acid, 60:30:10 (v/v/v) (Nayaka et al., 2013). The standard ochratoxin A solution was used as control. After migration, the plates were exposed to 365 nm UV light and analyzed according to Vujanovic & Ben Mansour (2011) protocol. To determine the presence of mycotoxigenic compounds in samples, the retention factor (Rf) was calculated for standard mycotoxins. This important parameter is calculated after chromatographic separation, and indicates the relative, specific displacement of each compound in the sample, along the solvent migration front. The Rf values are in between 0 and 1, while each TLC migrated compound has a different, specific value. However, the Rf can depend on the TLC parameters, such as temperature, layer thickness, degree of solvent saturation in the separation chamber, the volume of the sample, as well as the type of mobile and stationary phase. The formula for calculating the Rf value is given by the ratio of the compound migration compare to the migration front on the chromatographic plate, as follows:

$$Rf = \frac{\text{Distance traveled by the compound (cm)}}{\text{Distance traveled by the migration front (cm)}}$$

***In vitro* antagonistic assay**

Dual culture technique was performed to evaluate potential probiotic bacteria against blue mold development.

The test was performed *in vitro*, on PDA medium.

Eight bacterial strains were tested: *Bacillus* sp. B4, *B. subtilis* B5, B6, and *B. amyloliquefaciens* BW, BIR, BPA, OS15, and OS17.

The bacterial strains were inoculated as spots, four per plate, equidistant one from each other, and 2 cm away from the center of the plate. Then, the blue mold was inoculated in the center of the Petri dish.

Penicillium inoculum was prepared as conidial suspension inv sterile distilled water, supplemented with three drops of Tween 80.

The suspension was prepared as 10^7 cfu/ml, and 25 µl conidial suspension was used to inoculate each plate.

Fungal control was also prepared, were the blue mold was grown on PDA, and incubated in similar conditions.

The antifungal activity was evaluated after ten days of incubation at room temperature, by measuring the fungal growth in both control and test plates and calculating fungal inhibitory efficacy, according to Boiu-Sicuia et al. (2021).

RESULTS AND DISCUSSIONS

Fungal contaminants quantification

Fungal load in the Sudanese feed samples was quantified as 10^3 cfu/g of sample. Only molds were counted, while yeasts were not taken into consideration (Figure 1).

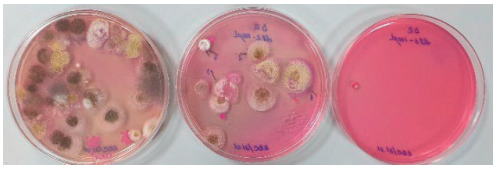

Figure 1. Mold colonies grown from different dilution of D2 Sudanese feed sample on RBC media

Blue molds enumeration

Each colony with typical color and morphology isolated from one of the poultry feed samples was considered an isolate.

At the end of the incubation period, which lasted 4 days at 28°C, the blue-like molds were purified. Based on the macroscopic features and colony morphology, only four strains (A3-2, A3-4, C1-1, and C3-1) were taken as potential blue molds (Figure 2).

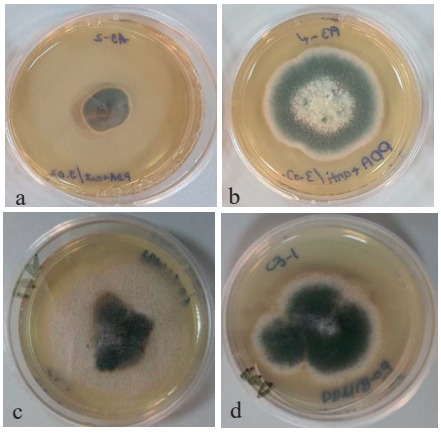

Figure 2. Blue molds contaminants isolated from Sudanese feed sources on PDA:
a. A3-2; b. A3-4; c. C1-1; d. C3-1 strains

Under microscopic evaluations, only A3-4 strain revealed *Penicillium* sp. characteristics, showing brush shape conidiophores (Figure 3a). Two other strains (C1-1 and C3-1) revealed vesiculated conidiophores (Figure 3b), while the A3-2 strain developed branched, brown pigmented, conidiophores (Figure 3c).

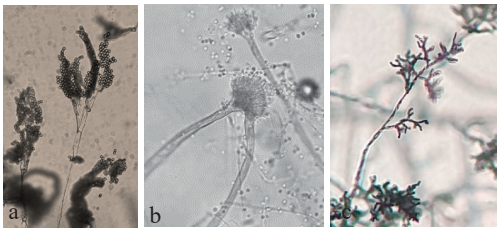

Figure 3. Conidiophoses and conidia:
a. *Penicillium* sp.; b. *Aspergillus* sp.; c. *Cladosporium* sp.

In other African countries, different multi-mycotoxin producing fungi were found as post-harvest contaminants of sorghum grains. *Aspergillus* and *Penicillium* are among the fungal contaminants recovered from infected grains (Mohammed et al., 2022; Deligeorgakis et al., 2023).

Generally, the *Cladosporium* species are not included among the major toxigenic fungi.

However, as contaminant is mentioned to be present of the sorghum grains produced in Africa (Pambuka et al., 2021).

Molecular identification

Based on the sequence analysis of the ITS1-5,8S-ITS2 region the mold strains A3-2, A3-4, C1-1, and C3-1 were attributed to 3 species (Table 2).

Fungal identification based on molecular means confirmed the microbiologic results, highlighting the presence of fungal species with mycotoxigenic potential.

Table 2. Fungal strains identification

Fungal strain	Molecular identification	Sequence length	Reference strain/ NCBI accession no.	Query Cover	Identity Percent
A3-2	*Cladosporium tenuissimum*	524 bp	BFMY-2 / MT573533.1	100%	100.00%
A3-4	*Penicillium polonicum*	503 bp	DUCC5746 / MT582786.1	100%	99.60%
C1-1	*Aspergillus nidulans*	523 bp	CBS 114 63 / MH858232.1	99%	99.81%
C3-1	*Aspergillus nidulans*	530 bp	CBS 129375 / MH865286.1	100%	100.00%

Molecular identification of mycotoxin encoding genes

Several genes encoding for mycotoxin synthesis were screened by classical PCR technique. The four tested fungal strains revealing the lack of *nor-1* (*aflD*), *ver-1* (*aflM*), *omt-A* (*aflP*), and *aflR* genes. As these genes are encoding for key enzymes involved in aflatoxin biosynthetic pathway, the results suggest that the studied strains are not aflatoxin producers. These findings are highly encouraging, considering that *A. nidulans* could produce the carcinogenic sterigmatocystin mycotoxin (encoded by *omt-A* gene), which is a precursor of the highly dangerous aflatoxins (Keller et al., 1994).

The other two studied genes, *ota.nps*, encoding for ochratoxin A, and *patN*, encoding for patulin were both found to be present in *P. polonicum* A3-4 strain (Figure 4 a, b).

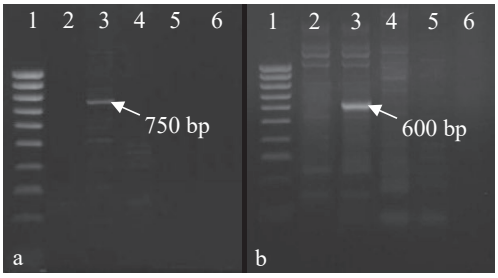

Figure 4. Highlighting the presence of the *ota.nps* (a) and *patN* (b) genes in *P. polonicum* A3-4 Sudanese strain
Legend: Line 1 = Molecular marker of 100bp; Line 2 = A3-2 strain; Line 3 = A3-4 strain; Line 4 = C1-1 strain; Line 5 = C3-1 strain; and Line 6 = Negative Control

Considering that *P. polonicum* is used as biosynthetic source of ochratoxin A (Mantle et al., 2016), the presence of *ota.nps* gene is not surprising. Beside ochratoxin A, *P. polonicum* fungi could produce various other mycotoxigenic metabolites, such as anacine, patulin, penicillic acid, verrucosidin, and 3-methoxyviridicatin (Núñez et al., 2000; Ding et al., 2013). Therefore, finding *patN* gene that encodes for patulin synthesis is considered to be a regular characteristic.

Mycotoxin analysis

Performing the TLC methodology for ochratoxin A detection, in the fourth studied fungal strains, confirmed the results of the molecular analysis. Ochratoxin A being found present only in the crude extract of *P. polonicum* A3-4 strain (Figure 5). Nether of the other strains of *C. tenuissimum* or *A. nidulans* revealing the presence on this toxin in their crude extracts.

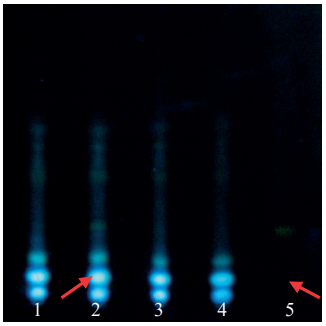

Figure 5. TLC chromatogram exposed to UV light revealing ochratoxin A compound (red arrows)
Legend: Line 1 = A3-2 strain; Line 2 = A3-4 strain; Line 3 = C1-1 strain; Line 4 = C3-1 strain, and Line 5 = ochratoxin A standard

Based on the calculated Rf value for the mycotoxin standard used, the green spot of 0.27 Rf is confirming the presence of ochratoxin A, only in the crude extract of A3-4 strain.

***Penicillium* biocontrol solution**

Bacterial biocontrol potential against mycotoxigenic *P. polonicum* A3-4 isolate was evaluated *in vitro* conditions.

Most of the tested strains, previously characterized as biocontrol strains (Sicuia, 2013) revealed good antifungal activity against *P. polonicum* A3-4, showing 78.09 to 82.02% mycelia inhibitory efficacy (Table 3).

Table 3. *P. polonicum* mycelia growth inhibition

Bacterial strain	Clear inhibition zone	Bacterial efficacy in fungal growth inhibition
B4	1.5 cm	54.67%
B5	2.0 cm	78.16%
B6	1.0 cm	69.34%
BW	0.5 cm	78.09%
BIR	1.5 cm	82.04%
BPA	3.5 cm	79.41%
OS15	2.5 cm	79.41%
OS17	1.5 cm	79.41%

Although some of the bacterial tested strains, such as B4 and B6, were promising biocontrol agents against other toxigenic fungi (Grosu et al., 2015), they revealed lower antifungal activity on *P. polonicum* A3-4 compared to the other tested strains.

CONCLUSIONS

The prevalence of fungal contaminants in Sudanese poultry feed revealed to be 10^3 cfu/g of sample. Among the fungal contaminants only 4 isolates revealed blue-like appearance. One of these isolates (A3-4) for identified as *P. polonicum* and found to be ochratoxin A and patulin producer. To reduce the growth of this pathogen, several *B. amyloliquefaciens* strains were found efficient inhibitor. Best results (82.04% efficacy) were obtained with the BIR biocontrol strain.

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RESEARCH INTO INCREASE BEE PRODUCTIVITY USING COVER CROPS

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Abstract

The general objective of the research work was to identify species of plant crops with honeydew potential during the July-October period when bee food is reduced, and it is necessary to supplement the bee family with artificial food. After harvesting the main commercial crops (barley, oats, wheat), honey plants were cultivated on the respective plots: buckwheat, mustard, phacelia, peas and camelina, which bloom during this period. After flowering, all these crops were incorporated into the soil by mechanized plowing in order to increase the amount of humus and keep the soil green, thus ensuring an ecological method of sustainability (cover crops). To determine the honeydew capacity of the selected crops, the capillary method was applied throughout the flowering period: or 3 repetitions were performed for each crop during the period of maximum nectar accumulation capacity, indicated by the specialized literature.

Key words*: cover crops, rural area, agricultural ecosystem, honey base, soil enrichment.*

INTRODUCTION

Cover crops play a crucial role in improving the humus content of the soil, contributing to its health and fertility, under the conditions of climate change (Pisante et al., 2015; Harrison et al., 1995), under which there are also Romania (Râșnoveanu et al., 2023; Drăgan et al., 2022a).
Several studies have determined the influence of cover crops on soil properties in a wide range of soil types and climates. Islam & Sherma (2021), showed 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. Furthermore, Farmaha et al., (2021) analyzed soil nutrients in 196 fields in the southeastern United States and reported improved soil health in no-till and cover cropping systems compared to the tillage system.
Cover crops such as legumes, crucifers and grasses add organic matter to the soil through their biomass (Drăgan et al., 2022a; Drăgan et al., 2022b).
According to Sharma et al. (2018), cover crops help to reduce soil water evaporation, thereby conserving soil moisture for the next crop. Through plowing incorporation technologies, cover crops are allowed to decompose in the soil, the plant material turns into humus, enriching the soil with essential nutrients.
Through tillage technologies, cover crops are allowed to decompose in the soil, the plant material turns into humus, enriching the soil with essential nutrients.
Cover crops stimulate microbial activity in the soil (Drăgan et al., 2022b). Microorganisms are responsible for breaking down organic matter and turning it into humus. Cover crop roots and residues provide food for these microorganisms, encouraging healthier soil (Islam & Sherman (Eds.), 2021; Farmaha et al., 2021).
Cover crops protect the soil from erosion caused by wind and water (Drăgoi et. al., 2020). By reducing erosion, they prevent the loss of the upper soil layer, which is rich in organic matter and humus (Stănilă et al., 2011)
Cover crops can suppress weed growth, reducing the need for herbicides and soil disturbance (Bîscoveanu et al., 2023). According to the studies developed by Drăgoi et al. (2020), this was found to allow the soil to remain more stable and able to support humus-forming processes.

