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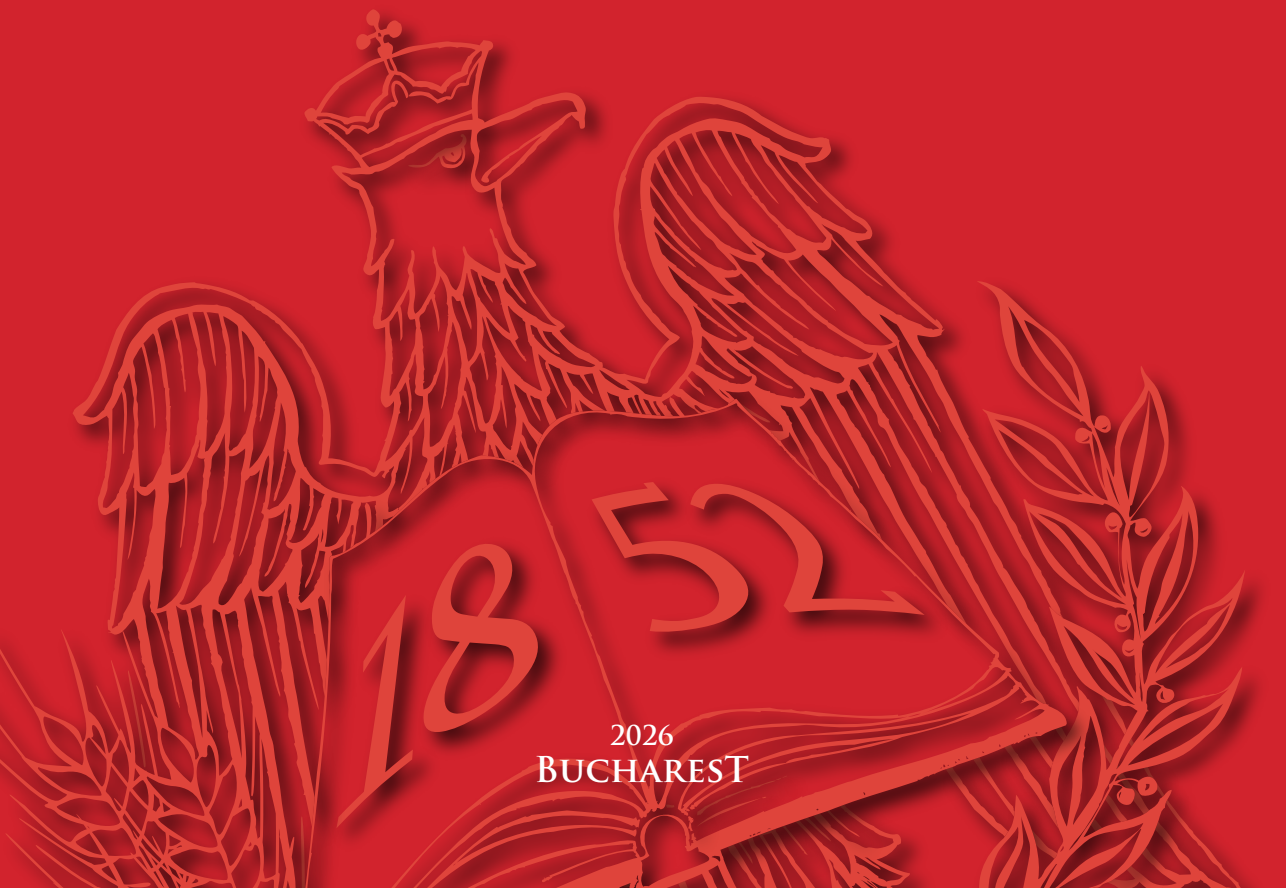

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SUMMARY

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INVESTIGATIONS ON THE USE OF EGG PROTEIN DERIVATIVES AND HYDROLYSATES FOR PUDDING FORTIFICATION

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Abstract

In the context of growing need for high-quality protein products, the fortification of foods with complete protein sources represents a sustainable strategy to prevent protein malnutrition. The present study aimed to develop a high-protein dessert, by fortifying a commercial starch-based pudding mix with egg-derived protein ingredients and their hydrolysates. The fortification level was decided in such to obtain "high-protein" products, whereas maintaining desirable sensory properties. Protein fortification of pudding with egg powders significantly altered the colour parameters. Among all fortified samples, the highest total colour difference compared to the control was noticed in case of the puddings prepared with yolk proteins and hydrolysates (ΔE of 7.15±0.00 and 8.93±0.02, respectively), highlighting the strong pigmenting effect of carotenoids. The egg white proteins addition significantly improved water-holding capacity and resulted in high sensory acceptability of the pudding. The use of egg proteins hydrolysates resulted in reduced overall acceptance. Although egg proteins hydrolysis ensured significant reduction of the antigenic properties, the bitter aftertaste of the peptides limited the use of the hydrolysates for puddings fortification at such levels to allow the high protein claim.

Key words*: egg proteins, pudding, protein hydrolysates, functional properties, rheological behaviour.*

INTRODUCTION

Processed foods, which include desserts, are usually low in protein and contain large amounts of sugars and refined carbohydrates. Therefore, the diets rich in highly processed foods is usually unbalanced and can lead to obesity and metabolic disorders (Wali et al., 2020). Food fortification is an efficient strategy for a healthy diet and the quality of nutrients is of crucial importance when attempting to balance the nutritional profile. Particular attention needs to be paid to the nutritional value and functionality of the proteins.

The EC Regulation No. 1924/2006 establishes that foods can be labelled as "source of protein" or "rich in protein", if at least 12% or 20%, respectively, of the total energy value is provided by the protein content.

The main products which can be targeted for fortification with proteins are dairy products and desserts, bakery and pastry products, protein drinks or smoothies, snacks like bars or chips, starch-based desserts, etc. Among these products, pudding is a promising matrix, suitable for supplementation with proteins of various origins. Commercial puddings are appreciated for their pleasant taste and creamy texture, but they often have an unbalanced nutritional profile, being rich in starch and refined sugars. Fortifying puddings with egg protein or protein hydrolysates allows balancing the nutritional profile, whereas supporting health benefits and sensory appealing. Reaching the protein threshold for obtaining puddings which can be labelled "rich in protein" requires fortification with high-quality protein ingredients.

Eggs and their derivatives are excellent sources of proteins with complete amino acid profile, high digestibility, and versatility for various food formulations. Moreover, the egg protein hydrolysates are advantageous ingredients due to their reduced antigenic properties and high antioxidant activity (Brumă (Călin) et al., 2024). In powdered state, eggs are easy to process, store and can contribute to the prevention of malnutrition among vulnerable populations, being a practical solution for low- and middle-income countries (Pirkwieser et al., 2022).

The egg proteins have valuable functional properties. The whole egg (WE) proteins have

good gelling ability, egg yolk (EY) have great emulsifying properties, whereas the egg whites (EW) are appreciated for the excellent foaming capacity (Mine, 2002).

Food manufacturers prefer to use pasteurized egg products over fresh eggs to avoid safety issues, and because of their reduced cost, and simplicity in handling and storage. The pasteurized liquid eggs and egg powders are more frequently used in processed and precooked foods. WE are used especially used for preparing baked products, mayonnaise, different type of pasta and pastries (Miranda et al., 2015). According to a recent review of Matsuoka and Sugano (2022), EW proteins have a score of 100 for amino acids, and serves many health purposes. Because of the good bioavailability, high levels of essential amino acids, and exceptional foaming, coagulating, gelling and emulsifying properties, EW are used as a key ingredient for obtaining a large variety of food products (Chang et al., 2018; Razi et al., 2023; Li et al., 2024). EY have historically been used mostly in cakes, mayonnaise, and other baked goods, due to their superior gelling and emulsifying properties (Xiao et al., 2021).

Many commercially products that contain eggs are available in the market (puddings, deserts, etc.). Compared to products made with traditional recipes, the egg-based products made with new or modified recipes have a much smoother and more refined texture (Asai et al., 2022). The production of protein enriched puddings with desired texture and stability can benefit from the special gel structure of EW, which can be mainly assigned to the albumen proteins.

Khatun et al. (2018) developed puddings supplemented with different amounts of egg proteins, and concluded that products with the highest amount of eggs exhibited the best nutritional value. On the other hands, the analysis of physical and organoleptic properties showed that puddings with lower eggs content were better accepted by the consumers, due to the more pleasant odour, colour, consistency, and texture properties (Khatun et al., 2018).

The role of the EY in the structure formation of creamy custard pudding was investigated by Nomura et al. (2012). They reported that EY contributed to the structural stability of the pudding, as well as heating and increasing the content of milk fat cream (Nomura et al., 2012).

The aim of this study was to develop a high protein content pudding, enriched with egg protein hydrolysates. The supplementation of the starch-based pudding with egg protein hydrolysates was performed in such a way as to ensure a final protein content higher than 16%. The proposed pudding can become an attractive alternative for consumers, contributing to a balanced diet that combines nutritional benefits with sensory acceptability.

MATERIALS AND METHODS

Materials

Three types egg-based products in powder form (whole eggs – WE - Ovomix P5001 LF, egg yolk – EY - P6003 and egg white – EW - Ovomix P4000H) were purchased from Ovostar LTD (Kiev, Ukraine). The following enzymes (Merck, Darmstadt, Germany) were used to obtain the egg protein hydrolysates: proteinase K (30 mAnsonU/mg), trypsin (0.2 FIP-U/mg) and pepsin (0.7 FIP-U/mg).

The sunflower oil (Spornic, Prutul SA, Galati, Romania) was used to prepare emulsions. The commercially available vanilla-flavoured pudding powder (Belbake, Lidl Discount SRL Galati, Romania) was used as basis for developing of a new product with a high protein content. The following ingredients were additionally used to prepare the vanilla-flavoured puddings: refined white sugar and cow milk of 1.5% fat, both purchased from Lidl Discount SRL, Galati, Romania.

Preparation of Egg Protein Hydrolysates

Pepsin, trypsin and proteinase K were used to hydrolyse the EY, EW and WE protein derivatives over 24 h at 37°C, as previously reported by Brumă (Călin) et al. (2024). The degree of hydrolysis of the egg protein derivatives with various tested enzymes ranged between 2 and 20%, as indicated by Brumă (Călin) et al. (2024). The obtained protein hydrolysates were subjected to freeze drying prior to analysis. The codification of the protein hydrolysates is provided in Table 1. For each type of analysis, a control consisting on the unhydrolyzed protein derivative was prepared.

Table 1. Samples codification and chemical composition

Codification of the hydrolysates prepared with				Chemical composition (%)	
Control	Pepsin	Trypsin	Proteinase K	Proteins	Fats
EY	EY-P	EY-T	EY-PK	35.4	51.0
EW	EW-P	EW-T	EW-PK	82.0	0.03
WE	WE-P	WE-T	WE-PK	48.1	43.9

Physical and Functional Characterization of the Protein Hydrolysates

The colour properties (lightness L*, parameter a* and parameter b*) of the samples were measured with the Chroma Meter CR410 (Konica Minolta Sensing Americas Inc., Ramsey, NJ, USA). The colour differences in respect to the corresponding controls (ΔE) and chroma (C*) were calculated as mentioned by Brumă (Călin) et al. (2023).

The water activity (a_w) of the samples was measured using a Fast Lab aw meter (GBX Instruments, Loire, France).

The foaming properties, emulsifying activity index (EAI) and emulsion stability index (ESI) were determined according to Brumă (Călin) et al. (2023). The emulsions prepared with 0.5 sunflower oil fraction and 6% protein or hydrolysate solution were characterized in terms of rheological properties using the AR 2000ex rheometer (TA Instruments, New Castle, DE) with a cone-plate geometry. Small amplitude oscillatory strain sweeps (frequency of 0.4 Hz and strain of 0.1-100%) were first carried out to check and viscoelastic regions of the samples, and then frequency sweeps (0.1 to 100 Hz) were employed to characterize the emulsion samples.

Pudding Preparation and Characterization

The vanilla-flavoured pudding was supplemented with protein derivatives and hydrolysates prepared with proteinase K at such level to obtain final products which can be labelled as "source of protein" or "rich in protein".

The puddings were prepared in the laboratory following the instructions provided by the manufacturer. The ingredients were mixed using a Braun mixer (De'Longhi, Neu Isenburg, Hessen, Germany) for 2 minutes at the highest speed, then the mixture was heated on a water bath for 2 minutes.

The ingredients used to obtain the vanilla-flavoured pudding used as control (Table 2) are: 100 ml milk, 6.93 g refined white sugar, 6.41 g vanilla-flavoured pudding powder.

The supplementation level of the control sample with egg derivatives or their hydrolysates varied with the type of ingredient (Table 2). The energy value (EV, kcal/100 g) and the percentage arising from the protein content in case of each variant was theoretically calculated, depending on the intake of each ingredient.

Table 2. Ingredients used to prepare vanilla-flavoured pudding samples with high protein content (P – control pudding, P-WE, P-EY, and P-EW –pudding enriched with whole eggs, egg yolk and egg white powder, respectively; P-WE-K, P-EY-K and P-EW-K puddings enriched with protein hydrolysates prepared with proteinase K from whole egg, egg yolk and egg white, respectively)

Codification of pudding samples	Ingredients									Energy value, kcal/ 100 g	% of total energy value assigned to proteins
	Milk, ml	Sugar, g	Pudding powder, g	WE, g	EY, g	EW, g	WE-K, g	EY-K, g	EW-K, g		
P	100	6.93	6.41	-	-	-	-	-	-	91	13.81%
P-WE	100	6.93	6.41	4.16	-	-	-	-	-	115	17.33%
P-EY	100	6.93	6.41	-	5.64	-	-	-	-	124	16.00%
P-EW	100	6.93	6.41	-	-	2.43	-	-	-	99	20.20%
P-WE-K	100	6.93	6.41	-	-	-	4.16	-	-	115	17.33%
P-EY-K	100	6.93	6.41	-	-	-	-	5.64	-	124	16.00%
P-EW-K	100	6.93	6.41	-	-	-	-	-	2.43	99	20.20%

Characterization of Pudding Samples

The evaluation of the vanilla-flavoured pudding samples was performed after tempering the pudding samples to room temperature, and was carried out by determining the colour properties, as described before. In order to estimate the tendency of the samples to exhibit syneresis behaviour, the liquid released by the gel network was measured while subjecting it to centrifugation for 10 min at 3500 rpm.

The sensory analysis of pudding samples was performed by 11 panellists, in agreement with the decision by the Dunarea de Jos University Ethics Commission no. 38/15 July 2025, and was focused on comparing the fortified products with the control sample.

The following essential sensory characteristics for the final acceptability of the product were assessed by the panel: appearance, consistency, taste, odour, creaminess, mealiness, mouthfeel, adherence to the teeth (stickiness), aroma and aftertaste. The evaluation of sensory attributes for each sample was performed using a rating system from 0 (absent) to 5 (very strong). The samples were served to the panellists in a specially designed testing area, and were assessed at room temperature (20°C). The panellists evaluated the samples and were instructed to rinse their mouths with water between samples to minimize any residual effect. Experimental samples were performed in duplicate and measurements were made at least twice.

Statistical analysis

Duplicate measurements were performed on each sample and the results are presented as mean ± standard deviation values. Statistical analysis was carried out with Minitab v19 (Minitab Inc., State College, PA, USA) software. Significant differences among samples were determined using the one-way ANOVA method, and Tukey test, at 95% confidence.

RESULTS AND DISCUSSIONS

Colour Properties of Protein Hydrolysates

The results of the colour parameters are presented in Table 3. As expected, EY presented the lowest L* value of 79.88±0.01 and the highest C* of 47.47±0.06 among all tested egg protein derivatives. Strong yellow tones were also identified on EY (b* of 46.95±0.06). On the other hand, EW presented the lowest C* of 17.29±0.01, the highest lightness (L* of 97.78±0.02) and a* value of -3.18±0.01, suggesting the presence of mild green tones.

The WE and EY hydrolysates presented higher L* values compared to the corresponding control samples, meanwhile the EW-derived hydrolysates showed lower lightness. When comparing with the controls, the a* (red-green) and b* (yellow-blue) chromatic coordinates showed significantly lower values ($p< 0.05$), suggesting an overall change in colour tones after enzymatic hydrolysis. The least importance colour change was noticed in case of WE-P (ΔE of 5.75 ± 0.13), while EY-T had the highest ΔE of 24.38 ± 0.00 (Table 3). In addition, C* decreased in all enzymatically hydrolysed samples compared with the corresponding controls. The most significant decreases were observed in case of EW hydrolysates, with C* decreasing from 17.29±0.01, specific to the control sample, to 4.36±0.01 in case of hydrolysate produced with trypsin. The modification of the size of the molecules and particles in the hydrolysate samples can influence their distribution and can cause the colour changes. Moreover, particle structure may be modified upon enzymatic hydrolysis, which could increase pigment oxidation caused by light (Gu et al., 2021; Shen et al., 2020).

Table 3. Colour properties and a_w of whole egg (WE), egg yolk (EY) and egg white (EW) powders, before and after enzymatic hydrolysis with pepsin (P), trypsin (T) and Proteinase K (PK)

Samples	L*	a*	b*	ΔE	C	a_w
WE	88.95 ±0.04^{d}	3.07 ±0.01^{a}	28.89 ±0.07^{a}	-	29.05 ±0.07^{a}	0.258 ±0.001^{c}
WE-P	89.76 ±0.14^{c}	-0.95 ±0.04^{b}	24.86 ±0.20^{b}	5.75 ±0.13^{c}	24.88 ±0.20^{b}	0.205 ±0.003^{d}
WE-T	94.44 ±0.00^{a}	-1.25 ±0.01^{d}	14.82 ±0.00^{c}	15.71 ±0.00^{b}	14.87 ±0.00^{c}	0.403 ±0.002^{b}
WE-PK	93.99 ±0.01^{b}	-1.10 ±0.02^{c}	13.82 ±0.01^{d}	16.43 ±0.02^{a}	13.86 ±0.01^{d}	0.440 ±0.001^{a}
EY	79.88 ±0.01^{c}	6.99 ±0.03^{a}	46.95 ±0.06^{a}	-	47.47 ±0.06^{a}	0.383 ±0.001^{b}
EY-P	77.72 ±0.02^{d}	3.43 ±0.01^{b}	35.15 ±0.00^{b}	12.51 ±0.01^{c}	35.32 ±0.00^{b}	0.372 ±0.003^{c}
EY-T	90.9 7±0.00^{a}	0.75 ±0.04^{c}	26.16 ±0.01^{c}	24.38 ±0.00^{a}	26.17 ±0.01^{c}	0.497 ±0.001^{a}
EY-PK	86.12 ±0.09^{b}	0.48 ±0.03^{d}	25.90 ±0.04^{d}	22.90 ±0.02^{b}	25.90 ±0.04^{d}	0.361 ±0.001^{d}
EW	97.78 ±0.02^{a}	-3.18 ±0.01^{d}	17.00 ±0.01^{a}	-	17.29 ±0.01^{a}	0.286 ±0.001^{d}
EW-P	92.33 ±0.04^{d}	-1.11 ±0.01^{c}	8.75 ±0.01^{b}	10.11 ±0.02^{c}	8.81 ±0.01^{b}	0.368 ±0.002^{c}
EW-T	97.46 ±0.04^{b}	-0.90 ±0.00^{b}	4.27 ±0.01^{d}	12.94 ±0.01^{a}	4.36 ±0.01^{d}	0.426 ±0.004^{a}
EW-PK	95.53 ±0.02^{c}	-0.70 ±0.00^{a}	6.06 ±0.02^{c}	11.45 ±0.02^{b}	6.10 ±0.02^{c}	0.384 ±0.004^{b}

ΔE – colour difference; C – colour saturation; Water activity – a_w. Mean values within the same column followed by different lowercase letters (a, b, c, d) are significantly different ($p < 0.05$), based on Tukey's test.

Regardless of the egg protein substrate, our results, in terms of ΔE values, suggest better stability of the colour when pepsin was used for hydrolysis. Higher ΔE value suggests higher degree of browning. It is also important to note that the colour change was influenced by both the substrate and enzyme used for preparing the hydrolysates. We can observe a significant loss of natural colour in case WE-T and WE-PK samples, which exhibited the increase of L* and the decrease of C*. The colour changes are very

important for industrial applications because egg powders are frequently evaluated based on their colour. Therefore, extensive hydrolysis with some proteases may limit the use of egg proteins hydrolysates as functional ingredients in food systems where appearance is important, such as baked goods or instant mixes.

Although the EY displayed the strongest colour intensity, proteolysis significantly changed the colour particularly when using trypsin (ΔE of 24.38±0.00). These changes might be caused by structural reorganizations of lipoproteins and protein complexes which are abundant in the yolk and highly susceptible to proteolytic cleavage (Xu et al., 2024).

Our observations agree with Liu et al. (2023), who showed that lightness and chromatic coordinates were affected by the structural modifications and pigment degradation caused by enzymatic hydrolysis. The EW hydrolysates showed increased green tones (lower a* value) and EY hydrolysates showed higher ΔE values and decreased C* values (Liu et al., 2023). As a result, both sets of data consistently point to enzymatic hydrolysis as a key contributor to colour changes of the egg protein derivatives.

Water Activity of the Protein Hydrolysates

Water activity (a_w) has been recognized as a crucial factor for food safety and quality, as well as for the functional stability of powders made from eggs, both in their natural and enzymatically hydrolysed forms.

Egg powders have low moisture content and a_w level below 0.6, which contribute to their microbiological stability (Farakos et al., 2013). The a_w values of the egg powders used in the experiment were 0.258±0.001 for WE powder, 0.383±0.001 for EY powder and 0.286 ± 0.001 for EW powder (Table 3). The higher a_w registered for EY powders compared to WE and EW samples might be due to the higher content of lipids, which interact differently with water molecules during dehydration. Upon enzymatic hydrolysis we observed some changes in a_w which suggesting that peptide release and structural disruption may have an impact on the water binding capacity.

Our results agree with Obara et al. (2006) who reported a_w values of 0.320 for spray-dried WE powder and 0.330 for EY powder, while Rao et al. (2013) determined a_w of 0.380 for EW powder and 0.270 for EY powder. The products can be considered safe from a microbiological perspective, because the pathogenic bacteria are generally unable to grow at a_w below 0.6 (Enache et al., 2017).

Foaming Properties of the Protein Hydrolysates

The functional properties of the egg protein derivatives and hydrolysates used in this study were evaluated by assessing their foaming properties (Figure 1), emulsifying activity and the rheological behaviour of the emulsions.

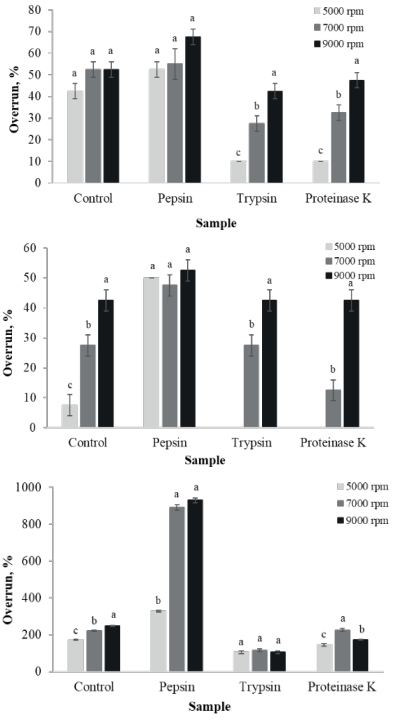

Figure 1. Effect of enzymatic hydrolysis with pepsin, trypsin and proteinase K, and of homogenization speed, on the foaming properties of whole egg (a), egg yolk (b) and egg white (c) proteins. Different lowercase letters placed on top of the bars indicate significant differences among results obtained under different homogenization speeds for the enzyme used for hydrolysis ($p < 0.05$), according to Tukey's test at a 95% confidence level.

Foams are colloidal systems of high importance for the texture, mouthfeel and flavour of the products. They are created by air bubbles dispersed in solid or water-soluble phases. However, they are thermodynamically unstable due to their high mechanical energy requirements (Razi et al., 2023).

Among all tested protein derivatives, the EW showed the highest overrun (Figure 1). The good foaming properties of the EW are due to their structural flexibility and quick diffusion to the air-water interface. On the other hand, the significantly lower foaming characteristics of WE and EY protein-based samples can be explained by their higher lipids content, which not only competes with proteins for interfacial adsorption, but also destabilizes the air–water interface, making foaming and foam stabilization more difficult.

The results presented in Figure 1 illustrates that all the egg protein samples hydrolysed with pepsin, trypsin and Proteinase K exhibited improved overrun when the homogenization speed was increased from 5000 to 7000 and 9000 rpm. This trend suggests better air incorporation into the protein solutions can improve stability and foam formation. Higher shear rates can accelerate the protein adsorption and is essential for foaming process. This finding aligns with Dumitraşcu et al. (2023), who reported that increasing the homogenization speed from 5000 to 9000 rpm notably improved the foaming capacity of the protein suspensions.

WE sample presented better foaming properties when homogenization was done at lower speed of 5000 rpm compared to the WE-T and WE-PK (Figure 1a). The extensive proteolysis negatively affected the overrun which was lowered by 76% and the foam stabilization of WE proteins. This might be the result of the inferior interfacial activity of the smaller peptides. Pepsin hydrolysis had no significant influence on the foaming properties compare to the control. This observation indicates that structural modifications induced by pepsin cleavage might not always result in functional changes for WE proteins.

It was observed that when the homogenization speed increased from 5000 to 9000 rpm, samples hydrolysed with trypsin and proteinase K demonstrated a noticeable increase of foaming ability. Additionally, proteinase K hydrolysates at 9000 rpm achieved foaming values that were comparable to or even higher than the control sample, suggesting that even highly hydrolysed proteins can restore or enhance their technological functionality under ideal processing conditions.

The increase of the homogenization speed allowed significant improvement ($p < 0.05$) of the foaming properties of EY proteins and hydrolysates as well. At lower homogenization speed of 5000 rpm, only EY-P exhibited foaming ability (Figure 1b).

The results shown in Figure 1c demonstrate that, regardless of the homogenization speed, the EW-P exhibited exceptional foaming properties compared with the control. On the other hand, the EW treatment with trypsin and proteinase K affected negatively the foaming capacity of EW proteins.

Chen et al. (2012) showed that the trypsin assisted hydrolysis of EW proteins up to hydrolysis degree of 12% affected the foaming and emulsifying properties of the proteins. The release of smaller peptides through enzyme assisted hydrolysis might affect the adsorption and migration at the air-water interface, and therefore the functional properties.

Razi et al. (2023) found that enzymatic treatment of EW proteins typically resulted in a decrease in foaming capacity. They attributed this result to the partial protein cleavage and the consequent alterations in interfacial activity. The hydrolysates samples had more stable foam than the controls. This means that the smaller peptides might be able to move around at the air-water interface more easily, stabilising the foam. Liu et al. (2023) showed that enzymatic hydrolysis significantly reduced the stability and foaming capacity of the EW proteins.

Emulsifying Ability of the Protein Hydrolysates

The emulsifying activity (EAI) and emulsion stability index (ESI) of the egg protein derivatives and their hydrolysates (Table 4) are important functional properties that indicate their suitability for various complex food systems.

In the present study, the enzymatic hydrolysis of egg proteins (WE, EY and EW) caused important changes in EAI and ESI (Table 4). The hydrolysates obtained with pepsin exhibited superior emulsifying properties compare to the control samples. This might be the result of limited protein proteolysis leading to more flexible peptides, with better ability to adhere to the surfaces. On the other hand, the extensive hydrolysis with trypsin and proteinase K led to lower emulsifying capacity, likely due to the

prevalence of low-molecular-weight peptides that cannot create cohesive and elastic interfacial layers. In agreement with the literature, our results suggest that the interfacial film strength and emulsion stability is influenced by peptide chain length (Klompong et al., 2007; Peighambardoust et al., 2021).

Table 4. Emulsifying activity index (EAI) and emulsion stability index (ESI) values of emulsions prepared from whole egg (WE), egg yolk (EY) and egg white (EW) proteins, before and after enzymatic hydrolysis with pepsin, trypsin and Proteinase K

Sample	EAI, m^2/g	ESI, min
WE	47.99±1.86^{a}	ND
WE-P	17.52±0.40^{b}	38.58±1.59^{a,b}
WE-T	4.80±0.08^{c}	17.76±0.03^{b}
WE-PK	21.20±4.49^{b}	68.87±17.95^{a}
EY	38.24±2.21^{b}	108.53±3.11^{a}
EY-P	59.90±3.20^{a}	ND
EY-T	20.24±0.27^{c}	37.42±2.65^{b}
EY-PK	32.62±4.77^{b,c}	ND
EW	11.38±0.16^{a}	62.66±1.36^{b}
EW-P	9.71±0.68^{b}	86.38±9.68^{a}
EW-T	4.72±0.04^{c}	23.11±0.07^{c}
EW-PK	4.28±0.04^{c}	20.38±0.40^{c}

ND – not determined due to the occurrence of coalescence. Mean values within the same column followed by different lowercase letters indicate significant differences among results obtained for the same substrate ($p < 0.05$), according to Tukey's test.

Enzymatic hydrolysis generally affected EAI by reducing peptide size and decreasing interfacial adsorption efficiency (Liu et al., 2023). Our results demonstrated a similar trend in case of trypsin and Proteinase K hydrolysates, in which case EAI values were lower than the control samples. When using EY proteins as substrate, the pepsin assisted hydrolysis resulted in increased EAI, unlike the results presented by Liu et al. (2023). This difference could be explained by the variability in the hydrolysis conditions, lipid-protein interactions in yolk systems or the specificity of the enzymes.

A different trend in terms of emulsifying properties was observed in case of the hydrolysates produced from WE and EW. Among all tested samples, the EW-PK and EW-T had the lowest EAI of 4.28±0.04 m^2/g, and 4.72±0.04 m^2/g, respectively, which was more than 50% lower than in case of EW (11.38±0.16 m^2/g). A similar trend was observed in the ESI results, with some exceptions. The WE-PK resulted in lower EAI (21.20±4.49 m^2/g), even if the emulsion was the most stable (68.87±17.95 min). The stability of the EW-T and EW-PK based emulsions was very low (23.11±0.07 min and 20.38±0.40 min, respectively). The most stable was the emulsion prepared with EW-P (86.38±9.68 min), significantly higher compared to the control (62.66±1.3 min).

Our research results demonstrate that the emulsifying properties of the egg protein hydrolysates highly depend on the substrate and enzyme.

Chen et al. (2012) showed that the emulsifying capacity was greatly reduced due to the extensive disruption of protein – protein interactions caused by proteolysis. The research made by Razi et al. (2023) validates that enzymatic hydrolysis of EW proteins can reduce EAI. Moderate hydrolysis can enhance ESI by increasing peptide flexibility, consistent with findings from a study examining EW peptides released with pepsin and WE with Proteinase K. Both studies demonstrated that hydrolysis with trypsin significantly reduced EAI, due to the inadequate migration of smaller peptides at oil-water interfaces. The findings indicate that Proteinase K hydrolysates enhance ESI, whereas yolk hydrolysates obtained with pepsin exhibited higher EAI, but led to unstable emulsions.

The investigation made by Ai et al. (2019), who examined the effects of six different proteases on EW proteins, demonstrated that ESI and EAI depended on pH of the sample. The enzymatic hydrolysis conducted with Flavourzyme at pH 3.0 produced the highest EAI value of 2.78±0.08 m^2/g. In case of trypsin hydrolysates obtained at pH 5.0, the lowest EAI value of 0.21±0.02 m^2/g was obtained. The peptides are more soluble and have a higher surface charge at low pH (Ai et al., 2019).

Gao et al. (2019) used the neutral and alkaline proteases for testing the influence of hydrolysis on the emulsifying and thermal stability of EY proteins. They demonstrated that alkaline protease led to more stable and heat-resistant hydrolysates, because of the reduced molecular weight of the peptides. The stability of the emulsion was due to the soluble proteins and peptides laid at the oil-water interface. The structural changes occurring after hydrolysis made the molecules more flexible, with better ability to integrate on the interfacial layers and to prevent coalescence. This indicate that proteases may enhance the functionality of yolk proteins in food emulsions (Gao et al., 2019).

Rheological Behaviour of the Emulsions Prepared with Protein Hydrolysates

The rheological parameters collected upon running oscillatory strain and frequency sweep tests on the emulsions prepared with egg protein derivatives and hydrolysates are presented in Table 5. These measurements provide valuable insights into the mechanical behaviour of the emulsions and are directly related to the strength and stability of the interfacial films formed during emulsification. Analysis of the strain sweep tests (Table 5) revealed that emulsions prepared with control samples exhibited a predominantly elastic response, as it is indicated by storage modulus (G′) values exceeding those of the loss modulus (G″). This behaviour is consistent with the formation of a more structured and interconnected protein network at the oil–water interface. In this way they exhibit better capability of resisting to deformation and contribute to the improved stability. Such elastic-dominant systems are generally associated with stable emulsions that can withstand mechanical stress and gravitational separation.