Cover crops, especially low-diversity mixtures that include buckwheat and *Phacelia* spp., provide a high abundance of flowers throughout the summer, resulting in excessive bee visitation rates, with *Phacelia* spp. being more attractive to honeybees and bumblebees, while sunflowers and local wildflowers are more attractive to solitary bees (Khalifa et al., 2021).

Phacelia crops (*Phacelia* spp.) have become increasingly popular due to their ecological and agricultural benefits (Râșnoveanu et al., 2023; Bîscoveanu et al., 2023; Lee-Mäder et al., 2018). These plants with attractive flowers are known for their ability to attract pollinators such as bees and other beneficial insects. Thus, they contribute to the promotion of biodiversity and the pollination of other agricultural crops. Phacelia crops are also remarkable for their ability to fix atmospheric nitrogen, thereby providing organic fertilizers to the soil and improving its fertility.

White mustard (*Sinapis alba* L.) is originally from the Mediterranean region, but due to its phenotypic plasticity, it is currently cultivated on all continents. This oilseed crop has numerous agronomic advantages over canola, including greater tolerance to drought, moisture, heat, frost, and pests (Jankowski, et al., 2020).

Bees are surrounded by several variables that affect their role as pollinators, such as pathogens, nutritional deficiencies, climate change and deforestation (Pătruică et al., 2017). Spraying of agrochemicals such as fungicides, insecticides and pesticides causes contamination, toxicity and decrease in the quality and quantity of nutrients in pollen and nectar, leading to poor colony health and therefore threatening bee survival (Khalifa et al., 2021).

The present study is based on the achievement of objectives pursued, during the years 2021 - 2024, of 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 and the completion of doctoral research studies, experimental mellifer potential cultures were established in the basic apiary from Cornetu (Călărași county, Romania),using nectar taken from staggered blooming flowers over 2-3 weeks.

MATERIALS AND METHODS

The experiments were carried out in locations located in the S-E of Romania, during 3 years of cultivation respectively: 2021-2022, 2022-2023, 2023-2024 in the following farms: Moara Domnească Didactic Farm from Găneasa, Ilfov county (area 1,000 sq m/m for each crop), Stupina Cornetu from Ilfov county (area 500 sq/m), Scurtu Mare commune, from Teleorman county (5,000 sq/m for each crop), and Ștefan cel Mare commune from Călărași county (5,000 sq/m each culture).

The cover crops selected in this research were represented by the following species: white mustard (*Sinapis alba* L. variety Myria), phacelia (*Phacelia tanacetifolia* L.), buckwheat (*Fagopyrum esculentum* L. Zita variety), spring peas (*Vicia sativa* L.) and camelina (*Camelina sativa* - Mădălina variety), by the fact that they demonstrated a good ability to fix atmospheric nitrogen in the soil as a result of symbiosis with rhizobial bacteria. For the establishment of camelina crops, the recommendations published by Dobre et al. (2014), as a result of the research carried out by them at Moara Domnească, on the use as a second effective crop on the same land area, were taken into account.

To measure nectar secretion, the capillary method was used (Ion et al., 2007) which involved the following steps:

- collection of nectar directly from flowers in the field at 9, 12 and 17 hours, the flowers being isolated 24 hours before starting the collection procedure using graduated capillaries;
- measuring the volume of nectar collected to determine the amount of nectar produced per flower;
- the comparative analysis of the data obtained to evaluate the performance of each crop in terms of nectar secretion.

The calculation of the carbohydrate index (mg/flower) needed to assess the honeydew value was carried out using the formula recommended by Ion et al. (2007):

Carbohydrate index (mg/flower) = [Nectar secretion (mg/flower) x sugar concentration (%)] / 100

RESULTS AND DISCUSSIONS

From the experiments carried out, the following was found:
- the plots sown with buckwheat, offered the highest floral coverage, total bee visitation rates increased for all cover crops during the flowering period, an important result noted by the beekeepers in the project was the elimination of the addition of bee families with artificial food.

The values recorded at the level of the samples collected from the cover crops of the Moara Domnească Didactic Farm, for the assessment of honeydew potential in the 2021 research year, were the following:

For buckwheat (*Fagopyrum esculentum* L. 'Zita') culture:
- the amount of nectar was 0.1-0.2 mg/flower
- honey production of 20 kg/ha

For white mustard (*Sinapis alba* L. variety 'Maryna') culture:
- the amount of nectar 0.04 - 0.1 mg/flower
- honey production 15 kg/ha

For camelina (*Camelina sativa* L. 'Mădălina') culture:
- the amount of nectar was below 0.02 mg/flower
- honey production below 8 kg/ha

Phacelia (*Phacelia tanacetifolia* L. 'Stala'), the culture did not emerge uniformly and due to the weather conditions of November 2021, the plant did not bloom, the collection of samples for nectar determinations were not sufficient and edifying.

The autumn pea (*Vicia villosa* L.) crop did not emerge uniformly and due to the weather conditions of November 2021, the plant did not bloom, the collection of samples for nectar determinations were not sufficient and edifying.

The values recorded at the level of the samples collected from the cover crops located in the Cornetu Commune and from the Moara Domnească Didactic Farm, for the assessment of the potential of the honeydew in the 2022 research year, were the following:

For buckwheat (*Fagopyrum esculentum* L. 'Zita') culture, the amount of nectar was 0.2-0.25 mg/flower, honey production 25 kg/ha.

And for white mustard (*Sinapis alba* L. variety 'Maryna') culture, the nectar quantity 0.1 - 0.15 mg/flower, honey production 20 kg/ha.

The cover crops camelina, phacelia, autumn pea did not withstand the water stress of July 2022.

Buckwheat and mustard crops have shown a good ability to develop under severe drought conditions.

Among the 5 crops of honey plants established to cover the ground at the basic apiary in Cornetu commune, only buckwheat (*Fagopyrum esculentum* L.) and white mustard (*Sinapis alba* L.) yielded quantifiable results, comparable to the data from the specialized literature.

The 2023 research year took place in the basic apiary in Cornetu commune, Moara Domnească Didactic Farm, Scurtu Mare commune, Teleorman county and Ștefan cel Mare commune in Călărași county.

In the experiments initiated with buckwheat culture (*Fagopyrum esculentum* L.), the results of which are presented in Table 1, the establishment of plant cultures was carried out in July 2023, for the purpose of testing for cover crops, and for this reason the determined values are different from the cultivation conditions of a main crop.

Plots sown with buckwheat provided the highest floral covers compared to the other crops studied. This suggests that buckwheat may be an excellent option for attracting pollinators due to its high flower density.

So, for mounth August and September of the year 2023 for the buckwheat crop (*Fagopyrum esculentum* L.), a production in the amount of approx. 40 kg/ha-honey, this value being located very close to the one in the specialized literature for buckwheat, through the average results recorded in the 3 series of determinations of honey collection/evaluation of honey potential (kg/ha), carried out at the 4 farms of the project.

Table 1. The values calculated in BRIEF between the hours of 9-12.30-16.30 at the level of the samples collected from the buckwheat crop, for the estimation of the honeydew harvest/evaluation of the honeydew potential (kg/ha) on Average duration of flowering = 26 days from 14 September 2023

BUCKWHEAT SERIES	Average number of flowers/ha	Glycemic index	The entire flowering period	Daily honeydew potential	Honeybee/culture potential
		(mg/flower)	(number of days)	(kg/ha/days)	(kg/ha)
SERIES 1 (9.00 a.m. (14.09.2023, Scurtu Mare)	475,200,000	0.0028	26	1.6333	42.4650
SERIES 2 (12.30 p.m. (14.09.2023, Scurtu Mare)	475,200,000	0.0029	26	1.7084	44.4177
SERIES 3 (4.30pm (14.09.2023, Scurtu Mare)	475,200,000	0.0011	26	0.6383	16.5956
Average values Scurtu Mare		0.0023		1.3267	34.4928

(with variation limits between 230,400,000 and 720,000,000)

For the months of August of the year 2023 for the white mustard crop (*Sinapis alba* L.), a production in the amount of approx. 32 kg/ha-honey, a quantity of nectar of 0.03-0.1 mg/flower, this value being very close to the one in the specialized literature for white mustard (*Sinapis alba* L.), through the average results recorded at the 3 series of determinations of honey which are presented in Table 2. The collection/evaluation of honey potential (kg/ha), are carried out at the 2 farms of the project.

Table 2. Values calculated in Ștefan cel Mare for the 9-12.30-17 hours interval, for the samples collected from the white mustard crop (*Sinapis alba* L.), for the estimation of the honey collection/evaluation of the honey potential (kg/ha) on Average duration of flowering = 26 days from 05 August 2023

WHITE MUSTARD SERIES	Average number of flowers / ha	Glycemic index	The entire flowering period	Daily honeydew potential	Honeybee/culture potential
		(mg/flowers)	(number of days)	(kg/ha/days)	(kg/ha)
SERIES 1 (9.00 a.m. (15.09.2023, Ștefan cel Mare)	475,200,000	0.0027	26	1.5939	41.4403
SERIES 2 (12.30 p.m. (15.09.2023, Ștefan cel Mare)	475,200,000	0.0019	26	1.1001	28.6031
SERIES 3 (4.30pm (15.09.2023, Ștefan cel Mare)	475,200,000	0.0016	26	0.9537	24.7958
Average values Ștefan cel Mare		0.0021		1.2159	31.6131

(with variation limits between 230,400,000 and 720,000,000)

In 2022, soil analyzes were carried out in the S.C. ALCHIMEX laboratory in Herești, Giurgiu county. The amount of humus determined from the Cornetu apiary was 2.70% (Figure 1).

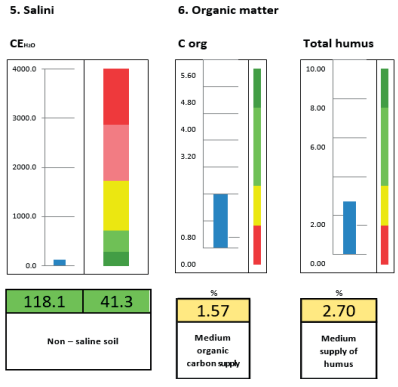

Figure 1. Soil analysis bulletin 2022, Cornetu, Ilfov county

The 2023 cover crop with humus-rich soil benefited from very good nutritional conditions. The analyzes were repeated in 2024, in March, the amount of humus increased to 4.27%. (Figure 2).

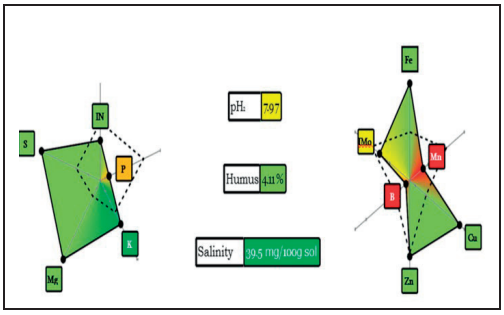

Figure 2. Soil analysis bulletin 2024, Cornetu, Ilfov county

The 2023 cover crop with humus-rich soil benefited from very good nutritional conditions. The amount of humus in the course of 2 years of research has doubled through the use of technologies for incorporating the plant mass resulting from the cultivation of "cover-crops" plants, proving indicators of soil fertility and the formation in the soil of humus mineralization (its destruction), a process through which it reproduces varying amounts of nutrients to the soil.

CONCLUSIONS

Due to the severe drought in the last 3 years on the territory of Romania, the local honey base has been seriously affected.

This research helps the specific area of beekeeping to prevent effective economic measures. Thus, the study carried out during the 3 years had the role of extending the honey and pollen harvesting period and supporting the beekeeping sector after the spring and summer periods, by increasing the values of the hive products, by using agricultural crops beneficial to bees and pollinators in accordance with the agro-environmental conditions, as well as increasing the annual production of honey and pollen.