Table 5. Rheological parameters recorded during strain and frequency sweep tests performed on emulsions prepared from whole egg (WE), egg yolk (EY) and egg white (EW) proteins, before and after enzymatic hydrolysis with pepsin, trypsin and Proteinase K

Emulsion samples	Strain Sweep Test	Frequency Sweep Test
		G* (Pa) la 1 Hz
WE	G' > G''	0.374±0.029^{a}
WE-P	G'' > G'	0.128±0.009^{b}
WE-T	G'' > G'	0.084±0.011^{b,c}
WE-PK	G'' > G'	0.054±0.007^{c}
EY	G' > G''	0.057±0.007^{a}
EY-P	G'' > G'	0.050±0.005^{a,b}
EY-T	G'' > G'	0.053±0.006^{a,b}
EY-PK	G'' > G'	0.031±0.001^{b}
EW	G' > G''	0.132±0.014^{a}
EW-P	G'' > G'	0.122±0.016^{a}
EW-T	G'' > G'	0.091±0.019^{a,b}
EW-PK	G'' > G'	0.051±0.001^{b}

Mean values within the same column followed by different lowercase letters indicate significant differences among the results obtained for the same substrate subjected to enzymatic hydrolysis ($p < 0.05$), according to Tukey's test.

Emulsions samples prepared with egg proteins hydrolysate displayed predominantly viscous behaviour, with G″ values higher than G′ values (Table 5). This phenomenon suggests that the peptide bonds hydrolysis effected the protein network at the interface, making it less stable and less able to resist deformation. In this way the emulsions acted more like liquid systems having also a limited ability to recover their structure after shear.

The overall resistance of the emulsions to deformations was also assessed through the complex modulus G* (Figure 2), whose value was calculated based on the moduli G' and G''. Thus, the G* modulus integrate both the non-recoverable and recoverable components for each sample (Dumitrașcu et al., 2023).

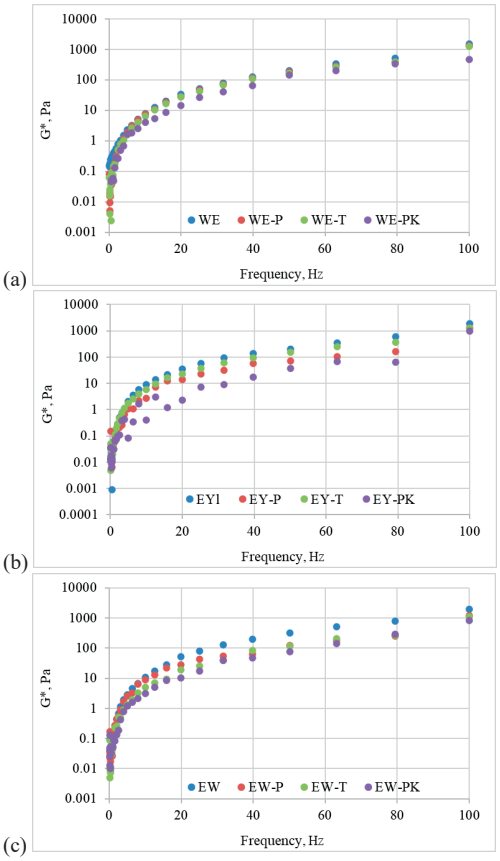

Figure 2. The evolution of the complex modulus G* as a function of frequency for emulsions prepared from (a) whole egg (WE), (b) egg yolk (EY) and (c) egg white (EW) proteins, before and after enzymatic hydrolysis with pepsin, trypsin and Proteinase K

For all tested emulsions, the results presented in Figure 2, indicate the high dependence of the G* values on the frequency. As expected, the emulsions prepared with egg protein hydrolysates exhibited lower G* values compared to the corresponding control samples.

Characterisation of the Pudding Samples

The egg protein derivatives and hydrolysates prepared with proteinase K were further used to enrich the vanilla flavoured puddings (Figure 3). Our previous investigations revealed that the use of proteinase K for egg proteins hydrolysis allows obtaining egg protein-based ingredients with improved antioxidant activity and low allergen levels (Brumă (Călin) et al., 2024).

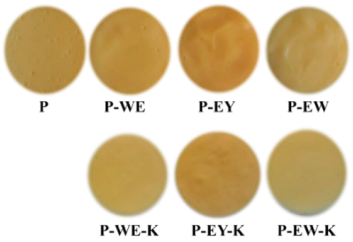

Figure 3. Appearance of the vanilla-flavoured pudding samples (P) enriched with whole egg (WE), egg yolk (EY) and egg white (EW), before and after enzymatic hydrolysis with Proteinase K (WE-K, EY-K and EW-K)

As it can be seen in Table 2, the fortified samples showed significant increases in energy values assigned to the protein content, from 13.81% (control) to 20.2% (fortified products). Of particular importance are the pudding enriched with egg white proteins or hydrolysates, which exceeded the threshold of 20% of energy arising from protein, and could be legally labelled as "rich in proteins", in agreement with EC Regulation 1924/2006.

The colour is directly related to the consumer opinion concerning freshness and quality. The colour properties of the pudding samples are presented in Figures 3 and 4. The obtained results indicated that the addition of various proteins and hydrolysates affected the appearance of the pudding samples ($p < 0.05$).

No significant difference in terms of lightness (L*) was observed when supplementing the control with WE (Figure 4). The addition of EW-K resulted in puddings with higher brightness (L* of 72.47±0.44), meanwhile the control sample (P) presented the darkest colour (L* of 69.04±0.07). A different trend was observed when EY was used for pudding supplementation: higher brightness was observed in case of P compared to samples P-EY and P-EY-K samples (L* of 66.42±0.02, and 68.15±0.00 respectively). The brightness reduction when adding egg yolk is most probably due to the presence of pigments (e.g. carotenoids).

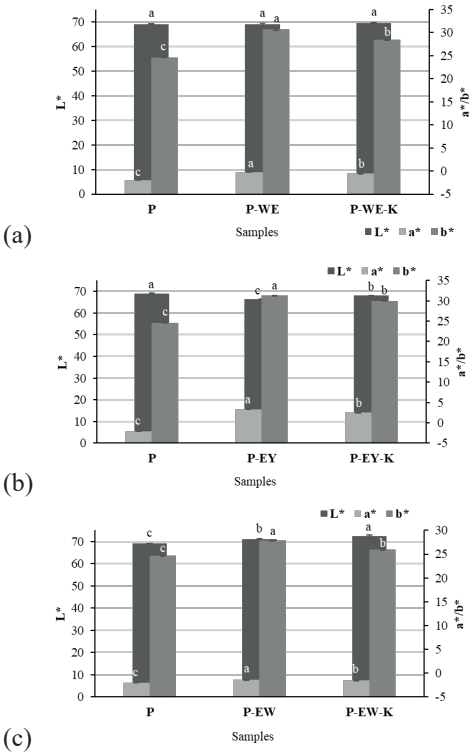

Figure 4. Colour properties of pudding samples (P) enriched with (a) whole egg (WE), (b) egg yolk (EY) and (c) egg white (EW) proteins, before (Control) and after enzymatic hydrolysis with Proteinase K

Regarding the a* and b* parameters indicating the presence of red-green and yellow-blue tones, respectively, the overall intensity of the analysed samples decreased compared to the control sample. P-EY presented the highest values of the parameters a* of 3.25±0.00 and b* of 31.33±0.04. The highest a* values were obtained when supplementing the pudding with EY proteins or hydrolysates, because of the presence of carotenoids, such as canthaxanthin or astaxanthin, which can induce reddish hues, increasing the a* value in the CIELab system and slightly reducing the L* (luminosity) (Dansou et al., 2023). The intensity of the colour can vary depending on the concentration of the carotenoids (Dansou et al., 2023; Kojima et al., 2022).

The a* parameter registered for the samples enriched with EY and EY-PK indicate the presence of red tones. The negative a* values of the P, P-WE, P-WE-K, P-EW, P-EW-K indicate the presence of green tones. All pudding samples presented positive values of the b* parameter, indicating the presence of yellowish tones, more intense in the case of P-EY or P-EY-K. Eggs contain carotenoids, natural pigments that give the yolk shades from pale yellow to deep orange (Abeyrathne et al., 2022). Carotenoids, such as lutein, zeaxanthin and β-carotene, influence the colour of the yolk in the CIELAB system. The b* parameter (yellow hue) can increase significantly due to yellow-orange pigments, and a* can indicate reddish shades depending on the type of carotenoid (e.g. canthaxanthin).

It was observed that when compared to the control sample (P) the total colour differences (ΔE) are greater than 3, which indicates visible colour differences. The sample P-EY presented the highest total colour difference (ΔE of 8.93±0.02), being followed by the P-WE (ΔE of 6.34±0.42). The smallest total colour differences (ΔE of 4.10±0.07, 3.83±0.12, and 3.71±0.47) are observed in samples P-WE-K, P-EW and P-EW-K respectively. Also, the colour saturation (C*) of vanilla-flavoured pudding samples reflects a higher colour intensity compared to the control sample.

The liquid retention capacity of the control pudding (P), which includes corn starch as the main component, was rather good. The P samples released only 2.14±0.10% of liquid when subjected to centrifugal forces. Anyway, the supplementation of the pudding samples with protein derivatives (WE, EY, EW) and WE-K resulted in excellent water retention capacity, with no liquid separated on the top of the centrifuged samples. These results are supported by the study of Scott et al. (2023), who conclude that protein-starch interactions significantly influence the water retention in food products. On the other hand, P-EW-K and P-EY-K samples exhibited extremely low water release (1.67±0.28 and 1.03±0.01) at the surface of the centrifuged sample. Anyway, these puddings presented significantly better water retention capacity compared to the control. Thus, the use of hydrolysed egg proteins, in combination with starch, optimizes the texture and stability of the pudding, reducing starch retrogradation and improving the quality of the final product (Scott et al., 2023).

One of the main functional characteristics of EW proteins is their ability to form gels following heat treatment. Egg proteins might unfold during the thermal treatment, becoming prone to aggregation and forming dense three-dimensional network. This has a good contribution to gelation properties, especially for the texture of protein-enriched puddings. The gelation process can be influenced by protein composition and processing conditions. The particular ability of EW proteins to form stable gels might contribute to improving the sensory qualities of the pudding products, making them valuable (Razi et al., 2023). Gelling property contributes to improving the consistency of food products, facilitating the retention and controlled release of nutrients and flavours, while providing a specific texture to foods (Su et al., 2015). The study conducted by Su et al. (2015) demonstrated that EW, when mixed with soy protein isolate, significantly contributed to increasing the water retention capacity, especially when the ratio between the two proteins is 1:1. This results in the formation of a compact and uniform three-dimensional network, which reduces liquid loss and maintains the stability of the gel over time. Wang et al. (2020) showed that EW proteins glycosylation contributed significantly to improving gelling properties and water retention capacity of the products. These molecular modifications lead to a more stable three-dimensional network, with better capability to retain water (Wang et al., 2020).

The results of the sensory evaluation of vanilla-flavoured pudding samples supplemented with egg proteins and hydrolysates are presented in Figure 5. Sensory attributes like consistency, creaminess, and overall appearance were better appreciated by the panellists on the pudding samples enriched with egg proteins, compared to the samples including protein hydrolysates (Figure 5).

The control pudding was preferred by the consumers. These results can be explained by the more homogeneous texture and a balanced sensory profile, being therefore preferred by the panellists, although the serum retention capacity was poorer (2.22%) compared to the egg protein-enriched samples.

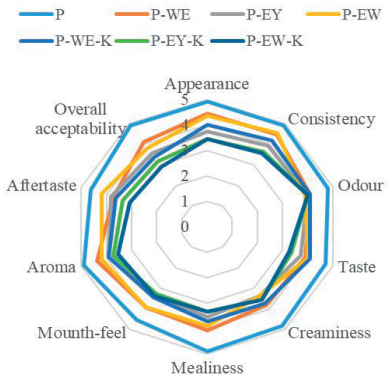

Figure 5. Sensory characteristics of vanilla-flavoured pudding samples(P) enriched with whole egg (WE), egg yolk (EY) and egg white (EW), before and after enzymatic hydrolysis with Proteinase K (WE-K, EY-K and EW-K)

Among the samples enriched with egg proteins, P-EW obtained the highest values for most of the sensory attributes, as follows: consistency (4.55), appearance (4.36), aroma (4.18), aftertaste (4.18) and pleasant taste (4.00). These results suggest that the use of non-hydrolysed EW might contribute favourably to the sensory profile of the pudding, providing a creamy texture and a pleasant aroma, without negative influences on the taste. The fact that, following the water retention capacity analysis, the released water content was not detectable, the sample has a stable texture, which was appreciated by the panellists.

CONCLUSIONS

The hydrolysis of the whole egg, egg yolk and white proteins with pepsin, trypsin, and proteinase K caused changes of important functional properties like foaming and emulsifying properties. The foaming properties of the egg white proteins were improved after treatment with pepsin. Proteins from yolk and whole egg exhibited the best emulsifying properties. The pepsin assisted hydrolysis of the egg yolk proteins was the only treatment which led to the improvement of emulsifying properties. The rheological measurements performed on the emulsions indicated the transition from the solid-like to the liquid-like behaviour upon the enzyme assisted hydrolysis of the egg proteins. Egg protein derivatives and hydrolysates prepared with Proteinase K were employed for the fortification of the pudding as such levels to obtain products which can be labelled as “rich in proteins”. The use of EW to fortify the vanilla-flavoured puddings allowed obtaining proteins enriched products with the most pleasant sensory characteristics among all supplemented samples. The protein enriched samples had creamy consistency, a pleasant appearance and a balanced flavour, being the most appreciated among the samples enriched with egg proteins.

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SILICON-BASED ELICITORS FOR SUSTAINABLE CROP PROTECTION

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Abstract

Silicon and its nano-formulations have emerged as powerful elicitors that activate plant immune responses and improve resistance against a wide range of pathogens in crops. Recent studies demonstrate that silicon-based products are able to penetrate root and leaf tissues, trigger systemic acquired resistance in a dose-dependent manner, and rely on salicylic acid signalling to confer broad-spectrum disease protection. Field and greenhouse trials across diverse species show that these formulations can reduce disease indices through physical reinforcement of cell walls, enhanced production of antioxidant enzymes, and priming of pattern-triggered immunity. In addition, to direct pathogen suppression, Si-based products act as biostimulants that modulate the plant microbiome, lower the need for synthetic pesticides, and thus diminish chemical residues in the environment, aligning with One Health goals that link plant health, ecosystem integrity, and human well-being. This review synthesizes current knowledge on the mechanisms of Si-elicited immunity, evaluates the efficacy of Si-based formulations for post-harvest and field protection of crops, and outlines research gaps and regulatory considerations needed to advance Si technologies as safe, sustainable tools within an integrated One Health framework.

Key words*: crops, elicitors, One Health, pathogens, silicon.*

INTRODUCTION

Global food security is under increasing pressure from a growing world population, climate change, and the mounting environmental costs of intensive agrochemical use. The demand for sustainable alternatives to synthetic pesticides and fungicides has intensified focus on naturally occurring compounds capable of bolstering plant immunity without direct toxic effects on target organisms or non-target species (Savvas & Ntatsi, 2015; Verma et al., 2024). In this setting, silicon (Si) has garnered significant interest from the scientific community and commercial interest as a low-cost, environmentally compatible plant protectant with broad-spectrum efficacy across multiple stress categories. Nano-enabled silicon formulations are now recognised as transformative tools for precision agriculture (Kah et al., 2019; Naidu et al., 2023).

Ranking second in crustal abundance, silicon is ubiquitous in agricultural soils worldwide, yet it is not classified as an essential nutrient for the majority of higher plants (Epstein, 1999; Ma and Yamaji, 2006). Despite this, an extensive body of experimental evidence accumulated over the past two decades demonstrates that silicon supplementation confers significant agronomic benefits, particularly under conditions of biotic and abiotic stress (Debona et al., 2017; Mukarram et al., 2025; Etesami and Jeong, 2018). When absorbed by plants predominantly as monosilicic acid ($Si(OH)_4$), silicon is deposited in epidermal tissues as amorphous silica, where it strengthens cell walls, reduces pathogen penetration, and activates a cascade of biochemical and molecular defence responses (Ma & Yamaji, 2006; Wang et al., 2017).

The evolution of nanotechnology has added a new dimension to silicon's agricultural utility. Silicon dioxide nanoparticles (SiO_2 NPs), mesoporous silica nanoparticles (MSNs), and organosilica nanoplatforms combine the intrinsic biological activity of silicon with advanced delivery capabilities, enabling controlled co-release of immune inducers and fungicides, stomatal penetration, and systemic

priming at field-relevant concentrations (El-Shetehy et al., 2021; Yan et al., 2024; Liang et al., 2025). These nano-enabled formulations represent a frontier in precision agriculture and are increasingly evaluated within integrated pest management (IPM) frameworks and the broader One Health paradigm (Lombi et al., 2019; Schaller et al., 2024). This review synthesises current knowledge on the mechanisms by which silicon-based products elicit plant immunity, evaluates nano-enabled formulations and their efficacy across key crop-pathosystems, explores the role of silicon in supporting soil microbiome health and postharvest protection, and outlines future priorities for research, formulation innovation, and regulatory harmonisation essential to unlock the complete benefits of silicon as a cornerstone of resilient crop protection.

SILICON AS A PLANT ELICITOR

Definition of Elicitors vs. Biostimulants

In plant protection science, elicitors are broadly defined as molecules that, upon recognition by plant cells, trigger defence responses without causing disease. Classical elicitors such as microbe-associated molecular patterns (MAMPs) and damage-associated molecular patterns (DAMPs) are detected by plant pattern recognition receptors, activating the pattern-triggered immunity (PTI) response. Silicon occupies a conceptually distinct but functionally overlapping category: it is neither a pathogen-derived signal nor a classical biostimulant, yet it primes plant defences in a manner that closely resembles induced resistance phenomena (Fauteux et al., 2005; Van Bockhaven et al., 2013). The breadth of silicon's benefits, spanning physical barriers, biochemical signalling, and abiotic stress relief, makes it unique among plant protection inputs (Cao et al., 2025).

The distinction between elicitors and biostimulants is relevant here. Biostimulants are defined by their capacity to improve nutrient use efficiency, tolerance to abiotic stress, and crop quality, independent of their nutrient content. Silicon satisfies most criteria for both categories: it reinforces physical barriers (an elicitor-type effect), activates biochemical signalling pathways (a priming effect), and improves photosynthetic efficiency and water use under stress (a biostimulant effect). Savvas and Ntatsi (2015) were among the first to formally position silicon within the biostimulant framework for horticultural crops, a classification now broadly accepted in the literature.

Silicon uptake and accumulation in plants

The primary route of silicon absorption in plants occurs via monosilicic acid ($Si(OH)_4$) dissolved in the soil solution, with substantial interspecific variation in the efficiency of this uptake process. Silicon (Si) accumulation in plants varies significantly across species. Silicon accumulation reaches its peak in commelinoid monocots within the angiosperm clade, especially among representatives of *Poales* and *Arecales*, where shoot silicon levels far exceed those of other monocot lineages. The *Gramineae* and *Cyperaceae* families within *Poales* are consistently identified as the strongest accumulators. In contrast, most plants, especially dicots, are poor Si accumulators (Kumar et al., 2017; Katz et al., 2021). Cereal crops and grasses, with rice and wheat as prime examples, display a remarkable capacity for silicon accumulation, with tissue concentrations reaching 10% dry weight or more, while many dicotyledonous crops accumulate silicon at lower but still functionally relevant levels (Ma et al., 2002; Deshmukh et al., 2017). Root-level silicon uptake efficiency is the principal factor accounting for these interspecific differences in accumulation.

Following root absorption, silicon is translocated via the xylem stream and subsequently polymerises into amorphous silica bodies (phytoliths), which accumulate in epidermal cells, intercellular spaces, and the sub-cuticular layer. These deposits are metabolically inert but alter the physical properties of plant tissues in ways that have profound implications for resistance to pests and pathogens (Ma &Yamaji, 2006; Kim et al., 2002). The silicon distribution within leaves follows both symplastic and apoplastic pathways, with recent studies clarifying the molecular regulation of local silicon allocation in rice leaf tissues (Coskun et al., 2019).

Integration into Integrated Pest Management (IPM) and One Health

Silicon-based products align closely with the principles of integrated pest management (IPM): prevention over cure, reduced reliance on synthetic chemicals, and preservation of ecosystem services. Because silicon enhances plant resistance through physiological priming rather than direct toxic action, it does not select for pest or pathogen resistance in the manner of conventional pesticides and poses minimal risk to beneficial arthropods, soil organisms, and non-target microorganisms (Reynolds et al., 2016; Denarié et al., 2025).

One Health is a holistic framework that acknowledges the intrinsic connections between plant health, animal welfare, human well-being, and broader environmental integrity, treating them as components of a single, integrated system. Silicon-based elicitors reduce pesticide inputs, limiting chemical runoff and contamination of water bodies, while also strengthening plants without toxic residues, improving food safety and farmworker health. They further support rhizosphere biodiversity, supporting the sustained health of soil systems and agroecosystem resilience (Lombi et al., 2019; Schaller et al., 2024). From a regulatory perspective, silicon is generally regarded as a low-risk input, facilitating adoption within organic and reduced-input production systems (Rajput et al., 2022).

MECHANISMS OF SI-ELICITED IMMUNITY

Physical Reinforcement of Barriers

The most consistently documented mechanism by which silicon enhances plant resistance is the physical reinforcement of cell walls and epidermal tissues. Silicon deposition under the cuticular layer and within epidermal cell wall architecture enhances tissue rigidity, reducing the efficiency of penetration by fungi that rely on mechanical force (e.g., appressorium-generated turgor) or enzymatic degradation (e.g., cell wall-degrading enzymes produced by necrotrophic pathogens) to breach host tissues (Wang et al., 2017; Islam et al., 2020).

Evidence from rice systems indicates that the structural consolidation of cell walls driven by silicon application leads to a significant decrease in appressorial penetration by *Magnaporthe grisea* (Kim et al., 2002; Seebold et al., 2001), while in wheat, silicon accumulation at infection sites impedes the development of *Blumeria graminis* (Bélanger et al., 2003). SiO_2 NPs have more recently been demonstrated to inhibit sporulation and pathogenicity of *Fusarium brachygibbosum* infecting olive trees through membrane disruption and morphological damage (Belhedi et al., 2025).

Beyond fungal pathogens, silicon accumulation increases tissue abrasiveness, reducing the feeding efficiency of chewing insects by increasing the mechanical cost of herbivory (Reynolds et al., 2016; Alhousari & Greger, 2018; Acevedo et al., 2021). The modified surface properties of silicon-amended plants also affect sucking insects by modifying phloem accessibility and increasing the physical resistance of leaf surfaces (Singh et al., 2020; Denarié et al., 2025). Silicon as an IPM component for managing insect pests was comprehensively reviewed by Suganthy et al. (2024), confirming its potential as a pesticide-free alternative in several cropping systems.

Biochemical defence responses

Beyond its structural role, silicon modulates a suite of biochemical defence pathways. Silicon-treated plants consistently show elevated activities of antioxidant enzymes, including superoxide dismutase (SOD), peroxidase (POD), catalase (CAT), and phenylalanine ammonia-lyase (PAL), which collectively mitigate oxidative stress during pathogen attack and maintain cellular homeostasis under biotic unfavourable conditions (Etesami & Jeong, 2018; Liang et al., 2003; Verma et al., 2024). Enhanced production of phenolic compounds and phytoalexins has also been documented in silicon-amended wheat infected with powdery mildew, indicating that silicon promotes secondary metabolite production beyond simple enzyme activation (Rémus-Borel et al., 2005).

Under abiotic adversity, including salinity, drought, and heavy metal exposure, silicon-supplemented plants mount a more robust enzymatic antioxidant response, characterised by heightened SOD, CAT, and APX (ascorbate

peroxidase) activities that work in concert to quench excess ROS (reactive oxygen species) and prevent oxidative cellular damage. These effects overlap substantially with patterns recorded under biotic stress, pointing that silicon primes a generalised stress response network rather than pathway-specific immune circuits (Liang et al., 2007; Ranjan et al., 2021; Mariyam et al., 2024).

Molecular priming and signalling

Silicon operates at the molecular level by sensitising plant immune circuits, simultaneously enhancing PTI responsiveness and triggering SAR (systemic acquired resistance) through signalling pathways in which salicylic acid serves as the principal mediator. Silicon supplementation upregulates SA signalling, driving the expression of pathogenesis-related (PR) proteins and a cascade of downstream defence genes whose coordinated activity underpins the establishment of systemic resistance (Van Bockhaven et al., 2013; Wang et al., 2017). Silicon additionally fine-tunes the reciprocal interactions between SA, JA (jasmonic acid), and ET (ethylene) hormonal circuits, conferring a versatile priming state that renders plants simultaneously fortified against both biotrophic and necrotrophic infection strategies (Ranjan et al., 2021; Song et al., 2021).

Silicon does not function as a classical elicitor by binding to specific pattern recognition receptors, but rather operates as a 'priming agent' that potentiates faster and stronger defence activation upon challenge. This priming effect reduces disease severity without constitutively activating costly immune responses, representing an energetically efficient strategy for enhancing resistance (Fauteux et al., 2005; Coskun et al., 2019). Nano-silicon formulations appear to amplify this priming capacity by enabling more efficient cellular uptake and sustained release of bioavailable silicon, as demonstrated in rice sheath blight models using MSN-based delivery systems (Liang et al., 2025).

Interaction with the Plant Microbiome

Silicon exerts significant indirect effects on plant protective responses through its documented ability to shift the composition of soil microbial assemblages in the root zone. Silicon-treated soils show enrichment of beneficial bacterial taxa including *Sphingomonas* spp. and *Streptomyces* spp., which contribute to disease suppression through the release of bioactive compounds capable of suppressing fungal growth and competition with pathogens residing in the soil matrix (Verma et al., 2022; Song et al., 2021). Biocontrol organisms such as *Trichoderma* spp. and *Pochonia chlamydosporia* are preserved or enhanced in silicon-treated rhizospheres, providing synergistic disease suppression without disrupting existing IPM programmes (Verma et al., 2021).

Applied silicon, whether as soluble silicic acid or SiO_2 nanoparticles, degrades into monosilicic acid and is ultimately incorporated into the natural silicon cycling of the soil, supporting beneficial soil microorganisms and maintaining rhizosphere health rather than accumulating as a persistent pollutant (Mukarram et al., 2022; Schaller et al., 2024). This property is particularly relevant from a One Health perspective, as it ensures that silicon application does not disrupt soil biodiversity or the microbial services upon which sustainable crop production depends.

NANO-ENABLED SILICON FORMULATIONS

Silicon Dioxide Nanoparticles (SiO_2NPs)

Silicon dioxide nanoparticles represent the most extensively studied class of nano-silicon formulations in agriculture. The nanoscale dimensions of SiO_2 NPs - typically ranging from 10 to 100 nm - generate a disproportionately large surface area relative to particle volume, a property that underpins their superior capacity for dissolution, chemical interaction, and translocation within plant tissues when set against conventional silicon sources (Yan et al., 2024; Mukarram et al., 2022). Their reduced particle dimensions enables penetration through stomata and root cell walls, facilitating direct interaction with cellular components and achieving more uniform silicon distribution within plant tissues compared to conventional silicate fertilisers (El-Shetehy et al., 2021).

El-Shetehy et al. (2021) demonstrated in a landmark investigation published in Nature Nanotechnology that hat when SiO_2 NPs were supplied at rates of 50-200 mg L^{-1} enhanced disease suppression capacity in *Arabidopsis thaliana* towards *Botrytis cinerea* and *Pseudomonas syringae* through SAR operating via salicylic acid-dependent regulatory circuits, with efficacy comparable to or exceeding that of chemical resistance inducers. Comparable results have been documented in wheat (Abdelrhim et al., 2021) and rice (Cai et al., 2008), with SiO_2 NPs activating antioxidant enzyme networks and reducing pathogen-induced oxidative damage. The capacity of SiO_2 NPs to prime systemic immunity makes them particularly attractive for foliar application in high-value crops where uniform systemic coverage is difficult to achieve with soil-applied silicon.

Mesoporous Silica Nanoparticles (MSNs)

Silica-based nanostructures featuring a well-defined mesoporous framework constitute characterised by a highly ordered pore structure with large specific surface areas (typically 500-1200 m^2 g^{-1}) and tunable pore sizes, making them excellent nanocarriers for the controlled delivery of agrochemicals, immune inducers, and biological control agents (Liang et al., 2020). MSNs are particularly valued for their dual capability: harbouring immune-priming agents such as salicylic acid (SA) within their mesoporous interior while simultaneously permitting surface functionalisation with stimuli-responsive gatekeeping systems- an advancement that has redefined possibilities in precision crop protection. (Liang et al., 2025).

A particularly innovative development is the MSN-ss-SA system reported by Liang et al. (2025), in which SA is covalently conjugated to disulfide-doped mesoporous silica nanoparticles (MSNs-ss-NH_2) via amide bonds. This architecture enables dual stimuli-responsive release activated by glutathione (GSH) and amidase, both being elevated at infection sites in plant tissues. Given their approximate particle diameter of 90 nm, MSNs-ss-SA can access the internal leaf environment of rice by passing through stomatal pores and deliver SA and bioavailable silicon concurrently, significantly enhancing resistance to rice sheath blight by activating physical barrier formation, antioxidant defence, and key immune genes including CsNPR1, CsPR1, CsERF004, and CsWRKY50.

Organosilica nanoplatforms

Organosilica nanoplatforms extend the capabilities of conventional MSNs by embedding organic bridging groups into the silica skeletal structure, enabling the co-delivery of chemically distinct actives with independent, controllable release kinetics. The CYM@MON-SA system developed by Zhang et al. (2025) exemplifies this approach: the plant immune inducer SA is grafted onto disulfide-bridged mesoporous organosilica nanoparticles (MON-NH_2), while the fungicide cymoxanil (CYM) is loaded into the mesopores. Dual stimuli-responsive release triggered by GSH and amidase enables synergistic disease management, with the SA component simultaneously priming plant immunity while CYM suppresses fungal growth. Importantly, the CYM@MON-SA formulation demonstrated a 3.22-fold increase in CYM photostability compared to technical-grade CYM, addressing a key limitation of conventional fungicide formulations. The triple-stimuli-responsive organosilica systems described by Song et al. (2025) represent further advances in this direction, offering pH, GSH, and chitinas-responsive pesticide release specifically triggered by pathogen infection.

Similar dual-function systems have been developed using pH, redox, and enzyme-responsive nanocarriers loaded with strobilurin fungicides and delivered to mitochondrial targets, offering improved selectivity for fungal pathogens and reduced toxicity to plant cells (Wu et al., 2025). The convergence of nano-delivery technology with plant immunity induction represents a paradigm shift in crop protection, moving away from single-mode-of-action inputs towards multifunctional, precision systems aligned with IPM principles.

Nano-Silicon Chelates and Nano-Silicon Foliar Sprays

Nano-silicon chelates and nano-silicon foliar sprays represent a more commercially accessible tier of nano-enabled silicon application, bridging the gap between

laboratory-scale nanoparticle synthesis and farm-scale implementation. Field-relevant concentrations of 50-200 mg L^{-1} of nano-silicon applied as foliar sprays have demonstrated significant effects on salt and drought stress tolerance, ion homeostasis, and disease indices in multiple crop species including feverfew, sugarcane, and citrus (Esmaili et al., 2022; Mvondo-She et al., 2021; Rachappanavar et al., 2024). The high surface area and improved bioavailability of nano-silicon relative to bulk silicates contribute to more consistent responses at lower application rates, reducing the material cost of silicon supplementation. The comparative efficacy of different silicon compound formulations delivered by foliar route has been systematically evaluated, confirming that nanoformulations generally outperform conventional silicate sprays for disease suppression at equivalent concentrations (Laane, 2018).

EFFICACY ACROSS CROPS AND PATHOSYSTEMS

Cereal crops

Graminaceous crops, particularly rice and wheat, have been the primary focus of silicon efficacy research given their naturally high silicon accumulation capacity. In rice, silicon supplementation consistently reduces blast severity caused by *Magnaporthe oryzae*, with disease index reductions of 30-60% reported across multiple studies (Seebold et al., 2001; Cai et al., 2008). Silicon application also suppresses sheath blight caused by *Rhizoctonia solani*, bacterial blight caused by *Xanthomonas oryzae*, and brown planthopper infestations through combined physical and biochemical mechanisms (Verma et al., 2024).

In wheat, silicon reduces powdery mildew (*Blumeria graminis*) severity through cell wall silicification and the induction of antifungal compounds, among which phenolic compounds and pathogen-suppressing phytoalexins feature prominently (Bélanger et al., 2003; Rémus-Borel et al., 2005). Both root-applied potassium silicate and foliar silicon sprays are effective delivery routes for wheat, with the efficacy dependent on timing and concentration. The effect of silicon on *Fusarium crown* and root rot in tomato (Huang et al., 2011) and *Fusarium wilt* in banana (Fortunato et al., 2012) further extends the documented spectrum of silicon-mediated resistance to economically significant soilborne diseases.

Horticulture and fruit crops

In horticultural systems, silicon has demonstrated efficacy against powdery mildew in cucumber (Liang et al., 2005), grape (Bowen et al., 1992), and multiple other species, with both root-applied and foliar silicon treatments reducing mycelial development and sporulation. In citrus, silicon-based treatments have shown promise against *Penicillium digitatum* (Liu et al., 2010; Moscoso-Ramírez & Palou, 2014) in storage-phase settings, while field applications have suppressed various biotic stresses associated with citrus production (Mvondo-She et al., 2021).