Thus, from the observations made, it was found that bees visit mustard flowers intensively, especially in the morning when nectar secretion is abundant. Throughout September and until October 18 of the year 2023, the bees had a source of collecting nectar and pollen.

Results obtained within the framework of the research thesis and the development of the AGROAPIS project have resulted in the creation of a database accessible for free online https://agroapis.polenizare.ro. by using the APIA database, where farmers report all the agricultural crops established in the current year, it is possible to see in real time all the honey-bearing agricultural crops from the entire territory of Romania, evaluating the honey-bearing potential of the location of any apiary.

The information found on the online database will be updated periodically and will thus provide beekeepers with a method to protect their bees and the production of bee products by avoiding the loss caused by bee colonies with reduced health that are at risk of not surviving. the winters.

Reducing the expenses generated by the need to use artificial sources of food to maintain their own beehive, will motivate farmers to inform themselves in order to obtain economically advantageous methods of increasing crop productivity.

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THEORETICAL EXPLORING OF ANTIGENOTOXIC PLANT INGREDIENTS WITH ANTIAGING PROPERTIES

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Abstract

Aging could be considered a chronic disorder that comes with more devastating traits than we could imagine or see, both at cellular and molecular levels. The paper aimed to summarize the molecular mechanisms of ageing and to emphasize some plants which have antigenotoxic properties that can be linked to antiaging features. In cosmeceutical manufacturing, using natural and organic ingredients is the best way to maintain and offer skin rejuvenation qualities. Moreover, the synergy between plant phytochemicals can enhance the overall antiaging effect, addressing multiple aspects of skin aging simultaneously. There are tremendous antigenotoxic plants provided by nature, while few of them have antiaging features, such as: Trichilia emetica, Psoralea corylifolia, Ximenia americana, Schinziophyton rautanenii, and Hypericum perforatum. Thus, the review outlined their benefits in order to reveal their therapeutic potential in the cosmetic industry.

Key words*: aging and antigenotoxicity ingredients, Mafura butter, Ximenia oil, Manketti oil, vegan cosmeceuticals.*

INTRODUCTION

The whole human body bears the traces of time by natural aging. However, in two special ways, human skin suffers particularly aging trials over time: one by chronological or intrinsic/internal causes and another by extrinsic/external agents. The internal elements of skin aging are provoked by: (a) genomic unsteadiness; (b) cellular caducity and (c) decrease of telomers. The extrinsic aging is expedite continually exposing to environmental stresses and damages such as: (a) UV radiation; (b) pollution; (c) extreme temperature differences; (d) tobacco smoke and alcohol consumption.

Both of them have several serious consequences: (a) narrowing of both the epidermal and dermal layers by losing collagen and crashing of collagen fibrils; (b) increasing fragility; (c) undermining vascular support; (d) retarding injury healing and (e) augmenting vulnerability to cancer development, by appearing inflammatory environment. Hence, epidermal caducity exerts - beyond trivial cosmetic and aesthetic concern - an obvious bestowing to this ″chronic medical disorder″ named age-related skin issue(Quan, 2023).

Skin aging is a complex biological phenomenon that includes DNA, RNA, and protein damage, structural deterioration, and accumulation of reactive oxygen species at the cellular level. Going from basic molecular pathology and cell biology theory, which establish that there is an intrinsic connection between form and function, the skin morphology is the most important. For instance, diabetes patients' feet are suffered by thickening of the epidermis thus, increasing stiffness and being less able to distort, but more exposed to risk of cleaving and ulceration. (Lechner & al., 2019) In other words, changed form or structure of the cell leads to a modified function of it.

Mechanical skin traits, respectively adhesion strength of the dermo-epidermal junction could be improved by epidermal hydration (El Genedy-Kalyoncu et al., 2022). For centuries, human have used plant bioactive molecules to moisturize, smooth, and nourish their skin of

any age. Plant lipids are made up by 95% of mono and diglycerides and free saturated or unsaturated fatty acids and 5% of different substances in varying proportions: phosphorlipids, glycolipids, sphingolipids, waxes (saponifiable fraction) and hydrocarbons like squalene, pigments in the form of carotenoids and chlorophyll, vitamin E, phytosterols, polyphenols, and triterpene alcohols (non-saponifiable fraction). The latter one, triterpene esters are not only the principally reliable in anti-inflammatory, antitumor and antioxidant activities of plant lipids, but give them their unicity and specificity. On the other hand, essential oils are commonly volatile, concentrated, and vigorous, being claimed to have rejuvenated features and tremendous health benefits (Sarkar et al., 2017).

The goal of this theoretical investigation is to summarize the available molecular information about aging in humans and to emphasize the effects of selected plants against this unwanted ″chronical disease″ in order to propose a reliable cosmetic treatment.

SKIN AGING

Epidermal aging

Epidermal is composed of five sheets; basal, spinosum, granulosum, lucidum, and corneum. The basal sheet, also known as rete ridges or germinative area, is separated from the dermis by the basement membrane. The cells in this layer are cuboidal to columnar, mitotically active stem cells that constantly fabricate keratinocytes, but there are also melanocytes. (Yousef et al., 2024) Epithelial cells are fixed within basement mem-brane by multiprotein structures named hemidesmosomes, which are essential for final transformation of keratinocytes and their relocation through injury curing and carcinoma incursion (Walko et al., 2015).

Hence, in the rete ridges area there are interfollicular epidermis (IFE), melanocyte stem cells (McSCs) and hair follicle stem cells (HFSCs) that contribute permanently to the regenerating epithelium and are important in wound regeneration. Epiderma-dermal attachment requires type XVII collagen (COL17), a structural component of hemidesmosomes that have revealed more functions in homeostasis of the skin stem cells. COL17A1 encodes alpha chain of COL17, the protein produced by keratinocytes Thus, the decreasing of COL17A1 expression will affect adhesion of stem cells to the basement membrane, reducing keratinocytes and melanocytes turnover, leading to thinning epidermal layer and diminished hair follicle stem cells (HFSCs). All of them are the initial morphological traits of aging skin and, respectively, greying and losing hair. Moreover, a reduction in the expression of COL17A1 may also infirm the bond between the epidermis and dermis, conducting to other aging signs: skin fragility, blistering, retarding injuries healing, wrinkles emergence, and loosened skin (Quan, 2023).

In connection with narrowing of epidermal layer, Lintzeri et al. (2022) have published a systematic meta-analysis with epidermal thickness within 37 areas of human body. They have concluded that the epidermis is thinning by age with a mean of almost 20%. The most diminished part is cheek with 27.14%, while for lower leg is 14.10%, as is showed in Table 1.

Table 1. Diminishing of the epidermal thickness by age, adapted after (Linzeri & al., 2022)

No.	Skin area	Age	Thickness (μm)	Narrowing (μm)	Narrowing (%)	Epidermal reduction mean (%)
1.	forehead	23	83	20	24.10	**19.16**
		66	63			
2.	cheek	26	70	19	27.14	
		58	51			
3.	back	22	78	6	7.69	
		55	72			
4.	inner upper arm	23	89	12	13.48	
		63	77			
5.	volar forearm	27	81	17	20.99	
		60	64			
6.	dorsal forearm	24	79	11	13.92	
		60	68			
7.	abdomen	24	83	22	26.51	
		59	61			
8.	gluteal	29	88	17	19.32	
		59	71			

No.	Skin area	Age	Thickness (µm)	Narrowing (µm)	Narrowing (%)	Epidermal reduction mean (%)
9.	thigh	24	78	19	24.36	**19.16**
		59	59			
10.	lower leg	23	78	11	14.10	
		66	67			

Dermal aging

The dermis layer is composed by closely intertwined clusters of collagen wires forming an elaborate spatial array named extracellular matrix (ECM) where are dwelling dermal fibroblasts, that produce these collagen fibrils. Time leaves its marks in this environment as well, by showing adulteration in both collagen threads and fibroblasts, such as: disruption and disorder of collagen wires and its production. Still, in the same time an inflammatory medium appears, known as ″inflammaging″ (Quan, 2023).

All of these are triggers for similar chronic disease signs: heightened brittleness, deteriorated blood vessels, disrupted injury repairing and boosting tumor-like mutations. From molecular point of view there are four mainly incidents clearly reviewed by Quan et al., 2023:

(1) Metalloproteinases (MMPs) activity which causes collagen fragmentation. Human MMPs form a 20-member proteinase class that is involved in natural and pathological processes related to ECM degradation. MMPs are usually extremely present in tumor environment being implicated in metastasis by disrupting the balance between growth and antigrowth molecular signals and ECM degradation. In normal condition and young skin, MMP1 known as collagenase 1 and synthesized by dermal fibroblasts, is at the lowest level possible, by tearing the initial collagen threads. However, in aging skin, MMPs having an increased level are responsible for permanent disruption of dermal structural and mechanical part, encountering - beyond dermal thinning, uplifted fragility and retarding wound healing - jeopardy of carcinoma emergence.

(2) Deterioration of the transforming growth factor-beta (TGF-β) signalling due to the dermal thinning and conducting to decreased collagen production. Being a multifunctional cytokine TGF-β is involved in various cellular activities, including the control of cell growth and differentiation and regulates production of collagen and elastin from ECM. Once dermal fibroblasts encounter disruption by dermal thinning, they present a malformation by diminishing the expression of the TGF-β Type II receptor (TβRII), which ends up damaging TGF-β signalling by inhibiting collagen production, increasing the activity of MMPs, and facilitating inflammaging emergence. Hence, the skin loses elasticity, firmness, resilience and expresses weakening, wrinkles, fine lines, appearance inflammations and all aging signs.

(3) The impact of elevated cysteine-rich protein 61 (CCN1/CYR61) on the aging process. CCN1 is one of the six proteins family which controls cell adhesion and cell migration - chemotaxis, the production of inflammatory mediators, cell-matrix interactions, the synthesis of ECM proteins, and wound responses. Its raised level in caducity skin brings elevated secretion of proteases, growth factors, chemokines and cytokines. Accordingly, as it was already observed in senescence, there are lofty expression of pro-inflammatory markers like interleukin-6 (IL-6), tumor necrosis factor-alpha (TNF-α), Activator Protein 1 (AP-1), leading to a damaged and inflammatory microenvironment within dermis.

(4) Decline in autophagic activity. As in every cell, autophagy is an essential duty to maintaining homeostasis, especially in fibroblasts. In senescent dermal fibroblasts, undermined autophagy leads to the accumulation of lipofuscin, which causes age-related pigmentation irregularities (Quan, 2023).

Based on these molecular events, the aging effects could be viewed as mutagenic ones, hence ageing is not only a fancy beauty concept, even it could be considerate as a serious chronical disorder with similar tumors traits. While genotoxicity is a general term, which describes the capacity of noxious matter to induce DNA impairment, mutagenicity accurately does these DNA alterations in quantity and composition. Hence, genotoxic substances include mutagenic ones. In this context, any agent that reduce the DNA alterations can be is named antigenotoxic (Izquierdo-Vega et al., 2017).

There are tremendous antigenotoxic plants provided by nature but few of them have natural antiaging compounds that can reduce the DNA damages and stimulate the skin cell growth, including collagen production.

ANTIGENOTOXIC INGREDIENTS

Mafura butter

Trichilia emetica commonly known as the Natal mahogany is an evergreen tree belonging *Meliaceae* family and widely distributed growing naturally throughout sub-Saharan Africa from KwaZulu-Natal in the South, through Swaziland, Mpumalanga and Limpopo Provinces (South Africa), into Zimbabwe and northwards into Cameroon, Sudan and Uganda. *T. emetica* has multipurpose properties such as antibacterial, antiviral, antifungal, anti-inflammatory, antischistosomal, antiplasmodial, anticonvulsant, antioxidant, antitrypanosomal, antitussive, antimutagenic and hepatoprotective (Komane et al., 2011).

The main taxonomic markers of *Meliaceae* family are limonoids from *T. emetica.* The most important limonoids isolated from *T. emetica* are trichilin A, B, C, D, E, dregeana 4, trichilia substance Tr-B, nymania 1, rohituka 5 and seco-A protolimonoid, manifesting DNA damage repair, antifeedant qualities and obstructing insect extension. Interestingly, the extracts of *Trichilia emetica* with dichloromethane and methanol have displayed weak antimutagenic activities, unlike *Helichrysum* extracts, which have unveiled high antimutagenic properties compared to the positive control. However, an isolated sesqui-terpenoid from *Trichilia emetica*, named Kurubasch aldehyde - being tested on different cell lines - reduced the expansion of the breast tumor cells MCF-7 with IC50 = 78 μM and inhibited the augmentation of sarcoma cells S180 by being tested on murine cases with IC50 = 7.4 μM. Moreover, polysaccharides isolated from this plant were traditionally used in wound healing as they can stimulate the skin cells growth (Komane et al., 2011).