In banana and citrus, Si-based treatments reduced Fusarium wilt and Penicillium decay without adversely affecting populations of endophytic biocontrol fungi such as *Pochonia chlamydosporia* and *Trichoderma* spp., preserving ecosystem services essential for IPM (Verma et al., 2021). Silicon treatments in grape and citrus have also maintained ascorbic acid, phenolic, and flavonoid contents in fruit, preserving nutritional quality and extending shelf life without introducing synthetic residues - a direct benefit for food safety and human health within the One Health framework (Rachappanavar et al., 2024).

Postharvest protection

Postharvest decay is a major contributor to food loss globally, with fungal pathogens including *Botrytis cinerea*, *Penicillium digitatum*, and *Rhizopus stolonifer* causing significant losses in fruits and vegetables. Silicon-based postharvest treatments, including sodium silicate dips and potassium silicate sprays, reduce decay indices by inducing physiological resistance in harvested tissues and by exerting a degree of direct antifungal activity through membrane damage mechanisms (Bi et al., 2006; Liu et al., 2010). Unlike conventional synthetic fungicides, silicon does not leave toxic metabolites on fruit surfaces, making it compatible with organic

certification standards and aligned with consumer demands for pesticide-free produce.
Sodium silicate treatment of Hami melons reduced *Trichothecium roseum* and other postharvest pathogens while simultaneously activating antioxidant enzyme systems and reducing oxidative stress in harvested fruit (Bi et al., 2006). Potassium silicate treatments on oranges demonstrated preventive and curative efficacy against *Penicillium italicum* and *P. digitatum* (Moscoso-Ramírez & Palou, 2014). SiO_2 nanoparticle coatings on citrus and grape maintained ascorbic acid and polyphenol contents while inhibiting *Botrytis* development, extending marketable shelf life without synthetic residues (Rachappanavar et al., 2024).

SILICON-BASED PRODUCTS IN THE ONE HEALTH CONTEXT

Environmental health benefits

Silicon-based products and their nano-enabled formulations represent a unique class of biological actives that embed deeply within the One Health concept by simultaneously advancing environmental, human, and animal health while sustaining productive crop systems (Schaller et al., 2024).
Applied silicon, whether as soluble silicic acid or SiO_2 nanoparticles, is ultimately deposited as amorphous silica (phytoliths) in plant tissues, where it provides its protective functions, and subsequently cycles back into the soil silicon pool upon crop residue decomposition. This dynamic does not generate persistent chemical residues, contrasting sharply with the environmental fate of many synthetic pesticides that accumulate in soil, surface water, and aquatic ecosystems (Lombi et al., 2019).
Silicon amendments attenuate the phytotoxic impact of heavy metals such as arsenic and cadmium in polluted soils by trapping these contaminants within the rhizospheric zone, reducing their translocation to above-ground plant tissues, thereby protecting soil and water quality (Mariyam et al., 2024). By restoring natural silicon cycles disrupted in intensive agriculture - where repeated harvesting of silica-rich biomass depletes soil silicon pools - silicon-based products foster a circular-economy approach that supports long-term agroecosystem resilience (Schaller et al., 2024; Rodriguez & Rombola, 2025).

Human health benefits

Postharvest decay and pesticide residues are major contributors to food loss and chronic dietary exposure to hazardous chemicals. Silicon-based elicitors reduce both simultaneously. Unlike conventional fungicides, silicon does not leave toxic metabolites on fruit surfaces, ensuring safer consumption and facilitating access to organic and premium markets (Rachappanavar et al., 2024).
The reduction of pesticide use also lowers occupational exposure risks for farmworkers, a key human health benefit recognised within One Health frameworks (Lowry et al., 2019). Furthermore, silicon-enhanced plant immunity reduces crop losses to pathogens and pests, stabilising food supply chains and prices, particularly in developing regions where banana and citrus exports are economic lifelines.
Silicon supplementation in crops has been linked to enhanced nutritional quality of harvested produce, including higher ascorbic acid, phenolic, and antioxidant contents, with direct implications for dietary health (Rachappanavar et al., 2024). Nano-silicon formulations that avoid leaving residues on fruit surfaces while improving quality indices represent a promising alignment of agricultural innovation with public health goals.

Regulatory landscape

From a regulatory perspective, silicon-based products are generally regarded as safe inputs with low toxicity to humans and non-target organisms (Rajput et al., 2022). Most commercial silicon formulations (potassium silicate, sodium silicate, silicic acid) are registered as fertilisers or plant strengtheners rather than as pesticides in most jurisdictions, which simplifies regulatory approval but also limits the marketing claims that producers can make about their protective effects.
Nano-silicon formulations present additional regulatory challenges, as SiO_2 nanoparticles are subject to nanomaterial-specific assessment frameworks in the European Union (REACH) and comparable regulatory structures in other

jurisdictions (Ding et al., 2023; Iavicoli et al., 2017).

The development of standardised toxicological and ecotoxicological testing protocols for nano-silicon products, encompassing impacts on beneficial soil microorganisms and non-target biota, is a priority for facilitating market approval and building stakeholder confidence (Mukarram et al., 2025).

SILICON AND ABIOTIC STRESS TOLERANCE

Substantial experimental evidence has consolidated the view of silicon as a multifaceted agent capable of reinforcing plant tolerance to an array of abiotic challenges, from drought and salinity to heavy metal burden and nutritional imbalance (Debona et al., 2017; Etesami and Jeong, 2018; Thorne et al., 2020). These stress-mitigating effects contribute indirectly to crop protection by maintaining plant vigour and reducing susceptibility to secondary pest and disease outbreaks under stressful environmental conditions. Improved water use efficiency, enhanced photosynthetic stability, and reduced ion toxicity have all been reported in silicon-amended plants, supporting the view that silicon enhances overall crop resilience rather than targeting single stress factors (Cooke & Leishman, 2016; Verma et al., 2024).

Under drought stress, silicon maintains cellular water balance through regulation of stomatal conductance and accumulation of compatible solutes. Under salt stress, silicon reduces ion toxicity by immobilising excess Na^+ and Cl^- in cell walls and vacuoles, keeping ionic homeostasis and membrane integrity. Under heavy metal stress, silicon sequesters toxic metals in the rhizosphere and at the root level, reducing their translocation to shoots and fruit - a critical food safety consideration where contaminated soils are used for production of edible crops (Mariyam et al., 2024; Liang et al., 2007).

As climate change compounds the frequency and intensity of both biotic and abiotic stressors in agricultural systems, the multipronged protective action of silicon becomes increasingly pertinent, where crops are ever more exposed to combined stress scenarios. Silicon application that simultaneously reduces fungal disease pressure and improves drought tolerance offers a strategic advantage for climate-smart crop management, reducing the need for multiple separate inputs and lowering the overall agrochemical burden on farm systems (Schaller et al., 2025; Mukarram et al., 2025).

RESEARCH GAPS AND FUTURE PRIORITIES

Regardless of the extensive documentation base supporting silicon's role in crop protection, several critical knowledge and implementation gaps limit its widespread adoption as a mainstream component of sustainable agriculture.

Mechanistic research. Future work must employ advanced molecular profiling, including CRISPR-based gene knockout studies and phosphoproteomics, to fully identify the receptors mediating SiO_2 nanoparticle responses in fruit crops and to clarify the downstream hormonal (SA/JA/ET) and MAPK signalling pathways. A comprehensive molecular map of silicon-primed immunity would enable rational design of formulations targeting specific resistance pathways for particular crop-pathogen combinations (Coskun et al., 2019; Mukarram et al., 2025).

Formulation and delivery innovation. Innovative nanocarrier technologies, including chitosan-Si composites and stimuli-responsive organosilica nanoplatforms, must be further developed to protect SiO_2 NPs from environmental degradation and enable controlled-release, synergistic co-delivery with microbial biocontrol agents. Smart, pathogen-responsive application systems that release their active payload only in the presence of disease-specific microenvironmental cues (pH change, GSH elevation, pathogen enzyme activity) represent the frontier of precision crop protection (Liang et al., 2025; Zhang et al., 2025; Wu et al., 2025).

Regulatory harmonisation and safety assessment. The priority is to harmonise fragmented global regulatory standards for SiO_2 nanoparticles by developing rigorous, standardised protocols for toxicological,

ecotoxicological, and chronic exposure assessment, including impacts on beneficial soil microbes and non-target arthropods. Coordinated international standards would expedite market entry and build the confidence of growers, processors, and consumers in nano-silicon products (Iavicoli et al., 2017; Ding et al., 2023).

Economic viability and scalability. Cost-effective green synthesis methods for SiO_2 nanoparticles using agricultural residues such as rice husk ash, coupled with life-cycle cost-benefit analyses demonstrating how reduced pesticide reliance and premium market access outweigh investment costs, are needed to drive broad adoption, particularly among smallholder farmers in resource-limited agricultural settings (Rajput et al., 2022; Schaller et al., 2024).

Climate resilience integration. Research must evaluate silicon's efficacy under realistic, combined biotic-abiotic stress scenarios common in changing climate conditions, and develop climate-smart application protocols that account for the overlapping consequences of thermal pressure, drought, and pathogen pressure on silicon performance (Schaller et al., 2025; Mukarram et al., 2025).

Integration with digital agriculture. Artificial intelligence and IoT sensor networks should be leveraged to predict disease outbreaks, optimise silicon spray timing and dosage, and utilise remote-sensing platforms to provide real-time feedback on silicon-induced physiological changes (e.g., enhanced chlorophyll fluorescence) for precision application (Yan et al., 2024; Schaller et al., 2024).

Socioeconomic and extension support. Successful farmer adoption requires development of IPM decision-support tools, practical training on silicon's triple-action benefits (disease control, stress tolerance, quality enhancement), and policy incentives such as subsidies or premium price access to accelerate uptake by smallholder farmers in silicon-responsive cropping systems (Artyszak, 2018).

Silicon, especially in nano-enabled formulations, functions as a versatile elicitor that simultaneously fortifies physical barriers, boosts biochemical defences, and primes immune signalling across a broad spectrum of crop-pathogen interactions. The meeting point between traditional silicon science and nanotechnology has spawned a new era of precision formulations, including MSN-based co-delivery systems and dual stimuli-responsive organosilica nanoplatforms, that dramatically expand the utility and efficiency of silicon in crop protection.

Robust evidence across diverse crops, from cereals to horticultural and fruit crops, demonstrates silicon's capacity to lower disease indices, reduce insect herbivory, extend postharvest shelf life, and improve tolerance to abiotic stress, all without generating persistent chemical residues. These properties position silicon as a genuinely multi-functional input aligned with IPM principles and the One Health framework, linking plant health, ecosystem integrity, and human well-being within a single, coherent sustainability paradigm.

Silicon-based products are not a standalone solution: crop species with low silicon uptake capacity, suboptimal application timing, and incompatibility with certain soil conditions can limit their efficacy. However, within the broader architecture of integrated crop production systems complemented by biocontrol agents, digital disease monitoring, and precision delivery technology- silicon offers a scientifically validated, environmentally sound, and economically viable pathway towards sustainable, low-input agricultural production. Targeted research, innovative formulation, and coherent regulatory pathways are essential to realise silicon's full potential as a cornerstone of twenty-first-century sustainable crop protection.

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FOOD SAFETY RISK ANALYSIS BASED ON AN IMPROVED HACCP METHODOLOGY: AN APPLIED STUDY

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Abstract

Food safety is a core objective of the modern food industry, directly affecting consumer health and trust in the food supply chain. This paper presents an applied food safety risk analysis based on an improved Hazard Analysis and Critical Control Points (HACCP) methodology that addresses the limits of traditional qualitative approaches. The proposed method integrates a semi-quantitative framework that differentiates between inherent process risk and residual risk after control measures are applied. Hazards are assessed by probability and severity, while the performance of the food safety management system is evaluated separately using a Control Capacity Index (CCI), which considers monitoring automation level and human reliability factors. The methodology is demonstrated through a case study covering key production stages in bread manufacturing. The results show that differentiating between hazard assessment and control performance allows for clearer prioritization of risks and more precise identification of vulnerabilities. By incorporating indicators based on automation and human quality, HACCP evolves from a static compliance tool into a more complex, dynamic risk management system. The approach supports the requirements of ISO 22000 and Codex Alimentarius and promotes continuous improvement and evidence-based decision-making in the agri-food sector.

Key words*: Food safety, HACCP, risk analysis, Control Capacity Index (CCI), food industry.*

INTRODUCTION

Food safety represents one of the major concerns of the modern agri-food industry, having a direct impact on public health, consumer confidence, and the sustainability of the food supply chain (Rossi et al., 2025). Market globalization, increasing complexity of supply chains, diversification of raw materials, and growing regulatory and consumer requirements have driven the need for increasingly robust and effective food safety management systems (Michel et al., 2024).

In this context, the Hazard Analysis and Critical Control Points (HACCP) methodology remains the cornerstone of food safety management, being internationally recognized and promoted by the Codex Alimentarius Commission (Codex Alimentarius, 2003), the World Health Organization (WHO), and the Food and Agriculture Organization of the United Nations (FAO) (Herrera, 2004). International standards such as ISO 22000, FSSC 22000, and BRCGS incorporate HACCP as a central element of food safety management systems, enforcing a preventive approach based on the identification, evaluation, and control of significant hazards (Bomba & Susol, 2020).

The scientific literature highlights that, in practice, HACCP implementation is often limited to predominantly qualitative risk assessments based on simple probability and severity scoring (Ricci et al., 2017). Recent studies indicate that such approaches may lead to subjective results, over- or underestimation of certain hazards, and difficulties in clearly distinguishing between critical control points and operational prerequisite controls (Pal et al., 2016). Moreover, the actual performance of control measures is frequently assumed to be constant, without systematic or quantifiable evaluation.

The current regulatory framework places clear responsibility on food business operators to demonstrate effective hazard control. European

Union legislation, such as Regulation (EC) No 178/2002 (Reg. 178, 2002), and Regulation (EC) No 852/2004 (Reg. 852, 2004), requires not only hazard identification but also evidence of the effectiveness of applied control measures. Nevertheless, there is a notable lack of integrated risk analysis approaches that correlate the inherent level of hazard with the real capacity of food safety management systems to prevent, detect, and correct deviations in a timely manner, particularly in food processing units with complex or semi-automated operations (Glevitzky et al., 2025).

The food industry faces multiple challenges and risks, including microbiological contamination, emerging chemical hazards, foreign bodies, human error, process variability, and increasing dependence on organizational and technological factors (Hooda et al., 2025). While HACCP offers undeniable benefits - such as incident prevention, regulatory compliance, and enhanced consumer trust - its limitations become apparent when control performance, automation level, monitoring reliability, and human factors are not explicitly considered (Zarid, 2025).

Within the scientific community, diverging perspectives exist regarding the future evolution of HACCP (Popa et al., 2021), some authors advocate maintaining the simplicity of the methodology to ensure practical applicability, whereas others support the integration of semi-quantitative tools and performance-based indicators to transform HACCP into a dynamic risk management system (Zhao et al., 2025). This ongoing debate underscores the need for methodological solutions that preserve HACCP's fundamental principles while providing a more objective and reproducible assessment of food safety risks.

The main aim of this study is to propose and validate an improved HACCP methodology based on a semi-quantitative risk analysis approach that separates hazard assessment from the evaluation of control measure performance. The original contribution of this work lies in the introduction of a Control Capacity Index (CCI), designed to quantify the effectiveness of food safety management systems by integrating factors such as level of automation, and human factors. The proposed methodology is demonstrated through a case study covering key stages of a food production process.

MATERIALS AND METHODS

The applicative study aims to validate the functionality of the improved HACCP methodology by comparing traditional qualitative risk classification with the semi-quantitative CCI approach.

Classical Risk Analysis (HACCP Approach)

In the traditional HACCP methodology, risk evaluation is based on the combined assessment of two fundamental parameters: severity and probability of occurrence (Table 1). This qualitative approach aims to estimate the potential impact of a hazard on consumer health and the likelihood of its presence in the final product. Risk classification is determined by analysing: Severity (S) – the magnitude of the adverse effect caused by the hazard; Frequency/ Probability (F) – the likelihood that the hazard occurs or persists in the final product. Severity reflects the potential consequences for consumer health, ranging from minor quality defects to serious food safety incidents. Frequency represents how often the hazard may occur under normal operating conditions (Marin & Vultur, 2007).

Table 1. Risk Matrix (Decision Diagram)

Severity	Frequency		
	Low	Medium	High
High	3	4	4
Medium	2	3	4
Low	1	2	3

The classical HACCP method uses a decision matrix that combines severity and frequency scores to determine the overall risk class.

Methodologically, the classical HACCP matrix functions primarily as a qualitative, with limited semi-quantitative features, tool built on a conservative structure that tends to favour precautionary risk classification; it provides limited differentiation between intermediate levels of severity and likelihood, does not incorporate an explicit evaluation of control measure effectiveness, and relies on a static assessment of hazards without accounting for process dynamics or operational variability over time.

Definition of Risk Assessment Components

The proposed methodology is based on four core components used to evaluate food safety risks: Severity (G), Frequency (F), Personnel

Reliability (P), and Automation Level (A). Each component is assigned a numerical score on a three-level scale, allowing the calculation of the CCI.

This structured scoring system enables a semi-quantitative differentiation between intrinsic hazard magnitude and the operational capacity of the system to control that hazard.

a) Severity (G) represents the potential impact of a hazard on product quality and consumer safety. Severity is evaluated using the following scale: 1 – Low: The hazard has a minor effect and does not significantly compromise product quality or safety; 2 – Medium: The hazard may produce noticeable effects and may require corrective actions; 3 – High: The hazard has a severe impact and may lead to product safety compromise or regulatory non-compliance. Severity reflects the intrinsic seriousness of the hazard, independent of control measures.

b) Frequency (F) represents the probability of occurrence of a hazard within the production process. Frequency is classified as: 1 – Low: The hazard occurs rarely under normal operating conditions; 2 – Medium: The hazard occurs occasionally; 3 – High: The hazard occurs frequently and requires continuous monitoring. Frequency captures the likelihood of hazard manifestation before considering control system effectiveness.

c) Personnel Reliability (P) reflects the influence of human factors, including competence, experience, training level, and adherence to operational procedures. The scoring criteria are: 1 – High reliability: Personnel are well-trained, motivated, and consistently comply with established procedures, minimizing risk; 2 – Moderate reliability: Personnel are technically competent but show reduced engagement or inconsistent procedural compliance, potentially affecting control efficiency; 3 – Low reliability: Personnel are insufficiently trained or inexperienced, significantly increasing operational risk. This parameter introduces the human factor as a measurable component of control capacity.

d) Automation Level (A) reflects the degree to which the production process depends on human intervention. The classification is defined as: 1 – Fully automated: The process is highly automated, and risk is minimally influenced by human intervention; 2 – Semi-automated: The process combines automation with operator involvement, and risk may be influenced by human interaction; 3 – Manual process: The process depends entirely on human operation, increasing variability and potential risk. Automation level quantifies technological control capacity and its impact on risk stability.

These four parameters form the analytical basis for calculating the CCI, enabling a structured and semi-quantitative assessment of both hazard intensity and control system performance within the food safety management framework.

Mathematical Formulation of the CCI

The CCI was developed to integrate intrinsic hazard characteristics with operational control performance into a single semi-quantitative indicator. The model combines hazard severity and occurrence probability with human and technological control factors, enabling a more comprehensive risk evaluation framework. The CCI is defined as:

$$CCI = (G \times F) \times (1 + A + P)$$

where: G represents hazard severity; F - frequency (probability of occurrence); P - personnel reliability; A - the level of automation. The model is structured in two distinct components:

a) Intrinsic Risk Component (G × F). The product of severity and frequency represents the baseline hazard intensity. This component reflects the inherent risk level independent of control measures.

b) Control Adjustment Component (1 + A + P). The additive term adjusts the intrinsic risk according to system control capacity. Higher values of P (lower personnel reliability) increase overall risk. Higher values of A (lower automation level) increase process variability and risk exposure. The constant "1" ensures that intrinsic risk remains present even under optimal control conditions.

This multiplicative structure allows differentiation between structural hazard magnitude and operational control performance.

CCI Risk Classification and Interpretation

The resulting CCI values are interpreted according to the following intervals: 3-20: Low Risk. Risks are considered controlled and manageable through standard operational procedures and routine monitoring. 21-36: Medium Risk. Risks require enhanced

supervision, periodic corrective actions, and performance verification measures. 37-63: High Risk. Risks demand immediate intervention, strengthened control mechanisms, and potential redesign of the process or control strategy.

This classification enables quantitative prioritization of critical stages and supports dynamic decision-making within the food safety management system. Unlike traditional qualitative HACCP matrices, the CCI provides numerical differentiation that facilitates monitoring over time and evaluation of improvement strategies (Mitrea et al., 2003).

APPLICATIVE STUDY

Comparative Risk Analysis – Classical HACCP vs. CCI

Table 2 presents a comparative assessment of selected representative stages in the bread production process, aiming to contrast the qualitative hazard classification provided by the classical HACCP matrix with the semi-quantitative CCI. This comparison highlights the differences in residual risk evaluation arising from personnel reliability and automation levels, illustrating how operational controls modulate the overall risk beyond intrinsic hazard severity.

Table 2. Comparative Risk Assessment – Bread Production Stages

Production Stage	Main Hazards	Classical	Classical Interpretation	CCI	CCI Interpretation
Flour Reception	Microbiological contamination, damaged packaging	3	High	24	Medium
Flour Storage	Mold, insect infestation	3	High	36	Medium
Sieving	Foreign bodies, contamination	3	High	30	Medium
Mixing	Non-uniform dough, contamination	3	High	20	Low
Baking	Undercooked products, microbiological risk	3	High	12	Low
Distributio n	Contamination during transport	3	High	12	Low

Raw Material Stages (Reception and Storage)*:* Classical HACCP classifies all raw material stages as high risk. In contrast, the CCI framework reduces the risk to medium, reflecting the mitigating influence of personnel and automation. This demonstrates that control factors such as training and monitoring can meaningfully lower residual risk even when intrinsic hazards are high.

Processing Stages (Mixing, Baking)*:* While classical HACCP assigns uniformly high risk due to hazard severity, CCI shows risk levels reduced to low or medium, accounting for partial automation and trained personnel. This indicates that operational management significantly mitigates risk, and that the classical matrix tends to overestimate hazards.

Post-Processing Stages (Distribution)*:* Both classical HACCP and CCI show lower risk in these stages, but CCI introduces quantitative differentiation (e.g., CCI = 12–16). This enables more precise monitoring and prioritization over time, rather than a simple binary classification.

The classical HACCP matrix offers a conservative, qualitative snapshot that is useful for regulatory compliance and initial hazard identification.

The CCI framework integrates operational control factors (Personnel and Automation), allowing numerical differentiation, dynamic monitoring, and more precise prioritization of preventive actions. The observed discrepancies underscore the added value of semi-quantitative assessment, particularly in production stages with strong monitoring or partial automation.

Sensitivity Analysis of the CCI Model

A one-factor-at-a-time (OAT) sensitivity analysis was conducted to evaluate the robustness of the CCI. Each parameter (G, F, P, A) was varied individually by ±1 unit while keeping the others constant. The resulting CCI values and percentage changes were calculated as:

$$\Delta\% = (CCI_{modified} - CCI_{initial}) / CCI_{initial} \times 100$$

Using the initial values of G = 3, F = 1, P = 2, and A = 1, the CCI calculation proceeds as follows: $CCI_i = (3 \times 1) \times (1 + 2 + 1) = 12$.

Table 3 presents the sensitivity analysis of baking parameters on the CCI index, highlighting how individual variations of each parameter affect the final value and the percentage difference compared to the reference value.

Table 3. Sensitivity Analysis - Baking

Parameter	Initial Value	Value (±1)	Applied CCI Formula	Final CCI	Difference	Δ%
Baseline	G=3,F=1,P=2,A=1	—	(3×1)×(1+2+1)	12	0	0
G	3	2	(2×1)×(1+2+1)	8	−4	−33.3
G	3	4*	(4×1)×(1+2+1)	16	+4	+33.3
F	1	0*	(3×0)×(1+2+1)	0	−12	−100
F	1	2	(3×2)×(1+2+1)	24	+12	+100
P	2	1	(3×1)×(1+1+1)	9	−3	−25
P	2	3	(3×1)×(1+3+1)	15	+3	+25
A	1	0*	(3×1)×(1+2+0)	9	−3	−25
A	1	2	(3×1)×(1+2+2)	15	+3	+25

*Values 0 or 4 are hypothetical for sensitivity testing beyond the 1-3 scale.

Frequency (F) is the dominant driver of CCI, and a baseline low frequency (F = 1) makes the process highly sensitive, so any increase effectively doubles the risk. Severity (G) retains a proportional impact of ±33.3%, directly influencing the baseline hazard, while Personnel (P) and Automation (A) exert a moderate influence of ±25%, highlighting that managerial control can meaningfully reduce residual risk in partially automated stages. The baking stage exemplifies operational sensitivity: although intrinsic hazards are moderate, deviations in process control or monitoring produce disproportionate effects on overall risk.

Table 4. Key Insights

Parameter	Influence Type	Managerial Leverage
Frequency (F)	Structural × operational	High
Severity (G)	Structural	Moderate
Personnel (P)	Operational	Moderate
Automation (A)	Operational	Moderate

Table 4 summarizes the key insights regarding the influence of baking parameters on performance and the corresponding level of managerial control for each.

For safety-critical stages such as baking, maintaining a low frequency of deviations is essential. Personnel training and partial automation offer actionable levers to stabilize risk without altering intrinsic hazard characteristics. The CCI model clearly highlights where monitoring and control efforts should be concentrated.

DISCUSSIONS

The sensitivity analysis confirms that the CCI model behaves multiplicatively with respect to intrinsic hazard magnitude, that control parameters gain strategic importance in low-frequency environments, and that the framework effectively distinguishes between structural and operational vulnerability.

The results show that distinguishing between inherent risk and residual risk enables clearer hazard prioritization and more accurate identification of critical control points.

The proposed HACCP–CCI framework transforms traditional hazard classification into a dynamic, performance-sensitive evaluation system. By separating intrinsic hazard from control capacity, the model enables evidence-based prioritization, resource optimization, continuous improvement in alignment with ISO 22000, and enhanced managerial decision-making transparency. Unlike classical qualitative matrices, the CCI approach introduces measurable elasticity, scalability, and analytical depth while preserving operational simplicity.

In the context of food safety management, HACCP remains the foundational risk assessment framework mandated by regulatory authorities worldwide for systematic identification, evaluation, and control of hazards linked to food production processes (Rizzo et al., 2025). Its qualitative or semi-quantitative nature has driven researchers and practitioners to explore alternative or complementary methodologies that enhance prioritization, detection capability, and continuous improvement (Marhavilas, 2011).

One commonly integrated method is Failure Mode and Effects Analysis (FMEA), originally developed in engineering but increasingly applied in food systems for risk identification and quantification (Li et al., 2023). FMEA systematically reviews potential failure modes within a process and ranks them based on severity, occurrence, and detectability, often yielding a Risk Priority Number (RPN) that can guide corrective actions. Studies have shown that pairing FMEA with HACCP can deepen analytical insight: for instance, in edible-coated orange processing, both a qualitative risk matrix and FMEA identified critical hazards, but

FMEA provided additional emphasis on the detection capability of controls, thus offering enhanced prioritization of failure modes and validation of hazard analyses (Segovia Bravo, 2026). In pastry processing and other food production contexts, FMEA has been integrated alongside ISO 22000 standards to quantify processing risks and monitor the effectiveness of control actions. The application of FMEA allowed identification of the most significant failure modes and demonstrated that corrective measures could substantially reduce RPN values, reinforcing its role as a complementary tool in structured food safety management systems (Varzakas, 2011; Lee et al., 2021). Another study on ready-to-eat vegetable processing found that FMEA combined with cause-and-effect analysis identified key high-risk stages, and subsequent corrective measures brought RPN values below acceptable thresholds, corroborating the method's utility in structured risk assessment (Varzakas & Arvanitoyannis, 2009).

Beyond FMEA, continuous improvement methodologies like Kaizen and Lean principles are increasingly used to support risk assessment frameworks (Díaz Reza et al., 2025). Although not risk assessment tools per se, Kaizen emphasizes incremental, data-driven problem solving and waste elimination across the value chain, thereby enhancing process stability and quality outcomes (Lizarelli et al., 2025). In food safety contexts, Kaizen has been proposed as a means to strengthen HACCP implementation by fostering an organizational culture oriented toward systematic improvement of controls and preventive measures throughout production and distribution stages (Mureşan et al., 2020). A specific application of Kaizen principles to food safety quality management from wheat to bread illustrates how continuous improvement techniques can reinforce analytical frameworks by focusing on root causes and process optimization, leading to safer and more efficient operations (Hill, 2014).

The results obtained in this study are consistent with existing literature on semi-quantitative and hybrid food safety risk assessment approaches (Arvanitoyannis & Varzakas, 2008; Soman & Raman, 2016; Trafialek & Kolanowski, 2014; Shirani & Demichela, 2015; Surareungchai et al., 2022). In particular, the observed dominance of frequency and the significant role of operational factors such as personnel reliability and automation align with findings reported in FMEA-based HACCP extensions and human reliability studies in food production systems. Moreover, the demonstrated benefit of separating intrinsic hazard from residual risk confirms previous evidence that integrated frameworks improve hazard prioritization and support more effective control strategies. These similarities strengthen the validity of the proposed HACCP–CCI model and highlight its applicability within modern risk-based food safety management systems.

Comparatively, standard risk matrices like the classical HACCP chart provide a qualitative baseline for hazard classification, which is accessible, easy to interpret, and widely mandated in food safety legislation (Popa et al., 2023). In contrast, methods such as FMEA add granularity by examining failure detection capability and supporting semi-quantitative prioritization, while Kaizen and Lean tools operationalize ongoing control improvements rather than discrete hazard ranking (Gomaa et al., 2025). These approaches complement rather than replace HACCP; in integrated food safety management systems, combining HACCP with FMEA, Ishikawa diagrams, Pareto analysis, and continuous improvement tools has been shown to result in more robust hazard identification, validation of control measures, and actionable performance metrics (Lee et al., 2021).

The literature suggests that while classical HACCP remains central due to its regulatory acceptance and preventive orientation, the integration of FMEA and continuous improvement methodologies enhances the depth, responsiveness, and operational relevance of risk assessment in modern food production systems. These hybrid approaches align with holistic risk management standards such as ISO 22000, which emphasize both hazard analysis and performance evaluation throughout the supply chain (Szczyrba & Ingaldi, 2024).

CONCLUSIONS

The semi-quantitative HACCP–CCI framework improves risk discrimination compared to the classical HACCP matrix by integrating human

reliability and automation levels. This allows a distinction between intrinsic hazard magnitude and residual risk after operational controls, shifting HACCP from a static compliance tool into a dynamic risk management system aligned with ISO 22000 and Codex Alimentarius requirements and supportive of continuous improvement in the agri-food sector.

Sensitivity analysis highlights stage-specific behaviour: intrinsic hazard parameters dominate in flour storage, while operational factors such as personnel reliability and automation have greater influence in baking. This enables targeted interventions where they are most effective, optimizing monitoring, training, and automation efforts.

The HACCP–CCI model provides quantitative rigor, actionable insight, and alignment with ISO 22000 and Codex Alimentarius. It supports evidence-based prioritization, continuous improvement, and enhanced food safety governance in the agri-food sector.

The present study is based on a semi-quantitative modelling approach and relies on assumed parameter ranges for human reliability, automation levels, and hazard classification. As such, the results may be sensitive to the quality and representativeness of input data. In addition, the HACCP–CCI framework was not validated on large-scale industrial datasets, which may limit its generalizability across different agri-food sectors and production environments.

Future work should focus on validating the HACCP–CCI model using real industrial case studies and large-scale empirical datasets across different food production systems. Further research could also explore the integration of real-time monitoring technologies, such as IoT-based sensors and machine learning approaches, to dynamically update risk parameters. Additionally, extending the model to incorporate economic cost analysis and supply chain variability could further enhance its practical applicability and decision-support capability.

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BIOINDICATORS, A KEY TOOL IN ASSESSING TOXIC EXPOSURE

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Abstract

Biomonitoring uses living organisms - bioindicators - to assess environmental health, while biomonitors specifically provide quantitative data on pollution levels, such as accumulation rates. Bioindicators - plants and animals - can provide valuable information about ecosystem exposure to a wide range of pollutants, as they are able to absorb and accumulate specific toxins from soil, water and atmosphere. This paper provides an overview of the main aspects of the potential of biomonitoring as an effective tool for assessing exposure to toxic substances and for identifying interventions needed to mitigate it.

Key words*: bioindicators, biomonitoring, ecosystem damage, environmental contaminants, environmental pollution.*

INTRODUCTION

Biomonitoring is a key tool when it is necessary to identify the presence of pollutants in biological substrates, to quantitatively and qualitatively assess exposure, as well as to estimate the level of exposure (Tadic, 2025).

The concept of monitoring derives from the verb "to monitor" which originates from the Latin verb "moneo", which means to warn, remind or recommend (Stoddard et al., 2006).

Industrial and domestic human activities have led to environmental pollution, and industrial growth and the gradual transition to urbanization are the main causes of the increase in various types of environmental pollutants (Cozea & Tanase, 2022).

In Romanian, the term "monitoring" is used relatively recently, and, in a broader sense, represents the supervision of the evolution in time of a natural or artificial system, by determining, evaluating or warning about the exceeding of limit values of important indicators or parameters of the system (Maciuca, 2004).