Bakuchiol

Psoralea corylifolia (synonym *Cullen corylifolium*) is an herbaceous plant from the *Fabaceae* family that contains Bakuchiol (BAK) 1-(4-hydroxyphenyl)-3,7-dimethyl-3-vinyl-1,6-octadiene, a natural meroterpenoid. This terpenoid is present in the plant cold-pressed oil obtained from seeds in a percentage of 6.24%. BAK presents powerful biological features such as antiaging, anti-inflammatory, and antibacterial and can be a cosmetic ingredient as a natural alternative for retinoids without side effects or irritation. Because they contain a 4-hydroxystyryl moiety, both bakuchiol and resveratrol have analogous structural. Moreover, the pyrone, chromene, and quinazoline derivatives linked to the styryl moiety proved strong biological features useful in the cosmetic industry. Having a similar pharmacophore, the bakuchiol may express similar traits as resveratrol (Barna et al., 2023). BAK was found to have an effect comparable with retinol, proved by a randomized, double-blind trial performed in 2018 for 12 weeks. This clinical study with 44 volunteers demonstrated that BAK is comparable with retinol in its ability to improve photoaging effects while showing better skin tolerance. The researchers noted fewer wrinkles, increased skin firmness, and lower hyperpigmentation (Dhaliwal et al., 2018).

Bakuchiol inhibits oxidative stress by preventing mitochondrial lipid peroxidation and protecting other enzymes from oxidative stress. Being an electron-donating group, it is supposed to diminish its phenolic moiety's bond dissociation enthalpy, enhancing its antioxidant activity (Krishna et al., 2022). Moreover, BAK has been proved to inhibit the cell proliferation of prostate cancer cells (Miao et al., 2018).

Ximenia oil

Ximenia americana is a shrub or small tree up to 7 meters from the *Olacaceae* family, found in the tropics under popular name yellow plum. By aqueous decoction extraction, several phytochemicals were found, such as alkaloids, flavonoids, anthraquinones, cardiac glycosides, saponins, tannins, and terpenoids which exhibited high cytotoxicity against MCF-7, the breast tumor cell line (Sawadogo et al., 2012).

One lectin extracted from *X. americana* kernels that revealed antineoplastic activity is riproximin. (Horrix & al., 2010) Riproximin is a cytotoxic type II ribosome inactivating protein with high selectivity for colorectal and pancreatic tumor cell lines (Adwan et al., 2014).

In traditional medicine, the roots of *X. americana* are used to treat different skin problems, while the bark powder or decoction form can treat skin ulcers and burning. Moreover, the oil from seeds is marketed in cosmetics and useful for a variety of purposes: emollients, conditioners, skin softeners, body and hair oils, as well as ingredients in soaps, lipsticks, and lubricants. Xymelys 45 is a drug that protect ultrasensitive skin and contains bark extract from ximenia (Medeiros & Medeiros, 2018)

Manketti tree oil

Schinziophyton rautanenii named mongongo, is a large spreading tree species within *Euphorbiaceae* family, that grows in the semi-arid Kalahari region of southern Africa, reaching 15 m high.

It makes fruits with a sweet and edible pulp surrounded by a hard nut and holds 1 or 2 seeds or kernels. It is an essential food for elephants and mongongo nuts have been consumed by local communities for centuries. Local people from Zambia, Zimbabwe and elsewhere in southern Africa extract the oil from the kernels for cooking and cosmetics as a body rob (Chidumayo, 2016).

From these nuts, the comestible oil is expelled and holds beyond the linoleic, oleic, and linolenic acid, a special fatty acid, namely, alpha-eleostearic acid (α-ESA), with great value potential in both health and cosmetics (Cheikhyoussef et al., 2019). Natural phytochemicals, such as α-ESA provides excellent protection to the skin from damage caused by UV rays. Manketti oil contains high level of vitamin E and polyunsaturated fatty acids that are effective not only for moisturizing the skin but also for restructuring and rejuvenating the epidermis. Moreover, α-ESA can enter the central nervous system (CNS) through the brain-blood barrier, which is conducive to this axis of CNS pathology-CDGSH iron-sulfur domain 2 (CISD2) - nuclear factor κB (NFκB). This phytochemical is exerting neuro-modulatory effects on CISD2 elevation and can be considered to be applied in CNS injuries and diseases (Kung & Lin, 2021).

The melanoma cell line UACC-62 displayed sensitivity towards the aqueous bark and root extracts of *S. rautanenii*, especially the organic aqueous root extract that displayed the highest IC50 values of 315.5 µg/ml and 444.8 µg/ml against the human fetal lung fibroblast cell (Dushimemaria, 2014).

St. John's wort extract

Hypericum perforatum is the most popular medicinal herb from the family *Hypericaceae*. Even if it is a considered a noxious weed, *Hypericum perforatum* holds numerous biologically active components, such as naphthodianthrone derivatives (e.g., hypericin), phloroglucinol derivatives (e.g., hyperforin), flavonoids, procyanidins, tannins, essential oils, phenylpropanoids, xanthones, and other hydro soluble compounds. Hyperforin (HF) is one of the major constituents of St. John's wort, a substance that is collected in its leaves and flowers and composed of a phloroglucinol skeleton with lipophilic isoprene chains, being responsible for antimutagenic effect. HR was analyzed by Ames test using *Salmonella typhi-murium* (TA97, TA98, and TA100) bacterial strains as standard and showed the reduction of gene mutations. During the evaluation ″*in vitro* mammalian chromosome aberration test″, HF had anticlastogenic impact on both tumor cell line HepG2 and normal cell line VH10 (Imreova et al., 2017).

H. perforatum extract showed collagenase, elastase and hyaluronidase inhibitory activities and can be used together with *H. calycinum* extract as potential agents for anti-aging and skin-whitening purposes (ERSOY, 2019). Moreover, Wölfle et al. (2014) proved that hyperforin helps reduce transepidermal water loss by differentiating keratinocytes and protecting the skin barrier.

CONCLUSIONS

Aging is associated with the decline of cellular function and the accumulation of cellular damage. Therefore, exploring antigenotoxic plant ingredients with antiaging properties is valuable for protecting DNA, offering natural alternatives, promoting healthy cellular function, reducing oxidative stress and inflammation, preventing age-related diseases, and supporting sustainable practices. By identifying plant bioactive molecules with antigenotoxic properties, it can help find natural

ways to protect DNA and enhance its repair mechanisms, potentially slowing the aging process and reducing the risk of genetic mutations. Moreover, phytochemicals often work synergistically to enhance each other's effects and provide other benefits, such as antioxidant activity, anti-inflammatory effects, and promotion of collagen synthesis.

Exploring antigenotoxic plant ingredients with antiaging properties opens new ways for research and development in pharmaceuticals, cosmetics, and nutraceuticals. *Trichilia emetica, Psoralea corylifolia, Ximenia americana, Schinziophyton rautanenii,* and *Hypericum perforatum* are potential candidates with natural antiaging compounds that can reduce the DNA damages and stimulate the skin cell growth, including collagen production. Their exploration can lead to the discovery of novel compounds with unique mechanisms of action, fostering innovation and the development of next-generation products. Moreover, this research has the potential to improve human health, skincare, and the broader wellness industry by providing safer, more effective, and naturally derived solutions to combat aging.

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FOOD CONTAMINANTS INCIDENCE ON CEREAL VALUE CHAIN, A MINIREVIEW

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Abstract

In order to ensure effective protection of public health, it is mandatory that the level of food contaminants present in the cereal value chain to be below the maximum level established by the regulations in force, and not to be placed on the market or used as ingredients or mixed with other food products if they don't comply with these limits. Cereals can be contaminated through exposure to polluted environment, mycotoxins and heavy metals being a significant source of contamination. These contaminants are considered a public health concern, having an impact on the food security and the economy of many countries, their monitoring being important. There are certain situations in which food is contaminated with mycotoxins and heavy metals, but which can hardly be controlled at all or is only poorly controlled. Therefore, the aim of this review is to offer an update of the incidence of these contaminants on the cereal value chain, the accumulation in the cereal and cereal-based products, the mitigation of these contaminants and the impact on the human health.

Key words*: cereals, contamination, food safety, heavy metals, mycotoxins.*

INTRODUCTION

Food safety cannot become a real fact unless it constitutes a responsibility of all those involved in the food field, from professionals to consumers. Along the food chain, various procedures and control mechanisms are implemented, which ensure that the food that reaches the consumer's table is edible and that the risk of contamination is reduced to a minimum, so that the population is healthier as a result of the benefits brought by safe and healthy food.

Cereals are currently the primary source of carbohydrates in the human diet worldwide. The Food and Agriculture Organization (FAO) forecasted that world grain use in 2021/2022 will reach a record level of 2809 million tons. The FAO forecast for total wheat utilization is 777 million tons, which was 2.4% (18.5 million tons) higher than in 2020/2021 (FAO, 2021). Only three of these (rice, maize and wheat) provide about 60 percent of the world's food energy intake (FAO, 2017).

Heavy metal pollution became a global problem, degrading environment and posing a serious threat for the human health. The underlying causes of this persistent problem seem to be the increased rate of urbanization, land use changes and industrialization, especially in densely populated countries and under developed ones (Zhang et al., 2019).

Food contamination with heavy metals, including cadmium (Cd), lead (Pb) and arsenic (As), is frequent in the agricultural regions and is a major concern worldwide (Naseri et al., 2015; Wang et al., 2021). Many studies have shown that even low levels of exposure to heavy metals could lead to serious health problems (Tchounwou, 2003; Khan et al., 2015; Abtahi et al., 2017; Al-Saleh et al., 2017). Also, by eating contaminated food with heavy metals for a long period of time could lead to many types of diseases, such as cancer, leukaemia, genetic toxicity and so on (Nejabat et al., 2017). Worldwide, about 600 million people are affected annually by contaminated food with heavy metals (World Health Organization, 2015).

Mycotoxins are considered to be part of the most significant food contaminants in terms of impact on public health, food security and the economy of many countries. They are found in a wide variety of plant products before, during and after harvest. Mycotoxins naturally present in various food products constitute a serious

food safety problem, especially in certain regions of the globe where climatic conditions are favorable to molding or agricultural standards are poor. Due to the toxic action on the human and animal body (De Ruyck et al., 2015), they negatively influence the quality of the food; in addition, the economic losses they generate are not negligible. Currently there are 300-400 mycotoxins. Among them, aflatoxins, ochratoxins, trichothecenes, zearalenone and fumonisins are the most studied due to their effects on the human and animal health and their special agro-economic importance.

The main genera of fungi producing mycotoxins are *Aspergillus*, *Penicillium*, *Fusarium, Alternaria* and *Claviceps*. A certain fungus can produce several mycotoxins and a given mycotoxin can be produced by several fungi; this fact can lead to the synergism phenomena and potentiation of the toxic action.

Chemical origin of mycotoxins is very diverse, some derives from amino acids (ergot alkaloids, aspartic acid, acid cyclopiazolic, slaframine, glioxin, roquefortin, sporodesin), some are polyketoacids (aflatoxins, ochratoxin, patulin, citrinin, sterigmatocystin, zeararlenona), and others are their terpens derivatives (fusar enone, deoxynivalenol varidin, T-2 toxin). Mycotoxins are formed at the end of the exponential phases and at the beginning of stagnation phases of mould growth.

RESULTS AND DISCUSSIONS

Heavy metal contamination of cereals and derivative products

Contamination of crop plants may occur either through contact with contaminated soils, or through air pollution. The soil, as the fundamental support of food cultures, can be contaminated with heavy metals from different sources, such as irrigation with waste water, industrial effluents, mining activities and poor agricultural practices, as well as incorrect waste disposal. Heavy metal levels in soil can be raised also by using certain types of fertilizers, including organic manure, pesticides and herbicides which contain such compounds.

Air can also be a source of contamination representing a vehicular path for heavy metals and their deposition on the soil or plants (eg. lead emission from automobiles). Absorption of heavy metals by plants from contaminated areas depends not only on soil properties, such as temperature, moisture content, organic matter, pH and availability nutrients, but also on plant species.

Kumar et al. (2022) investigated the level of contamination with heavy metals and metalloids in irrigation water, soil and cereal plants in the area of Lucknow city from India. The highest concentration of these compounds was found in irrigation water, while in the majority of soils the levels were close to the maximum allowed concentration. Concentration levels significantly higher than the maximum allowed limits were found in the aerial parts of the studied plants.

A study conducted by Ali & Al-Qahtani (2012) showed exceeding limits values for some heavy metals' concentration in various vegetables, including, cereals, in four areas from Saudi Arabia. The highest amounts of heavy metals were determined in leafy vegetables, mostly for those collected from middle and eastern districts.

Pirsaheb et al. (2021) conducted a study to analyse the presence of chromium (Cr), cadmium (Cd), lead (Pb), nickel (Ni), copper (Cu), zinc (Zn), mercury (Hg) and arsenic (As) in wheat, rice, peas, split peas, lentil, corn and bean, which are high consumed cereals, from the market of Kermanshah city from Iran. The results of the study showed that the overall concentration was of 27.3 μg/kg for Cr, 6.85 μg/kg for Cd, 87.7 μg/kg for Pb, 21.2 μg/kg for Ni, 147 μg/kg for Cu, 459 μg/kg for Zn, 2.03 μg/kg for Hg and 1.80 μg/kg for As, all values reported to the dry weight.

Different cereal grains from Jos market (Nigeria) were analysed by Ikem et al. (2023) in terms of elemental composition and the results showed below allowed limit values for daily (for example Zn, Cu, etc.) and weekly intakes (for example Hg, Pb, Cd, etc.). Although the hazard index showed no hazard for adult consumers, the cumulative cancer risk (because of Pb, Cr, As and Ni) presented higher values that the established standard (10^{-5}).

A study performed by Wei & Cen (2020) aimed at determining heavy metal contamination and dietary exposure in different types of legumes, cereals and their products

from 16 districts in Beijing, China. The results of the study registered daily exposure doses of 0.0772 µg kg d^{-1} for Cd, 0.0051 µg kg d^{-1} for Cr, 0.3350 µg kg d^{-1} for Pb, 0.0119 mg kg d^{-1} for Cu, 0.0417 mg kg d^{-1} for Fe, 0.0367 mg kg d^{-1} for Mn and 0.0505 mg kg d^{-1} for Zn. In this study, the hazard index values showed that long term consumption could lead to non-carcinogenic adverse effects for the studied area residents.

A mining area from Nanyang Basin (Henan Province, China) was studied from heavy metal presence in soils and crops by Dong et al. (2023). Varying degrees of pollution were determined in crop roots. In maize fields, heavy metal concentration was higher compared to wheat fields and Cd gives the highest pollution in both fields.

Singh et al. (2022) investigated heavy metal concentration in vegetables and crops from an area in India where untreated wastewater has been discharged for a long period of time. The results showed exceeded safety limits in *Beta vulgaris* L. (5.35 µg/g DW for Cd and 58.41 µg/g DW for Zn) and *Triticum aestivum* L. (16.02 µg/g DW for Cr, 27.97 µg/g DW for Cu and 40.74 µg/g DW for Ni), also having the highest values.

Absorption and translocation of heavy metals in cereals

The degree of absorption of heavy metals varies between different cereal species, some crops showing a greater accumulation than others. This accumulation may have serious implications on consumers health because extended exposure to high levels of these toxic metals can lead to different health problems. The current maximum levels for heavy metals and mycotoxins in foods like cereals and derived products, are regulated according to Commission Regulation (EU) 2023/915.

The highest lead concentrations were identified in a rice sample in Thailand at 0.419 mg/kg, followed by Iran with a rice sample at 0.297 mg/kg and a sample of infant rice cereal from Spain, having a lead concentration of 0.116 mg/kg. Regarding cadmium, the highest concentration was identified in a rice sample from India with a concentration of 0.190 mg/kg, followed by a rice sample from Malaysia with a cadmium concentration of 0.160 mg/kg. For Arsenic, the highest concentration was identified in a rice sample from Nepal with a concentration of 0.180 mg/kg.

Mycotoxin contamination of cereals and derived products

Cereals along with their producs, nuts, fruits and also feed, present high risk of mycotoxin contamination, such as deoxynivalenol (DON), aflatoxin B1 (AFB1), nivalenol (NIV), zearalenone (ZEN), fumonisin Bs (FBs), ochratoxin A (OTA) and T-2 toxins, which are secondary metabolites produced by some fungi (Terzi et al., 2014; Sirbu et al., 2020; Khodaei et al., 2021; Tan et al., 2023), which are further presented.

Aspergillus genus produces aflatoxins: B1, B2, G1, G2, M1, M2, ochratoxin A, sterigmatocystins and cyclopiazonic acid.

The genus *Penicillium* produces patulin, citrinin, penitrem A, rubratoxin and other substances as well known as ochratoxin A and cyclopiazonic acid.

The genus *Fusarium* produces trichothecins and deoxynivalenol (DON, vomitoxin), 3-acetyldioxynivalenol, 15-acetyldioxynivalenol, nivalenol, zearalenone, fuminizins and moniliformin as well as other potentially toxic substances and unknown toxic substances.

The genus *Alternaria* produces a number of biologically active compounds such as tenuazoic acid, ethylmetal alternariol.

The exposure to mycotoxins can occur under different situations, such as food, working area or various environments. However, the ingestion of contaminated food (especially cereal and nut based foods) represents the main cause for mycotoxicosis (Karlovsky et al., 2016). The types and levels of mycotoxins in human diets vary depending on different factors such as geographic location, type and amount of grain and grain based products, spices and other ingredients, but also cooking or processing methods.

For example, in Africa and South America the main cereal in the human diet is maize, in North America and Europe wheat is preferred and rice in Asia. Regarding those cereals, the main mycotoxins present are aflatoxin, ochratoxin and deoxynivalenol. The level of mycotoxins can be reduced during food or feed

processing and/or cooking, resulting products that are relatively safe to eat (Paterson et al., 2010).

The contamination with mycotoxins can happen both in the field and during storage (Streit et al., 2013; Smeu et al., 2017). The climatic conditions, especially tropical and subtropical ones, are thought to be the main factors for aflatoxin occurence, above all in developing countries, where there is insufficient food and the conditions for storage are poor (Paterson et al., 2010). Aflatoxins (AF), deoxynivalenol (DON), fumonisins (FUM), ochratoxin A (OTA) represents the main identified mycotoxins, especially in maize grains (Streit et al., 2013).

The Food and Agriculture Organization of the United Nations (FAO) estimated that before 1985, about 25% of the world's food will have in its composition some form of mycotoxins (CAST. Mycotoxins, USA, 1989). An extensive review of the literature found the global prevalence of mycotoxins in food crops to be as high as 60-80%, although data vary widely, depending on a lot of factors like mycotoxin type, methods of analysis and results reporting (Eskola et al., 2020). According to this survey report, at a global level, the most frequent determined mycotoxins are deoxynivalenol (65%), fumonisins (64%) and zearalenone (48%) in the grain harvested in 2020 (Biomin World Mycotoxin Survey, 2020). Higher contents of AFB1 and OTA are often present in cereals produced in developing countries, which also have higher levels of mycotoxins, generally above regulatory limits. Some of these mycotoxins can be taken into cooked or processed food and food products when using highly contaminated cereals as ingredients, mainly due to the fact that common processing and cooking methods like sorting, cleaning, cutting, grinding, baking, etc.) can't eliminate them; however, these methods could significantly reduce their content (Karlovsky et al., 2016).

A study conducted by Andrade et al. (2020) focused on determining the probabilistic dietary risk in respect to mycotoxins for the Brazilian population. The results showed that for the general population and teenagers, safe limits were exceeded in respect to chronic exposure for fumonisins and DON.

Huang et al. (2022) performed a screening for mycotoxins in different stored cereals from Shanghai, China. The results showed the presence of 46 mycotoxins and their metabolites in 138 stored samples. Some of them were reported in cereals, fact that present a potential health risk for both humans and animals.

Quinoa and kaniwa (pseudocereals) were analysed by Ramos-Diaz et al. (2021) in comparison with cereals (wheat, oat, barley) in two areas, namely North Europe (Latvia, Finland, Denmark) and South America (Peru, Bolivia). The aim of the study was to determine mycotoxin contamination in these products. The results showed lower mycotoxin contamination in South America for the pseudocereals and higher in cereals compared to North European samples. The identified analytes presented various levels and were mainly produced by fungal strains belonging to *Aspergillus* spp., *Fusarium* spp. and *Penicillium* spp.

The toxic effects of mycotoxins can affect all humans, but infants are more susceptible to these effects, mainly due to their different physiology (lower body weight, high metabolic rate, etc.) (Piacentini et al., 2019). Ji et al. (2022) screened 820 samples of cereal-based foods for infants in China and the results showed the presence of deoxynivalenol, enniatin A, tenuazonic acid, enniatin B, enniatin B1, enniatin A1, zearalenone, alternariol, alternariol nonomethyl ether, ochratoxin A, fumonisin B1 and fumonisin B2 in low levels in the tested samples. A greater variety of mycotoxins was determined in wheat based products compared to rice based products.

CONCLUSIONS

Heavy metal contamination of cereals is a great challenge nowadays, which is extended to the entire world, having negative effects on human health and not only. Therefore, there is a need for technology development in order to remove heavy metals from food and feed, assuring this way the safety and security of all populations. Regarding mycotoxins exposure, it seems to be unavoidable. Mycotoxin contamination of grain will continue to be a major challenge for grain

producers as well as for food and feed industrials as grain can be contaminated with fungi both in the field and after harvest, especially during storage. Therefore, it is very important to implement mycotoxin control strategies before and after harvest to reduce the initial degree of contamination.

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THE RESULTS OF AN ACADEMIC EXERCISE OF USING *AI* AS AN TOOL FOR IDENTIFYING CERTAIN KEYWORDS IN USAMVB SCIENTIFIC ARTICLES

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Abstract

This article presents the results of an academic exercise aimed at selecting an AI-efficient working tool between AISA1 from TC1 and AISA2 from TC2 to identify LED's light, and plant keywords in scientific articles available on-line from publications of USAMV of Bucharest. Such a tool is necessary for students, teachers, and researchers who have scientific activity with implications in the use of LED's light to plants.
Each tool used three keyword search variants, namely: individual keywords, a succession of keywords, and an explicit request for identification (identifies in the scientific publications of USAMV of Bucharest the following keywords: LED's, light, and plants). The results obtained, for each of the three variants of keyword identification, indicate both the use of the AISA1 AI-based working tool from TC1 for quick identification of text-based information without identification by accurate academic citation of the source, as well as using the AISA2 AI-based tool from TC2 to quickly identify text information and sources as links that it must be accessed, studied and selected in established academic mode.
Both tools can provide information that through concatenation generates a basic image for the primary verification of the existence of LED's, light, and plant keywords, in scientific articles available on-line from publications of USAMV of Bucharest.

Key words*: artificial intelligence (AI), LED's, light, plants, scientific articles (issues).*

INTRODUCTION

The term Artificial Intelligence (AI), "is frequently applied to the project of developing systems endowed with the intellectual processes characteristic of humans, such as the ability to reason, discover meaning, generalize, or learn from past experience" (https://www.britannica.com/technology/artificial-intelligence).
AI is a part of computer science concentrated on creating systems capable of accomplishing tasks that typically require human intelligence (e.g., recognizing patterns, problem-solving, learning, understanding natural language, and making decisions). AI systems utilize algorithms and large data sets to identify patterns and make predictions or decisions without human intervention. There are various types of AI (AISA1, AISA2, etc.), ranging from narrow AI, designed for specific tasks, to general AI, which aims to understand and accomplish intellectual tasks that a human can (e.g., customer support, programming, language translation, education, creative writing, personal assistant, etc.).
AISA1 is "an AI language model created by TC1, designed to understand and generate human-like text based on the input I receive" and It's "purpose is to assist with a wide range of tasks, including answering questions, providing information, offering writing help, and engaging in conversations" (Source: Conversation with AISA1. Accessed at 10.05.2024).
AISA2, created by TC2 is "an AI companion. It can provide information, answer questions, and engage in conversation" and it "can also help with creative writing tasks, such as editing, rewriting, improving, translating, and optimizing content" (Source: Conversation with AISA2. Accessed at 10.05.2024).
Artificial Intelligence (AI) is one of the tools that the student, teacher, and scientific researcher use to identify useful information in the work they carry out. But, although in recent

years, information obtained through AI is accessible to almost anyone and anytime, when the information must be at a didactic or/and scientific level, it requires the use of AI that is based on professional-level sources.

One of the professional-level scientific sources accessible on-line consists of scientific articles.

The main types of scientific articles in the knowledge flow are reviews, research articles, and communications. However, the sources for these types of scientific articles are scientific publications made up both from volumes of scientific conferences and from specialized journals that publish using the peer-review system.

Amongst the scientific publications of the University of Agronomic Sciences and Veterinary Medicine of Bucharest (USAMV of Bucharest), containing scientific articles available on-line there are seven collections of scientific conferences specific to each of the seven faculties and a specialized journal that publishes using the peer-review system (https://agricultureforlife.usamv.ro/index.php/publications).