The fundamental reasons for using bioindicators are based on demonstrating how the biological effect of pollutants is integrated with the sensitivity of biological sensors and environmental climatic factors (Macoveanu, 2006).

By extrapolating the toxic risk from plants to humans, the toxic potential on humans is demonstrated and, implicitly, the possibility of standardizing working methods (Krishnaveni, 2013). The importance of biomonitors (Figure 1) is reflected in parameters related to human health, environmental quality, biodiversity and ecological significance.

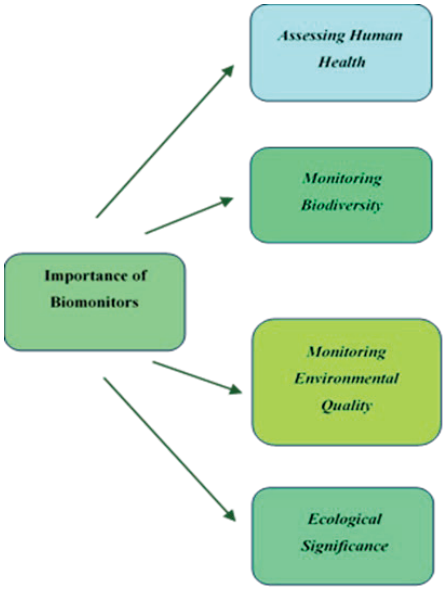

Figure 1. The importance of biomonitors (Holt & Miller, 2010)

This process can sometimes lead to a diagnosis of the current state and possibly the development of forecasts (Environmental Protection Agency, 2025).
This review provides a synthesis of the main aspects related to bioindicators as effective tools for assessing exposure to toxic substances and for identifying actions required to mitigate this exposure.

BASIC ACTIVITIES IN BIOMONITORING

Environmental monitoring is the systematic and continuous process of collecting and analysing data on natural resources - such as air, water, soil, and biodiversity - to assess environmental conditions, impacts, and ensure compliance with environmental standards (Holt & Miller, 2010).
Environmental monitoring consists of three basic types of activities:

- the first activity involves measurements and observations aimed at ensuring a correct description of the environment and the changes to which it may be subject;
- the second activity is represented by the evaluation of environmental data to determine possible directions of evolution and the development of an early warning system based on pre-established criteria, but which are in continuous evolution with the development of scientific knowledge;
- the third activity within of the system is to plan actions that prevent environmental deterioration and ensure optimal environmental management.

Significantly, monitoring provides not only the basis for the formulation of environmental policies, but also allows for the evaluation of the effectiveness of these policies. Data relating to the quality of environmental factors, processed through a cycle involving multiple vertical and horizontal levels, become information on environmental quality (Ahluwalia, 2025).
The vertical levels include the activities and operations that take place from the level of measuring/sampling stations to the users of information on the quality of environmental factors (Scheepers, 2025)
The horizontal data loop in biomonitoring based on bioindicators focuses on the spatial distribution of sampling and their analysis in direct relation to a pollution source or a given geographical area, ensuring horizontal coverage (mapping) of environmental quality (Aslam et al., 2012).
Environmental teams within environmental organizations involved in biomonitoring must collect, interpret, evaluate and report on deviations, the amount of data involved is often large. This makes the task time-consuming, especially when it is hampered by unsuitable tools for the task, such as spreadsheets, which can be time-consuming, error-prone, unreliable and lack data traceability (Kettrup, 2003).

PRIORITY OBJECTIVES OF BIOMONITORING

The national environmental quality monitoring system was established in the 1960s, but systematic observations began to be carried out in the 1980s, currently having as priority objectives:

- monitoring of environmental quality and determining the level of pollution;
- detecting cases of exceptional pollution of surface water, air and soil;
- preventing and reducing negative effects on the environment and the population;
- emergency notification of decision-making bodies regarding the exceptional degree of environmental pollution;
- systematic familiarization of the public with environmental quality (Environmental Protection Agency, 2025).

Thus, the marked persistence of pollutants in environmental media and its pronounced toxicity determine the increased danger of these pollutants for humans and wildlife. All these characteristics impose the need to implement measures to monitor pollutant levels in the environment and the impact on human health, especially young children (Barmashova & Lazutkina, 2019).
Human activity is a component of natural ecosystems and, over time, its influence on these ecosystems has undergone fundamental transformations. If initially the anthropogenic changes in ecosystems were imperceptible, with the advancement of human society, the effects have become increasingly visible. The degree of human interference in ecosystem functioning has progressively intensified, ultimately reaching the point of affecting the ecosphere (Kettrup, 2003).

The evidence of the negative effects of massive human intervention on ecosystems, which affected the very complex but also very sensitive balance of the biosphere, alarmed people, perhaps too late. As a result of anthropogenic activity, global climate changes have already occurred, as well as global biogeochemical cycles, with repercussions on all terrestrial ecosystems (Contrea & Contrea, 2000).
The implications of this state of affairs can become very serious, as human survival and continued development depend on the proper functioning of these ecosystems. With growing awareness of the risks posed by the ecosystem imbalances, efforts have been undertaken to develop systems for their monitoring (Abbott & Le Maitre, 2009).

BIOMONITORING SURVEILLANCE

Biomonitoring surveillance is essential for assessing human exposure to environmental and occupational hazards and evaluating associated public health risks. The surveillance implies two directions:

- the early detection of possible changes in the functioning and composition of biological systems, which could ultimately lead them to disorganization, collapse, or could commit them to an unfavourable direction of evolution. Early alerting of specialists enables decision-makers to take measures to remedy the situation, before irreversible negative effects occur;
- study the reaction and response of biosystems to global environmental change.

The information obtained from the surveillance systems is stored in databases, to allow comparisons over time (Weinhold, 2010).
Globally, there are several networks for monitoring ecosystems or only certain abiotic parameters. These networks have a varying density and extent and are equipped with more or less sophisticated equipment (Bellinger & Needleman, 2003).

INSTRUMENTAL OR BIOLOGICAL MONITORING

Biological monitoring or biomonitoring may complement and, in some cases, replace instrumental monitoring. Biomonitoring is preferable to instrumental monitoring, if there are not sufficient financial resources for the installation and maintenance of sophisticated equipment (as is generally the case in developing or underdeveloped countries). Biomonitoring is very convenient for cases where the long-term monitoring of large areas is sought (Creager, 2018).
Instrumental monitoring is not defined by time resolution alone; it also includes continuous monitoring systems. Instrumental monitoring also includes biotic parameters (e.g., DNA-based sensors, biochemical assays). Many instrumental methods provide derived, qualitative or semi-quantitative interpretations after processing (Tranfo, 2020).
Bioindicators are species, populations, or communities of species that, due to their variability (biochemical, physiological, ethological or ecological), allow the characterization of the state of an ecosystem and highlight, as early as possible, its natural or anthropogenic changes (Holt & Miller, 2010).
The idea of a bioindicator species was introduced when the indicator capacity of lichens was observed regarding the composition, quality and humidity of the air. In the second half of the 20th century, research generally aimed at finding indicators and developing methods that would provide information related to air, soil and water pollutants (Birsan & Luca, 2010).
Subsequently, as concerns regarding ecosystem degradation expanded, efforts were made to identify bioindicators that would provide information on ecosystem stability, biodiversity maintenance, sustainable management of forest or agricultural ecosystems - the effect of certain management measures or techniques on these ecosystems, or information on the response of ecosystems to global climate change (Cozea & Tanase, 2019).

BIOINDICATORS OF POLLUTION - OVERVIEW

A biological monitor or biomonitor is an organism that provides indirect information on the quality of the environment around it. Pollution indicators are physical, chemical or biological variables, and they can help assess contamination levels of air, water or soil, reflecting environmental quality and health risks (Pauline et al., 2007).

Regarding pollution indicators, there are two types:

- specifically sensitive, which indicate the presence of a pollutant through the appearance of lesions or malformations;
- specifically accumulator, which concentrate the pollutant in their body, which proliferate and become abundant in polluted areas.

Pollution indicators can be animal or plant, the latter being more numerous (Gavrilescu & Pavel, 2008).

Bioindicators for pollution have the advantage, compared to instrumental monitoring, of being able to provide an answer to the combined effect of certain pollutants, unlike instruments that measure the amounts of each pollutant separately. Bioindicators can give indications, following tissue analysis, related to very small amounts of pollutants in the environment, as well as the evolution of the pollutant over time, over longer periods (Baconi & Bălălău, 2001).

As mentioned, the first and most widely used species as indicators of air quality were lichen species. Their value as bioindicators has been recognized since a 100 years ago, but concrete methods for monitoring air pollution with sulphur dioxide, through lichens, have been developed and improved in the last 30 years (Barr, 2008).

BIOINDICATORS OF POLLUTION – PLANT

Plant species that have been used as bioindicators of ozone pollution, along with other indicators of air pollution, can be classified into two categories:

- introduced species, generally herbaceous, fast-growing, genetically uniform plants, generically called "sentinel species", used in active biomonitoring;
- species that grow naturally in a given area, are perennial plants, shrubs or trees, slow-growing and have a slow reaction to increasing pollutant concentrations, the effects appearing later, during the growth period; these species are generically called "detector species" or biomonitors, used in passive monitoring.

Examples of species in the first category are tobacco (*Nicotiana tabacum* L.) and stinging nettle (*Urtica urens* L.), and for species in the second category, it could be mentioned: American black cherry (*Prunus serotina* Ehrh.), yellow pine (*Pinus ponderosa* Laws.), American ash (*Fraxinus americana* L.), Pennsylvania ash (*Fraxinus pennsylvanica* Marsh.), quaking aspen (*Populus tremuloides* L.), tulip tree (*Liriodendron tulipifera* L.) (Dobrota, 2010).

By monitoring these sentinel plants - often planted in strategic locations like botanical gardens, nurseries, or near ports - researchers can identify early warning signs of threats that are otherwise difficult to spot. Sentinel species usually react quickly to increased ozone concentrations in the air, being used to signal its presence early. However, responses may be more pronounced in young plants and therefore plants should be reintroduced periodically. (Mansfield et al., 2019)

They are previously cultivated in clean air, free of pollutants and subsequently transplanted to monitoring sites; being genetically uniform, their reaction to the pollutant is relatively uniform (Grandjean & Landrigan, 2014).

Detector species are not subject to special care measures in the natural environment in which they grow. It should be noted that only some individuals in a population, namely the sensitive ones, react to elevated ozone levels. The reaction of individuals is also conditioned by other seasonal conditions (Krishnaveni & Magesh, 2014).

As some examples, these plant species are used as bioindicators for various pollutants: St. John's wort (*Hypericum perforatum*) for hydrofluoric acid, stinging nettle (*Urtica doica* L.) for ozone and peroxyacetyl nitrates, ryegrass (*Lolium* spp.) for hydrofluoric acid and heavy metals, valerian (*Valeriana officinalis*) for heavy metals, alfalfa (*Medicago sativa* L.) for sulfur dioxide, barley (*Hordeum vulgare* L.) for heavy metals and fluorinated compounds, corn (*Zea mays* L.) for hydrofluoric acid, sulfur dioxide, heavy metals. (Caliman et al., 2011).

BIOINDICATORS OF POLLUTION – INSECTS

Along with plants, insects are used as bioindicators of pollution, such as the elder aphid (*Aphis sambucci* L.) for sulfur dioxide, and among mammals, the rat (*Rattus rattus* L.) for nitrogen dioxide. As mentioned, biological

indicators are not only used in the case of pollution, but also for other purposes (Rosianu et al., 2022).

Thus, ants (fam. *Formicidae*, ord. *Hymenoptera*) are used as bioindicators in the conditions of ecological recovery in certain areas (areas degraded by mining activities, areas destroyed by fires) or as bioindicators of diversity. In general, the ant communities in the respective areas and their relationships with prey or predators are studied (Lutinski, 2024).

Other insect species, namely ground beetles (fam. *Carabidae*, ord. *Coleoptera*), are a reliable indicator of the distribution of vegetation: there are species characteristic of the alpine, subalpine or forest environment. These species have, in relation to abiotic (and implicitly biotic) conditions, very strict requirements (in fact, a condition for choosing bioindicator species is that they are stenotopic species); in addition, ground beetles are characterized by great mobility, so that any disturbance of their specific microclimate determines a rapid reaction and the movement of individuals to another, more convenient habitat (Dickie & Tucker, 2010).

The response of insects to environmental changes is faster than that of vegetation, for example. Inventorying, at regular time intervals, the communities of ground beetle species and storing this information in databases can provide, by comparison, information regarding the dynamics of ecosystems (Pont et al., 2006).

Other insects, such as dragonflies (ord. *Odonata*), can provide, by studying the evolution of their spatial distribution, indications about the occurrence of a disturbance in the functioning of the ecosystem of which they are part. Butterflies (ord. *Lepidoptera*) can provide information about the reappearance and succession of plant species on denuded land. Spiders (ord. *Araneae*) can also be used as bioindicators of ecosystem balance (Dickie & Tucker, 2010).

BIOINDICATORS OF POLLUTION – BIRDS

Birds play an important role as bioindicators: birds are conspicuous, relatively easy to observe, one of the best studied groups of organisms, and in the focus of public interest and care (Becker, 2003).

Birds are very good bioindicators (and in some cases, the only ones) of environmental changes, to which they react by modifying the composition of species within a biocenosis, by modifying their behaviour or appearance and reproductive capacity (Cotin, 2012).

Birds can be used to examine the long-term effects of their habitat fragmentation, the effect of introducing new species into the ecosystem, to monitor water quality, to obtain information on the health of fish populations, to identify pollutants such as organochlorine pesticides, heavy metals or radioactive substances (Aktar et al., 2009).

An advantage of using birds as bioindicators is that they have been studied in detail in the past and, as a result, there is already a lot of data available regarding their natural distribution, ecology and ethology, which can be compared with new data obtained from ecosystems possibly affected by degradation or various disturbances (Becker, 2003).

Thus, for the monitoring of water quality in mountain springs in forest ecosystems, the wagtail (*Motacilla alba*) is used as a bioindicator in the United States. It was chosen as a bioindicator for the stability of forest ecosystems that host springs, for establishing priority measures for the conservation of these ecosystems and for establishing objectives for ecological reconstruction, where appropriate (these ecosystems being affected by the fragmentation of forest areas and the acidification of waters due to mining drainage techniques). The wagtail was selected as a bioindicator because it is linked to both mountain water quality and large areas of mature forest (Brooks, 2000).

Various species of owls have also been used as sentinel species for early warning of ecosystem degradation. These species, like other predator species, have been used as biomonitors because they are widely distributed, have territorial behaviour, are not migratory, and a fast metabolism (Wendaisaul, 2015).

Owls, considered bioindicators of environmental health, excel due to their position as higher-order consumers - top predators - in the food chain. Due to this fact, they accumulate and concentrate large amounts

of pollutants ingested by prey - rodents, insects - a process known as bioaccumulation (Popescu, 2010).

Species used as indicators in various parts of the world, especially in North America (Canada and the USA), Europe (Norway, the Netherlands, Spain, Great Britain) and Africa (South Africa) are represented by several owl species (*Asio otus* L., *Tyto alba* L., *Bubo bubo* L., *Bubo virginianus* L., *Asio flammeus* L., *Glaucidium perlatum* L.) (Wendaisaul, 2015).

The use of species (such as raptors, peregrine falcons or various passerine species) as bioindicators for monitoring ecosystem health and pollution involves a multidisciplinary approach. These studies focus on the effects of contaminants on animal physiology and behaviour:

- reproductive behaviour changes – monitoring nesting success, chick feeding frequency and courtship behaviour, which can be affected by exposure to pesticides (e.g. organochlorines);
- study of eggshell thickness - a classic indicator of pollution, especially in the case of pesticides such as DDT, which cause the shell to thin, leading to the cracking of eggs during hatching;
- analysis of the activity level of liver enzymes (e.g. cytochrome P450) to assess exposure to xenobiotics and the body's ability to metabolize toxins;
- the use of non-killing samples, essential for the study of endangered species (Mitrofan et al., 2022).

Another bioindicator that has been frequently used and well studied is the peregrine falcon (*Falco peregrinus* L.), whose populations have experienced a drastic decline in the past due to exposure to dichlorodiphenyltrichloroethane (DDT) and other organochlorine insecticides. After the banning of these insecticides, populations of the species recovered in many countries and interest in this species as a bioindicator decreased, its place being taken by the various owl species mentioned above (Askeyev et al., 2019).

CONCLUSIONS

Bioindicators open up a wide field of research; numerous research projects are currently underway and being finalized, as there are still many aspects to be clarified and it is necessary to develop coherent methods for environmental monitoring using bioindicators.

Biological monitoring and biological effects monitoring can play a role in exposure assessment and health surveillance, helping to evaluate control measures and manage risks to human health and the environment in which they live.

For practical application, species must be carefully selected based on their ability to provide relevant information for specific monitoring objectives, as well as on well-documented relationships with environmental factors and other components of the biocenosis.