The aforementioned seven collections of scientific conferences are: Scientific Papers. Series A. Agronomy, Scientific Papers. Series B. Horticulture, Scientific Papers. Series D. Animal Science, Scientific works. Series C. Veterinary Medicine, Scientific Papers. Series E. Land Reclamation, Earth Observation & Surveying, Environmental Engineering; Scientific Bulletin. Series F. Biotechnologies and Scientific Papers Series Management, Economic Engineering in Agriculture and Rural Development, and they contain scientific articles previously presented within the framework of the International Conference "Agriculture for Life, Life for Agriculture" (https://agricultureforlife.usamv.ro/index.php/publications). Additionally, the relevant magazine that publishes using the peer-review system (AgroLife Scientific Journal) covers scientific issues about every field of activity of every seven faculties of USAMV of Bucharest and has two editions every year (https://agrolifejournal.usamv.ro/index.php/agrolife). In 2015, the United Nations (ONU), established as part of the 2030 Agenda for Sustainable Development, (https://sdgs.un.org/2030agenda), the 17 Sustainable Development Goals (e.g: end of hunger, achieve food security and improved nutrition and promote sustainable agriculture; sustainable cities and communities; clean/fresh water, etc.), in which contribute information from the scientific publications of USAMV of Bucharest (e.g.: Mitelut et al., 2022; Jerca et al., 2022; Chiselita et al., 2023; Drăghici et al., 2023: Geicu-Cristea et al., 2023; Lloha et al., 2023; Lelieveld, 2023; Vamanu & Dangnon, 2023; etc.). In this regard, an example of applying scientific information to support sustainable development is the use of LED's light to plants belonging to the following species: *Camelina sativa* L. (Podgoreanu et al., 2015); *Artemisia dracunculus* L. (Enache & Livadariu, 2016); *Lycopersicum esculentum* L. (varieties Buzău 4C, Rio Grande ST and Saint Pierre ST) (Livadariu & Dumitru, 2019); *Lycopersicum esculentum* L. (varieties Sonia de Buzău, Hera and Coralina) (Dănăilă-Guidea et al., 2020); *Taxus baccata* L. (Delian et al., 2022).

Subsequently, given both the above-mentioned and the attention paid in recent years to the use of LED's light for plant growth and development, this paper has the following academic purpose: selecting an AI-powered tool to identify LED's, light, and plant keywords in scientific articles available on-line from publications of the USAMV of Bucharest.

In order to reach this academic goal, we have designed an exercise using two AI types, one form TC1 (respectively, AISA1 = "Generative Pre-trained Transformer-based conversational AI") and one from TC2 (respectively, AISA2 = "an AI assistant developed by TC2").

Every working tool used three variants to identify LED's, light, and plants keywords from the scientific articles available on-line in the USAMV of Bucharest publications, as: individual keywords (LED's, light, or plants), sequence of keywords (LED's, light, and plants) and an explicit request for identification (identifies in the scientific publications of USAMV of Bucharest the following keywords: LED's, light and plants).

MATERIALS AND METHODS

The working **materials** consisted of a Lenovo IdeaPad S145-15IIL laptop with Operating System: Windows 11 Pro, internet access, AISA1 access, and AISA2 access.
The working **method** involved creating an exercise using **two working tools**. Among these, one working tool was one model AI created by TC1 (respectively, AISA1 Version-3.5) and one assistant AI (Source: conversation with AISA2. Accessed at 10.05.2024) created by TC2 (respectively, AISA2).
Each of these working tools used three variants of sources identification through:
- V1 = individual keywords (LED's, light, or plants);
- V2 = sequence of keywords (LED's, light, and plants);
- V3 = an explicit request for identification (Identify the following keywords in the scientific publications of USAMV of Bucharest: LEDs, light, and plants).

Variant V1 generated three assertions, as it follows:
- V1Q1 = LED's in publications of University of Agronomic Sciences and Veterinary Medicine of Bucharest;
- V1Q2 = Light in publications of University of Agronomic Sciences and Veterinary Medicine of Bucharest;
- V1Q3 = Plants in publications of University of Agronomic Sciences and Veterinary Medicine of Bucharest.

Variant V2 generated one assertion, respectively:
- V2Q4 = LED's, light and plants in publications of University of Agronomic Sciences and Veterinary Medicine of Bucharest.

Variant V3 generated an explicit request for identification, respectively:
- V3Q5 = Identify LED's, light and plants in publications of University of Agronomic Sciences and Veterinary Medicine of Bucharest.

Each of the three variant of searching key words was realizes in three iterations (V1, V2 and V3) (10 December 2023, 10 February 2024 and 10 May 2024). To elaborate the final form of the paper we used the 10 May 2024 iteration. The results obtained using AISA1 (according to the responses generated by AISA1, Version-3.5) and AISA2 (according to the responses generated by AISA2), have been noticed and assessed by comparison from the perspective of the established goal through the performed exercise.

RESULTS AND DISCUSSIONS

Results obtained following the exercise to use AI to identify LED's, light and plants keywords in scientific articles on-line USAMV of Bucharest publications are displayed in two panels subsequent to the two working tools (AISA1 from TC1 and AISA2 from TC2). And, within the framework of each tool, the results are sub-grouped on three variants made up from individual keywords (V1Q1, V1Q2 and V1Q3), sequence of keywords (V2Q4) and an explicit request for identification (V3Q5).

AISA1 from TC1. According to responses generated by AISA1, Version-3.5
(Source: Conversation with Chat Generative Pre-trained Transformer from TC1. Accessed at 10.05.2024)
V1Q1. Response to the V1Q1 statement starts with the following sequence of text: "Finding specific references to LED's technology within publications from a particular institution can be challenging without access to their internal databases or repositories. However, I can provide some general information about how LED technology might be featured in research or publications from the University of Agronomic Sciences and Veterinary Medicine of Bucharest (USAMV Bucharest)".
The response to this question provides information related to the current implications of LED's in: Greenhouse Technology, Animal Sciences, Energy Efficiency, and Aquaculture.
The response ends with:
"To find specific publications, you may need to search through academic databases like PubMed, Google Scholar, or the university's own library catalogue. Additionally, reaching out to researchers or faculty members directly could provide insights into their current

projects and publications related to LED technology".

V1Q2. Response to the statement V1Q2 starts with the following sequence of text: "Research related to light, including artificial lighting technologies like LEDs, can be found in various publications from the University of Agronomic Sciences and Veterinary Medicine of Bucharest (USAMV Bucharest). Here are some areas where you might find such publications".

Response provides information regarding current implications of the light in: Horticulture and Plant Sciences, Animal Welfare and Behaviour, Aquaculture, and Energy Efficiency and Environmental Sustainability.

The response end with a sequence of text which identically with the end of V1Q1, relates to the academic databases (PubMed, Google Scholar, or the university's own library catalogue), or contact faculty members directly.

The main difference between the final text from V1Q1 and V1Q2 is given by the reference to "... related to LED technology" compared to "... related to light and lighting technologies".

V1Q3. Response to the statement V1Q3 starts with the following text: "Publications from the University of Agronomic Sciences and Veterinary Medicine of Bucharest (USAMV Bucharest) cover a wide range of topics related to plants. Here are some areas where you might find publications related to plants".

Response provides information regarding current implications of plants in: Crop Science and Agronomy, Horticulture and Floriculture, Plant Pathology and Plant Protection, Plant Biotechnology and Molecular Biology, and Environmental and Ecological Studies.

Essentially, this response is different to the responses from V1Q1 (Greenhouse Technology, Animal Sciences, Energy Efficiency, Aquaculture) and V1Q2 (Horticulture and Plant Sciences, Animal Welfare and Behaviour, Aquaculture, and Energy Efficiency and Environmental Sustainability). Therefore, one can notice a differentiation of responses according to the keyword used as relevant element within the statements in variant V1, respectively: LED's in V1Q1, light in V1Q2, or plants in V1Q3.

The response ends with a text that differs only in the final part ("... publications in plant science"), from the final text in V1Q1 ("... publications related to LED technology"), and V1Q2 ("...publications related to light and lighting technologies").

V2Q4. Response to the V2Q4 statement starts with the text: "Research at the University of Agronomic Sciences and Veterinary Medicine of Bucharest (USAMV Bucharest) likely covers the intersection of LEDs, light, and plants across various disciplines such as agronomy, horticulture, plant physiology, and environmental science. Here's how these topics might converge in publications from USAMV Bucharest".

Response provides information regarding current implications LED's, light and plants in: LED's in Plant Growth and Development, Light Quality and Quantity Effects on Crop Production, LED's for Sustainable Agriculture, LED's in Plant Stress Physiology, and Optimization of LED's Lighting.

This response is different from the responses from V1Q1, V1Q2 and V1Q3, which provided information from a broader area as it used only one key word. Basically, this response reduces the information search area to a narrower level and it is more applied on a certain information direction as it uses a sequence of keywords.

The response ends with a text which, unlike the correspondent text sequence from V1Q1, V1Q2 and V1Q3, provides additional, more customized suggestions, narrowing down to just three areas of search research, respectively "... Additionally, reaching out to researchers or faculty members in departments related to agronomy, horticulture, or plant physiology may provide insights into their current projects and publications related to LEDs, light, and plants".

V3Q5. Response to the explicit identification request V3Q5 begins with the text: "To provide a detailed list of publications from the University of Agronomic Sciences and Veterinary Medicine of Bucharest (USAMV Bucharest) specifically focusing on LED's, light and plants, I would need access to their academic database or repository, which is not available to me. However, I can guide you on how to conduct a search to identify such publications".

This response is different from the responses to the V1Q1, V1Q2, V1Q3 and V2Q4 statements,

as it highlights the presence of certain information that "is not available" for AISA1 from Open AI. But the answer provides information about how to search for information by exploring within:
- University Repository or Library Database;
- Academic Databases;
- Faculty Profiles and Research Websites;
- Conference Proceedings and Journals;
- Collaborative Projects and Networks.

The response ends with the following text "By employing these strategies and carefully selecting search terms related to LEDs, light, and plants, you can identify publications from USAMV Bucharest that focus on this topic. Additionally, contacting the university's library or relevant faculty members for assistance in accessing publications or obtaining further information can be beneficial".

By guiding the identification of sources for accessing the desired information, it contributes to educating an inexperienced user in improving the documentation competence from scientific sources.

AISA2 from TC2. According to responses generated by AISA2
(Source: Conversation with AISA2. Accessed at 10.05.2024)

V1Q1. Response to V1Q1 statement starts with the following text: "Here are some resources related to LED's from the University of Agronomic Sciences and Veterinary Medicine of Bucharest".

Response to V1Q1 statement provides information regarding sources to access USAMV of Bucharest publications:
- Scientific Journals with eight Journals (https://www.usamv.ro/index.php/en/scientific-research/scientific-journal);
- Agriculture for Life Conference Publications (https://agricultureforlife.usamv.ro/);
- Bulletin of University of Agricultural Sciences and Veterinary Medicine (https://doaj.org/toc/1843-5378).

The eight Scientific Journals are:
- Agrolife Journal;
- Agronomy Journal;
- Horticulture Journal;
- Animal Science Journal;
- Veterinary Medicine Journal;
- Land Reclamation Journal;
- Biotechnology Journal;
- Management Journal.

The sources mentioned in the response to the V1Q1 statement may contain scientific information about LED's and links for direct access to the sources used. But the answer to question V1Q1 also contains additional sources, in addition to those already mentioned for this answer, namely: https://www.usamv.ro/index.php/en/home-eng; https://www.timeshighereducation.com/world-university-rankings/university-agronomic-sciences-and-veterinary-medicine-bucharest; https://www.timeshighereducation.com/sites/default/files/university/rankings/721492-en.json and https://agricultureforlife.usamv.ro/index.php/publications.

The response ends with:
"Please note that while these resources provide valuable insights, they may not directly contain information about LED's. For more specific publications, I recommend checking each journal's archives or using their search function, if available".

V1Q2. The answer to the statement V1Q2 begins with a text that differs from the similar text area from the statement V1Q1 only by replacing the word LED's with "Light".

The answer to the statement V1Q2 differs from the answer to the statement V1Q1 by explicitly mentioning only a number of just six Scientific Journals (https://www.usamv.ro/index.php/en/home-eng). The Scientific Journals mentioned are:
- AgroLife Scientific Journal;
- Agronomy Scientific Papers;
- Horticulture Scientific Papers;
- Animal Science Scientific Papers;
- Veterinary Medicine Scientific works;
- Land Reclamation, Earth Observation & Surveying, Environmental Engineering Scientific Papers.

Assessing the results from V1Q1 and V1Q2 statements, it can be seen that besides the difference between the number of Scientific Journals there is also a difference even at the level of their titles. This highlights the active role that AISA2 has in selecting the number and type of sources that may contain useful information based on individual keyword-based search.

But, the number of suggested additional sources is lower, being only two instead of four. The two additional suggested sources are: https://www.veterinarymedicinejournal.usamv.ro/ and https://agricultureforlife.usamv.ro/index.php/publications.

The response ends with a text that differs from the final text from the question V1Q1, only by replacing the word LED's with "Light".

V1Q3. Response to the V1Q3 statement begins with a text that differs from the similar text area from the statements V1Q1 and V1Q2 only by replacing the words LED's or "Light" with "Plants".

Response to the V1Q3 statement unlike the responses to the V1Q1 and V1Q2 statements brings information about two of the three sources of access to USAMV of Bucharest publications. The source which is no longer referred to is Bulletin of University of Agricultural Sciences and Veterinary Medicine (https://doaj.org/toc/1843-5378). Therefore, it is highlighted, once again the active role played by AISA2 in selecting sources that may contain useful information according to the search based on individual keywords. But, the number of suggested additional sources is lower, being only two instead of four. The two additional suggested sources are: https://creativecommons.org/licenses/by/4.0%29 and https://agricultureforlife.usamv.ro/index.php/publications.

It can be noticed that one of the two additional sources is common to one of the additional sources mentioned in the response for the V1Q2 statement.

The response ends with a text that is different from the final text in V1Q1 and V1Q2 statements, only by replacing the words LED's and "Light" by "Plants".

V2Q4. Response to the V2Q4 statement begins with a text that differs from the similar text area from the V1Q1, V1Q2 and V1Q3 statements by replacing the individual words "LED's", V1Q3, "Light" and "Plants" with the sequence of words "LED's, light and plants".

Response to the V2Q4 statement unlike responses to the V1Q1, V1Q2 and V1Q3 statements no longer provide information about the sources of access to the publications USAMV of Bucharest. Instead, it mentions specific information about two scientific articles, namely:

- "BIOCHEMICAL CHANGES, INDUCED BY LED LIGHT, IN TOMATO PLANTS, GROWN IN THE INTEGRATED MANAGEMENT SYSTEM (SMI) OF AGROECOSYSTEM RESISTANCE" (https://agronomyjournal.usamv.ro/index.php/scientific-papers/past-issues?id=1284)

and

- "LED Technology Applied to Plant Development for Promoting the Accumulation of Bioactive Compounds: A Review" (https://www.mdpi.com/2223-7747/12/5/1075).

By explicitly stating the two scientific articles one can save search time. But the disadvantage is that one of the two scientific articles do not belong to a USAMV of Bucharest publication.

The number of additional sources proposed is similar to the number of sources proposed for V1Q2 and V1Q3. The additional sources proposed are: https://www.mdpi.com/2073-4395/11/6/1106?src=444017 and https://doi.org/10.3390/plants12051075.

Similar to the answer itself, also in the response area with additional sources proposed, it is noticed that one of them is not from a publication of USAMV of Bucharest.

Response for V2Q4 statement ends with a text with two components that differ from the final text in V1Q1, V1Q2 and V1Q3 statement. The first component for V2Q4 statement consists in the stating that information "... while these publications provide valuable insights into the use of LED lighting in plant growth, they may not be directly affiliated with the University of Agronomic Sciences and Veterinary Medicine of Bucharest", but for V1Q1, V1Q2 and V1Q3 consists in the stating that information "... while these resources provide valuable insights, they may not directly contain information about LED's".

The second component for V2Q4 statement consists in stating that ("... I recommend checking their official website or academic databases"). This mention differs from that for V1Q1, V1Q2, and V1Q3 statements ("... I recommend checking each journal's archives or using their search function, if available").

Assessing the final response areas for V1Q1, V1Q2, V1Q3, and V2Q4 statements, the conclusion is that statements using only one keyword compared to the statement that used a keyword sequence generated more accuracy in identifying publications of USAMV of Bucharest. It can also be noted that only through the V2Q4 statement was explicitly identified a scientific article available on-line from publications of USAMV of Bucharest.

V3Q5. Response to the explicit request for identification V3Q5 begins/comprises (the same two scientific articles), and ends with a text that is similar to those in the corresponding areas in response to the V2Q4 statement.

From observing and analysing the results obtained by performing the exercise with the two working tools, it is noted that:

- each of the two working tools has advantages and limits,

and

- the main advantage of AISA1 from TC1 is that it can be used to generate information in the form of texts that have a type of NLP (Natural Language Processing), which is, closer to human expression/behaviour,
- the main advantage of AISA2 from TC2 is that it can generate texts in which it provides access to the sources it uses in generating the information, because it embedded also links in the answer.

By using the two AI tools, primary guidance can be achieved in identifying the sources for accessing the desired information, and it can help to educate an inexperienced user in improving the documentation competence from scientific sources.

CONCLUSIONS

The results obtained by performing the exercise of using two AI working tools (AISA1 from TC1 and AISA2 from TC2), highlight for the identification of LED's keywords, light and plants in scientific articles available on-line from publications of USAMV of Bucharest, the existence of peculiarities to be used according to the needs of the user.

AISA1 tool from TC1 generated the most useful information for responding to V2Q4 *versus* V1Q1, V1Q2 and V1Q3. Practically, the response to the V2Q4 statement reduces the search area of information to a narrower level and more applied to a particular direction of information because it uses a sequence of keywords as opposed to V1Q1, V1Q2 and V1Q3 that used only one key word and provided information at a broader and less applied level.

Meanwhile, the response of the AISA1 tool from TC1, at the explicit identification request V3Q5 is different from the responses from the V1Q1, V1Q2, V1Q3, and V2Q4 statements, respectively, by signalling the presence of certain information that for AISA1 from Open AI "is not available". But the answer provides information about how to search for information by exploring within a series of Academic Databases type.

AISA2's TC2 tool has generated the most targeted information, both for the response to the V2Q4 statement and for the response to the explicit V3Q5 identification request, unlike the V1Q1, V1Q2, and V1Q3 statements, because it mentions specific and relevant information, about a scientific article from a USAMV of Bucharest publication.

Responses provided by The AISA2 tool from TC2 to the V2Q4 statement and the explicit V3Q5 identification request, unlike the responses from the V1Q1, V1Q2, and V1Q3 statements, no longer provides information about sources of access to USAMV of Bucharest publications. Also, the responses of the same tool, to the V2Q4 statement and to the explicit identification request V3Q5, were similar.

On the overall, the results of the exercise indicate that according to certain specific academic needs of the user, each of the two AI tools must be analysed and compared before selecting one of them for the primary verification of the existence of LED's, light and plants keywords in scientific articles available on-line from USAMV of Bucharest publications.

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UNVEILING THE FUNCTIONAL PROPERTIES OF *Pelargonium graveolens*: INSIGHTS INTO ITS BIOLOGICAL ACTIVITIES

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Abstract

This article aims to present a review of Pelargonium graveolens, or rose-scented geranium, highlighting its diverse medicinal, aromatic, and industrial applications. Thus, P. graveolens is a multifaceted plant with significant medicinal, aromatic, and industrial applications The recent studies highlight its strong antifungal and antibacterial activities, particularly against resistant strains, with key compounds like β-citronellol, geraniol, and linalool contributing to these effects. The essential oil has shown efficacy in formulations for treating vaginal candidiasis and preventing biofilm formation on medical devices, demonstrating potential as a natural adjunct to conventional antifungal therapies. Additionally, P. graveolens exhibits significant antioxidant properties, with hydrosols proving more effective than essential oils in this regard. Its applications extend to pest control, as the oil has acaricidal and insecticidal effects, and it shows promise in treating conditions like diabetes and inflammatory diseases. Moreover, the plant's versatility is evident in its use across cosmetics and food preservation. This review emphasizes the need for further research to fully elucidate the pharmacological potential and to optimize the applications of P. graveolens in medicine, agriculture, and food industry.

Key words*: Pelargonium graveolens, bioactive compounds, pharmacological potential, citronellol, geraniol.*

INTRODUCTION

Pelargonium graveolens, also known as rose-scented geranium, is a versatile plant with significant medicinal, aromatic, and industrial applications. Traditionally used in South Africa, it has been burned and inhaled to treat asthma, while an infusion of its leaves is used for relieving abdominal cramps, diarrhoea, nausea, and insomnia (Meyers et al., 2006). The essential oil of *P. graveolens* is widely recognized in aromatherapy for its relaxing properties, with studies showing it can lower blood pressure, exert sedative effects, and stimulate brain activity (Meyers et al., 2006; Rajesh et al., 2023).

The oil also enhances the antifungal efficacy of ketoconazole and is used in personal aromatherapy blends to alleviate anxiety, often combined with oils like lavender and clary sage (Meyers et al., 2006; Naeini et al., 2011; Hamidpour et al., 2017). However, some rare cases of contact dermatitis and sensitization have been reported (Meyers et al., 2006).

Research has highlighted the oil's antioxidant and antibacterial activities, particularly those extracted during the flowering stage, which demonstrate high efficacy against food spoilage pathogens.

The oil's major compounds include β-citronellol, geraniol, and linalool, which contribute to its medicinal properties. Additionally, the phenological stage of the plant influences the oil yield, composition, and biological activities, suggesting that optimizing the harvesting time can enhance its functional properties for use in pathogen control and food preservation (Boukhris et al., 2015).

In Bosnia, the first detailed phytochemical analysis of *P. graveolens* revealed that its hydrosols possess stronger antioxidant activity than the essential oils, likely due to higher phenolic and flavonoid content. These findings suggest potential applications in antimicrobial and antiviral therapies (Ćavar & Maksimović, 2012).

Moreover, dry extracts of the plant have shown strong antioxidant and cytotoxic effects, indicating possible therapeutic use in treating metabolic diseases such as diabetes and dyslipidaemia (Neagu et al., 2018).

P. graveolens is not only valued for its therapeutic potential but also for its essential oil, which is a key ingredient in perfumes, cosmetics, and the flavour industry (Rajesh et al., 2023; Swanepoel, 2003). In India, efforts to improve agronomic practices, particularly nutrient management, aim to enhance oil yield and quality to reduce the country's dependency on imports (Rajesh et al., 2023; Upadhyay et al., 2022). The plant also shows promise in bioremediation as a phyto-accumulator for heavy metals (Mazeed et al., 2022).

The chemical composition of *P. graveolens* oil varies by region, with studies from Algeria (Boukhatem et al., 2013a) and Egypt (Rajesh et al., 2023; Ibrahim et al., 2021; Abd El-Kareem et al., 2020; Blerot et al., 2016), revealing differences in its primary constituents, which include citronellol, geraniol and linalool. These oils have demonstrated significant antimicrobial efficacy, particularly against Gram-positive bacteria and fungi, suggesting their potential as natural preservatives in the food industry (Ibrahim et al., 2021; Abd El-Kareem et al., 2020). Research also highlights the oil's antidiabetic (Hamidpour et al., 2017, Amel et al., 2022), antifungal (Hamidpour et al., 2017; Gucwa et al., 2018; Abd El-Kareem et al., 2020; Hsouna & Hamdi, 2012; Mahboubi & Valian, 2019; Juárez et al., 2016; Saraswathi et al., 2011; Narnoliya et al., 2019; Ghedira & Goetz, 2015; Gâlea & Hancu, 2014; Amel et al., 2022), and antioxidant properties (Hamidpour et al., 2017; Rahman et al., 2020; Jaradat et al., 2022; Ben Slima et al., 2013; Dimitrova et al, 2015; Saraswathi et al., 2011; Narnoliya et al., 2019; Ghedira & Goetz, 2015; Amel et al., 2022), with studies showing its ability to reduce blood glucose levels in diabetic rats (Hamidpour et al., 2017) and protect against oxidative stress (Rahman et al., 2020; Boukhris et al., 2012; Ben Slima et al., 2013).

Despite these benefits, the oil's toxicity must be carefully monitored to ensure safe therapeutic use (Hamidpour et al., 2017). Further research is necessary to fully understand the pharmacological potential of *P. graveolens* and to optimize its applications in medicine (Hsouna & Hamdi, 2012; Peterson et al., 2006), cosmetics (Swanepoel, 2003; Ghedira & Goetz, 2015; Jeon et al., 2008), and food preservation (Blerot et al., 2016).

ANTIFUNGAL ACTIVITY

The provided studies collectively demonstrate the significant antifungal potential of *P. graveolens* essential oil (PGEO) against various fungal species, with promising applications in both clinical and medical device contexts (Giongo et al., 2016). Thus, *P. graveolens* has demonstrated a large spectrum of antifungal activity, being a versatile option for fungal infections.

- **Vaginal Candidiasis**. A mucoadhesive hydrogel-thickened nanoemulsion containing PGEO was shown to significantly enhance antifungal activity against *Candida* sp., reducing the minimum inhibitory concentration (MIC) by up to 64 times. This formulation not only improves efficacy but also reduces irritant potential, making it a promising treatment for vaginal candidiasis (Dos Santos et al., 2020).
- **Medical Devices**. Nanoemulsions containing geranium oil (GO) were developed to prevent biofilm formation by *Candida* sp. on medical devices. These nanoemulsions (NEG) significantly inhibited biofilm formation, particularly against *C. albicans*, *C. tropicalis*, and *C. glabrata*, suggesting their potential to reduce microbial adhesion and prevent infections in catheterized patients (Giongo et al., 2016).
- **Combination with Antifungals**. The synergistic effects of PGEO with conventional antifungal drugs like Nystatin (Rosato et al., 2009) and Amphotericin B (Essid et al., 2015; Rosato et al., 2008) were explored. While PGEO showed limited synergy with Nystatin, it demonstrated a strong synergistic effect with Amphotericin B, potentially reducing the required dose of the drug and minimizing its side effects.
- **Antifungal spectrum**. In a broader antifungal evaluation, PGEO exhibited both fungistatic and fungicidal activities against *Candida* isolates. It also demonstrated synergy with Amphotericin B, indicating its potential as an adjunct in traditional antifungal therapy (Gucwa et al., 2018).
- **Anti-Pityriasis Versicolor**. PGEO showed significant antifungal activity against *Malassezia* sp., outperforming the conventional drug ketoconazole. The oil's major components, citronellol and geraniol, were identified as key

contributors to its effectiveness, suggesting PGEO as a promising natural alternative for treating pityriasis versicolor (Naeini et al., 2011; Hamidpour et al., 2017).

Antifungal potential of *P. graveolens* against *Fusarium proliferatum* and confirms, through in-silico and *in vitro* analyses, its suitability for developing natural antifungal agents (Grine et al., 2023).

Overall, these studies underscore the versatility and efficacy of *P. graveolens* essential oil as a potent antifungal agent with applications ranging from clinical treatments to infection prevention on medical devices.

ANTIBACTERIAL ACTIVITY

Pelargonium graveolens essential oil, rich in compounds like citronellol and geraniol, has shown significant antibacterial activity against various bacterial strains, including antibiotic-resistant pathogens (Elansary et al., 2018; Dumlupinar et al., 2020; Rosato et al., 2007). Its potential applications expand across medical, pharmaceutical, and industrial contexts (Fayoumi et al., 2022; Upadhyay et al., 2022; Okla et al., 2022; M'hamdi et al., 2024).

Antibacterial Efficacy of *P. graveolens* demonstrated high potential in growth inhibition of various bacterial strains.

• **Activity Against** *Helicobacter pylori*. The essential oil demonstrated a minimal inhibitory concentration (MIC) of 15.63 mg/ml against *H. pylori*, a major cause of peptic ulcers. When combined with the antibiotic clarithromycin (CLR), the oil showed a synergistic effect, reducing the effective dose of CLR needed and potentially decreasing the risk of side effects and antibiotic resistance (Ibrahim et al., 2021).

• **Synergistic effects with Standard Antibiotics**. Studies have shown that *P. graveolens* oil enhances the antibacterial activity of standard antibiotics like norfloxacin (Dumlupinar et al., 2020; Rosato et al., 2007; Choi et al., 2007) and erythromycin (Choi et al., 2007). For instance, the combination of the oil with norfloxacin resulted in a significant reduction in the MICs against *B. cereus* and *S. aureus*, including antibiotic-resistant strains of *S. pneumoniae* (Rosato et al., 2007). This synergy can lead to more effective treatments with lower doses of antibiotics, reducing the likelihood of resistance development (Choi et al., 2007).

• **Broad-Spectrum Antibacterial Activity**. The essential oil has demonstrated inhibitory effects against both Gram-positive bacteria, such as *Micrococcus luteus*, *S. aureus, L. monocytogenes* and *E. faecalis*, and Gram-negative bacteria like *P. aeruginosa* and *E. coli* (Wei et al., 2022; Rathore et al., 2023; Dos Santos et al., 2024). Notably, the oil showed comparable efficacy to some commercial antibiotics, particularly in the case of *S. aureus* and *C. albicans*, where complete inhibition of growth was observed (Gâlea & Hancu, 2014).

The applications are correlated with the antibacterial properties of *P. graveolens* oil. It is a promising candidate for developing alternative treatments for infections, particularly those involving antibiotic-resistant bacteria. Its use could be extended to topical formulations for skin infections, oral hygiene products, and as a natural preservative in pharmaceutical preparations (El Aanachi et al., 2020; Swanepoel, 2003).

At the same time, it can be used as cosmetic and personal care products (El Aanachi et al., 2020; Peterson et al., 2006; Ghedira & Goetz, 2015; Szutt et al., 2020; Jeon et al., 2008). The oil's antimicrobial properties, combined with its pleasant fragrance, make it ideal for inclusion in cosmetics, deodorants, and other personal care products, providing both antimicrobial protection and aromatic benefits. The antibacterial potential of *P. graveolens* essential oil is significant, offering a natural and effective alternative or complement to traditional antibiotics (Abd El-Kareem et al., 2020; Choi et al., 2007). Its application spans various contexts, from medicine to food preservation, and its role in combating antibiotic resistance highlights its importance in modern healthcare and food industry (Rajesh et al., 2023; Hamidpour et al., 2017). Further research and development are necessary to optimize formulations and ensure safe and effective use across these applications (El Aanachi et al., 2020; Ibrahim et al., 2021; Jeon et al., 2009).

OTHER PHARMACEUTICAL APPLICATIONS CORRELATED WITH OTHER *Pelargonium graveolens* ACTIVITIES

• **Antiacaricidal Effects**. *P. graveolens* has demonstrated acaricidal properties, notably against cattle ticks (*Rhipicephalus microplus*) (Pazinato et al., 2016) and storage food mites, as *Tyrophagus putrescentiae* (Jeon et al., 2009), *Dermatophagoides farinae* and *D. pteronyssinus* (Jeon et al., 2008). *In vitro* studies revealed that geranium oil, particularly its active compound geraniol, shows effective inhibition of tick oviposition and mite activity (Pazinato et al., 2016). Geraniol, identified as a primary active compound, has an LD_{50} of 1.95 µg/cm³, making it significantly more toxic than benzyl benzoate, which has an LD_{50} of 11.27 µg/cm³ (Jeon et al., 2009). For house dust mites (*D. farinae* and *D. pteronyssinus*), geraniol demonstrated high toxicity with LD_{50} values of 0.26 µg/cm² and 0.28 µg/cm², respectively (Jeon et al., 2008). These results suggest geranium oil could be a valuable alternative to synthetic acaricides for pest control (Jeon et al., 2008; Jeon et al., 2009).

• **Repellent and Insecticidal Activity**. The essential oil of *P. graveolens* exhibits notable repellent and insecticidal effects against adult house flies (*Musca domestica*) and blowflies (*Lucilia cuprina*). At 1% concentration, the oil shows significant repellent activity. In terms of insecticidal effectiveness, the lethal concentration (LC_{50}) is 3.0% for *M. domestica* and 2.5% for *L. cuprina* in direct surface applications. In impregnated paper tests, the LC_{50} values are 5.9% and 3.5%, respectively. The effectiveness is attributed to compounds like citronellol and geraniol, though it is less potent compared to synthetic insecticides like Diazinon (Saraiva et al., 2020).

• **Antidermatophyte Effects**. Geranium essential oil shows strong antidermatophyte activity. In a study performed by Mahboubi & Valian, among tested oils, the E20 sample, high in geraniol and citronellol, exhibited the highest efficacy in inhibiting fungal growth against dermatophytes such as *Microsporum canis* and *Trichophyton rubrum*. Geraniol is identified as a key antifungal compound, underscoring the oil's potential for treating fungal infections (Mahboubi & Valian, 2019).

• **Antileishmanial Activity**. *P. graveolens* essential oil, including its major compounds β-caryophyllene and geraniol, demonstrates significant antileishmanial activity against *Leishmania major* and *Leishmania infantum*. The oil showed IC_{50} values ranging from 0.05 to 0.28 µg/mL for *Leishmania* sp., outperforming the reference drug amphotericin B. The low toxicity against Raw 264.7 macrophage cells and the high selectivity index suggests its potential in developing new antileishmanial drugs (Martins et al., 2017).

• **Anti-inflammatory Effects**. The essential oil of *P. graveolens* exhibits moderate anti-inflammatory effects. In comprehensive studies, the crude extract and ethyl acetate fraction reduced levels of prostanoids like PGE2 and TXB2 at a concentration of 50 µg/mL. The effectiveness is attributed to polar compounds such as flavonoids, including rutin and kaempferol. Geranium oil has shown a significant reduction in edema in carrageenan-induced paw and croton oil-induced ear models, comparable to diclofenac, suggesting its potential for treating inflammatory conditions (Boukhatem et al., 2013b).

• **Antidiabetic and Anti-Alzheimer's Potential**. *Pelargonium graveolens* essential oil shows promise in managing type-2 diabetes and Alzheimer's disease due to its inhibitory effects on α-amylase and acetylcholinesterase. The high antioxidant capacity and hypoglycemic effects further support its use in diabetes management. The plant's extracts also offer potential in Alzheimer's disease treatment by inhibiting acetylcholinesterase (Ali et al., 2020, Fayoumi et al., 2022).

• **Cytotoxicity Against Cancer Cells**. The leaf extract of *P. graveolens* is used to synthesize palladium nanoparticles (PdNPs), which show dose-dependent cytotoxicity against K562 human leukemia cells. This eco-friendly synthesis approach highlights the potential biomedical applications of PdNPs produced using geranium extract (Li et al., 2017). Another study, revealed that *P. graveolens* leaf extract have cytotoxic effects using its gold nanoparticles (AuNPs) on human dermal fibroblasts from neonates (HDFn). The study also confirmed that *P. graveolens* AuNPs

displayed slight cytotoxicity (about 20%) at various dosages, with high biocompatibility observed in normal human fibroblasts. This aligns with earlier studies and underscores the increasing interest in nanotechnology-based pharmaceuticals, which have gained FDA approvals for therapeutic use (Asker et al., 2024).

• **Anti-proliferative effects**. The text outlines a study on the biological effects of essential oils, including *P. graveolens* (geranium), on human dermal fibroblasts in a simulated chronic inflammation environment. It highlights the anti-proliferative effects of geranium essential oil but does not provide details on its effects on specific biomarkers (Han et al., 2017)

• **Potential Anesthetic**. Geranium oil demonstrates anesthetic potential in aquaculture, reducing induction time and increasing recovery time in freshwater aquarium fish. The minimum effective concentration for deep anesthesia is 75 μL L^{-1}, suggesting geranium oil's potential as a pleasant-smelling anesthetic for aquatic species (Can et al., 2018).

• **Supplement for Aquaculture**. In aquaculture, *Pelargonium graveolens* essential oil has shown protective effects against pesticide-induced toxicity in common carp (*Cyprinus carpio*). GEO supplementation improved liver and kidney functions, enhanced immune responses, and reduced oxidative stress, indicating its potential as a dietary supplement to mitigate pesticide toxicity in fish (Rahman et al., 2020).

Pelargonium graveolens L. has the ability to remove the antibiotic tetracycline (TC) from soil and the effect of different concentrations on the plant. The researched study investigates the mechanism of antibiotic sequestration in the plant under both soil and hydroponic conditions, as well as the impacts on the plant's biomass, growth, and biochemical content. The research concludes that *P. graveolens* can tolerate tetracycline stress and can be used for the remediation of antibiotic-contaminated areas (Siddiqui et al., 2024).

Overall, *P. graveolens* exhibits a wide range of biological activities and applications, from pest control and antifungal treatment to anti-inflammatory, antidiabetic, and potential anesthetic uses (Can et al., 2018). The plant's active compounds, particularly geraniol and citronellol, are crucial in these therapeutic and practical applications, warranting further research and development (Narnoliya et al., 2019).

CONCLUSIONS

This review highlights that *Pelargonium graveolens*, or rose-scented geranium, exhibits a diverse array of biological activities, including significant antifungal and antibacterial properties, making it a valuable candidate for various therapeutic applications. Its essential oil, rich in compounds like citronellol and geraniol, shows promise in treating infections, enhancing traditional antifungal therapies, and addressing antibiotic resistance, while also being beneficial in areas such as pest control and anti-inflammatory treatments, warranting further investigation into its pharmacological potential.

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