When developing specific monitoring methods, the scale at which the determinations are made and the exact data that are collected must be taken into account, as well as the way in which these data are processed and stored, so that they are relevant and the interpretations made on their basis are as close to reality as possible.

Taking into account the financial difficulties existing in our country and other objective reasons, which make the instrumental surveillance of forest ecosystems difficult, biomonitoring represents a particularly interesting alternative (or a possible complement).

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HIGHLIGHTING THE INFLUENCE OF BROCCOLI MICROGREENS (*Brassica oleracea* var. *italica*) ADDED TO COW'S WHITE CHEESE (TELEMEA) ON THE PURCHASING PREFERENCES OF THE FINAL CONSUMER

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Abstract

The present study analyses the impact of the product obtained by incorporating broccoli microplants (microgreens/sprouts) into a cow's white cheese pie on the sensory acceptability and purchasing preferences of the final consumer. Broccoli microplants (microgreens/sprouts) were obtained under aseptic conditions and incorporated in different proportions into the pie, in compliance with food safety requirements. The product was evaluated by means of sensory analysis and an online questionnaire. The obtained results indicate a moderate influence on aroma and texture, without negatively affecting overall acceptability, while taste and colour were positively evaluated. Taste and perceived quality proved to be determining factors in the purchase decision, alongside a price level considered appropriate. The recorded results indicate a favourable acceptance of the product and highlight the potential of broccoli microplants (microgreens/sprouts) as a functional ingredient in formulations of cow's white cheese (telemea) pie.

Key words*: broccoli, cow's white cheese (telemea) pie, microplants (microgreens/sprouts).*

INTRODUCTION

Broccoli (*Brassica oleracea* var. *italica*) is recognized as a member of *Brassica oleracea* Broccoli Group (USDA, Agricultural Research Service, 2026a), with economic use as human food by flower buds and sprouts (USDA, Agricultural Research Service, 2026b). Broccoli flower buds have nutrient density supports and health benefits that are considered as a superfood (Drăghici et al., 2023). But, on the other hand, broccoli sprouts, by comparation with mature broccoli, have higher levels of metabolically active compounds and reduce chronic disease risks (Hong, 2020).

Broccoli sprouts may be processed into powders (Xu et al., 2013), capsules (Park, 2006) and granules (Jang, 2024), or may be used in functional foods for easy consumption and health benefits. Additionally, another advantage of broccoli sprouts is that support the marketability (Popa et al., 2022), and consumers show preference for functional broccoli sprout products (Geicu-Cristea et al., 2023). At the same time, there is a need for strict elimination of microorganisms during seed germination and sprout development to ensure that the sprouts are safe and suitable for consumption.

Taking into account the information presented above, broccoli microplants (microgreens/ sprouts; MMS) were obtained under aseptic conditions (Livadariu and Adam, 2025). By incorporating broccoli MMS into a cow's white cheese (telemea) pie, a new product was developed.

Consequently, the present study highlights the influence of broccoli MMS added to a cow's white cheese (telemea) pie on the purchasing preferences of the final consumer, assessed through sensory analysis and an online questionnaire.

The findings, supported by detailed charts and analyses, highlight the new product's sensory attributes, examine consumer acceptance and purchasing behaviour associated with the addition of broccoli MMS. These insights enhance the understanding of the market

potential for a functional new product consisting of the integration of broccoli MMS into cow's white cheese pie formulations (= the product cow's white cheese (telemea) pie with broccoli MMS) and provide key considerations related to consumer preferences.

MATERIALS AND METHODS

Method for obtaining broccoli microplants (microgreens/sprouts). Broccoli seeds *(Brassica oleracea* var. *italica)* were purchased from a commercial source and aseptically sterilized prior to inoculation (Di Bella et al., 2020; Hong, 2021; Livadariu and Adam, 2025). The substrate for seed germination and the growth of broccoli MMS was prepared under aseptic conditions (Barbu et al., 2024; Livadariu et al., 2025; Livadariu and Adam, 2025).

Broccoli microplants (microgreens/sprouts) were obtained under aseptic and growth conditions (Livadariu et al., 2025), which allowed harvesting of broccoli MMS after 14 days (Livadariu and Adam, 2025).

Method for obtaining the product cow's white cheese (telemea) pie with broccoli microplants (microgreens/sprouts). The technological process for obtaining the product cow's white cheese (telemea) pie with broccoli MMS consisted of six main stages: harvesting, dosing, combining, baking, cooling, and packaging. These stages were carried out within the facilities of SC PICCOLO ANGELO SRL.

The harvesting of broccoli MMS was conducted together with the edible substrate and included mixing them using a blender (Livadariu and Adam, 2025).

The dosing consisted of weighing the raw materials: flour, water, oil, vinegar, salt, cow's white cheese (telemea), fresh curd cheese (caș), eggs, yogurt, and broccoli MMS mixed with the growth substrate.

The combination of the raw materials included a sequence of sub-stages, namely:

a) kneading the sifted flour together with water, oil, vinegar, and salt until the dough was obtained;

b) dividing, rolling, and stretching the dough;

c) filling the dough with a mixture composed of cow's white cheese (telemea), eggs and broccoli MMS and

d) shaping the dough into a pie, placing it in baking trays and brushing with a mixture of yogurt and egg.

Baking was carried out at 200℃ (392°F) for 40 minutes, and cooling was performed by maintaining the product at room temperature for 15 minutes. Packaging was done in transparent containers with lids.

By completing the six main stages of the technological process the product cow's white cheese (telemea) pie with broccoli MMS was obtained. This product was portioned and tested by the final consumer through sensory analysis.

The **experimental variants** tested by the final consumer consisted of one control variant, which did not contain broccoli MMS and four variants containing broccoli MMS in different amounts, established as percentages of the total quantity of broccoli MMS obtained by mixing with the edible substrate, as follows:

- Control = cow's white cheese (telemea) pie without broccoli MMS;
- Variant 1 = the product cow's white cheese (telemea) pie with 25% broccoli MMS;
- Variant 2 = the product cow's white cheese (telemea) pie with 50% broccoli MMS;
- Variant 3 = the product cow's white cheese (telemea) pie with 75% broccoli MMS;
- Variant 4 = the product cow's white cheese (telemea) pie with 100% broccoli MMS.

The **method of statistical processing** used an online questionnaire completed by final consumers after the sensory analysis of the product, in order to explore the influence of broccoli microplants (*Brassica oleracea* var. *italica*) added to the cow's white cheese (telemea) pie, on the purchasing preferences of the final consumer.

The questionnaire was created using the Google Forms platform (Geicu-Cristea et al., 2023), and was distributed through the employees of the company Moesis by Angelo, in order to facilitate efficient and cost-effective data collection. It consisted of 22 questions, structured to collect information both for grouping the final consumers according to age and gender and for identifying the perception of the final consumer regarding the product cow's white cheese (telemea) pie with broccoli MMS.

The online questionnaire was divided into three main sections:

- the profile of the final consumer according to age and gender (through 2 questions);

- the sensory preferences of the final consumer (through 7 questions regarding aroma, colour, taste, and texture);
- the behaviour of the final consumer (through 3 questions related to the most important sense in choosing a pie-type product, the price for 1 kg of the product cow's white cheese (telemea) pie with broccoli MMS, and the dominant factor in the choice of a food product).

The sensory analysis was conducted with 56 evaluators randomly selected from the company's personnel. Each final consumer evaluated all 5 products. The samples were first labelled and marked with a code created for each sample individually, and subsequently served in increasing order of the broccoli MMS content. Prior to the start of the evaluation, the participants were informed about the sensory analysis procedure and GDPR regulations.

RESULTS AND DISCUSSIONS

After obtaining the new product cow's white cheese (telemea) pie with broccoli MMS, the final step in highlighting the influence of broccoli MMS added to the cow's white cheese (telemea) pie on the purchasing preferences of the final consumer consisted of conducting a sensory analysis, followed by an online questionnaire based on its results.

The first part of the questionnaire outlined the profile of the final consumer using information related to age and gender. The age of the final consumers ranged from 18 to over 58 years, with the highest percentage (37%) recorded in the 49-58 age group (Figure 1).

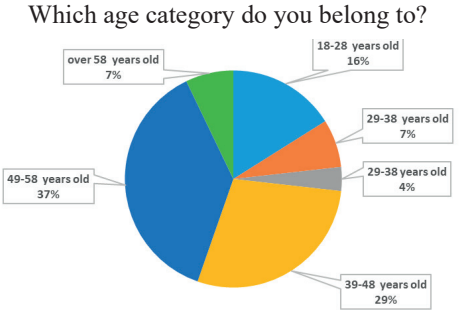

Figure 1. The results show that most respondents belong to the middle-age categories, indicating a mature consumer profile

Meanwhile, the gender distribution of the final consumers was predominantly female, accounting for 84% (Figure 2).

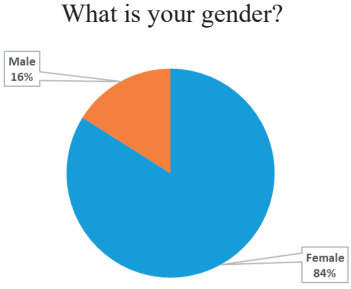

Figure 2. The sample is predominantly female, suggesting a higher interest among women in the evaluated product

The second part of the questionnaire highlighted the sensory preferences of the final consumer for the product cow's white cheese (telemea) pie with broccoli MMS, through information regarding aroma (Figure 3), colour (Figure 4), taste (Figures 5-8), and texture (Figure 9).

The aroma of the product cow's white cheese (telemea) pie with broccoli MMS (Figure 3) was perceived by the highest percentage of final consumers (38%) as being slightly changed. However, a proportion of the final consumers considered that the aroma of the product was changed to a large extent (21%), to a very large extent (20%), to a very small extent (14%), while only 7% indicated that they did not know. These data reflect the subjective perception of the final consumer and indicate the capacity of broccoli MMS to influence the aroma of the tested product.

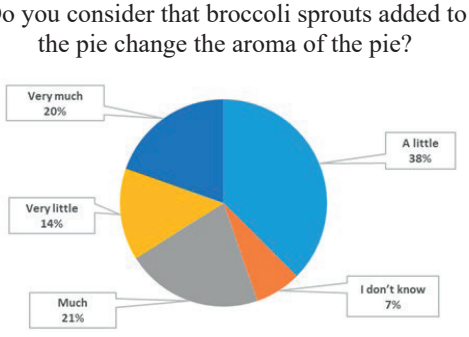

Figure 3 Broccoli sprouts are perceived to moderately influence the aroma of the pie

The colour of the product cow's white cheese (telemea) pie with broccoli MMS was perceived by the highest percentage of final consumers (84%) as pale green. However, some final consumers considered the product colour to be yellow-green (2%) or intense green (14%). These data indicate the stability of colour in the perception of the final consumer.

Do you prefer the product pie with broccoli sprouts to have a certain colour/shade?

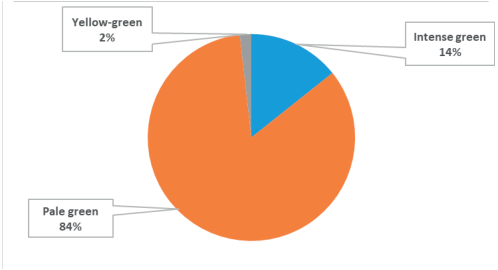

Figure 4. A pale green colour is the most preferred shade for the product

The taste of the product cow's white cheese (telemea) pie with broccoli MMS was perceived by the highest percentage of final consumers as very satisfactory (39%) (Figure 5), dominant (34%) (Figure 6), salty (55%) (Figure 7), and moderate (39%) (Figure 8). These data indicate the ability of broccoli MMS to positively influence taste.

In addition, by analysing the results presented in Figure 8, a preference of the final consumer can be observed for the experimental variant consisting of the product cow's white cheese (telemea) pie with 50% broccoli MMS (Variant 2).

How would you rate the taste quality of the product pie with added broccoli sprouts?

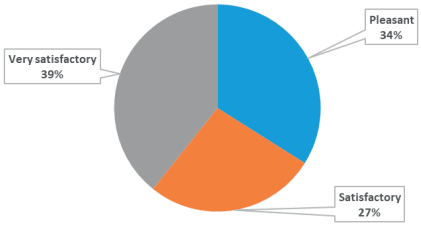

Figure 5. The taste quality of the product was rated positively by most consumers

What is the dominant taste of the pie with broccoli sprouts?

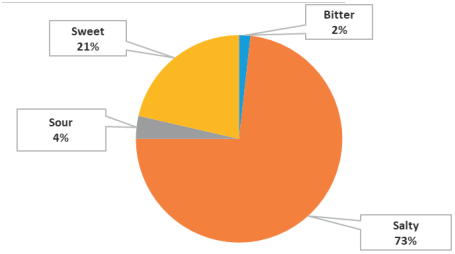

Figure 6. The dominant taste perceived by consumers is salty

Do you prefer the pie with broccoli sprouts to have a taste that is:

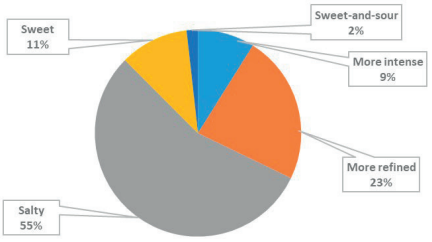

Figure 7. Consumers generally prefer a salty or more refined taste profile for the product

Do you consider that the product pie with broccoli sprouts should contain a certain amount of sprouts?

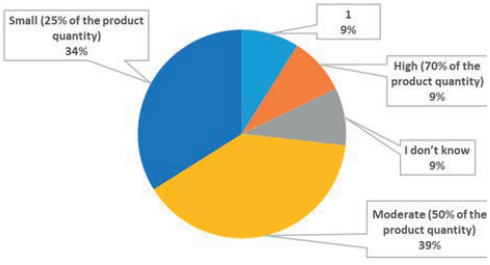

Figure 8. Consumers mainly prefer a small to moderate amount of broccoli sprouts in the product

The texture of the product cow's white cheese (telemea) pie with broccoli MMS was perceived by the highest percentage of final consumers as being slightly influenced (36%) by broccoli MMS However, a proportion of the final consumers considered that the texture of the product was changed to a very small extent (18%), not changed (18%), changed to a very

large extent (12%), or changed to a large extent (11%), while only 5% indicated that they did not know. These data indicate that broccoli MMS do not significantly influence the texture of the product. This aspect may contribute to easier acceptance of the product by potential final consumers who prefer the conventional texture of a cow's white cheese (telemea) pie.

Do you consider that the addition of broccoli sprouts changed the texture of the pie?

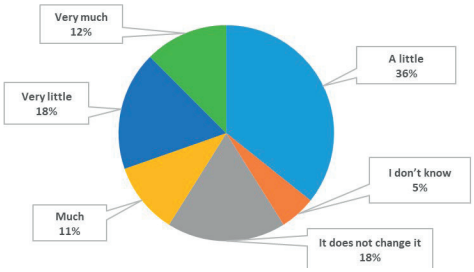

Figure 9. Most respondents consider that broccoli sprouts slightly affect the texture of the pie

The third part of the questionnaire highlighted the behaviour of the final consumer through information regarding the most important sense in choosing a pie-type product (Figure 10), the price for 1 kg of the product cow's white cheese (telemea) pie with broccoli MMS (Figure 11), and the dominant factor in the choice of a food product (Figure 12).

According to the opinion of the final consumer, the most important sense in choosing a pie-type product was taste (84%) (Figure 10), while the olfactory sense ranked second (7%), followed by the visual sense (5%) and the tactile sense/texture (4%).

The price for 1 kg of the product cow's white cheese (telemea) pie with broccoli MMS (Figure 11), in the opinion of the final consumer, was considered most appropriate at 43 RON (37%). While the values of 48 RON (34%), 38 RON (25%), 53 RON (2%), and 58 RON (2%) indicate a preference of the final consumer for a price range between 43 and 48 RON.

The dominant factor in the choice of a food product (Figure 12), according to the opinion of the final consumer, was quality (57%). However, a proportion of the final consumers considered price (21%), brand (7%), and packaging (6%) to be more important than the quality of a food product. These data indicate that the purchasing decision of the final consumer is influenced by quality and price as cumulative factors with a decisive role in the selection of a food product.

From your point of view, which sense is the most important when choosing a pie?

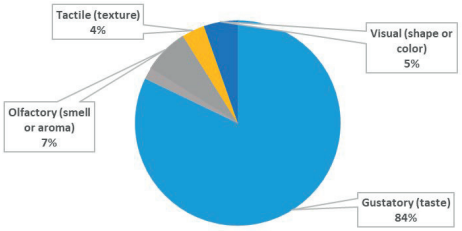

Figure 10. Taste is considered the most important sense when choosing a pie

What would be the maximum amount you would be willing to pay for 1 kg of the product pie with added broccoli sprouts?

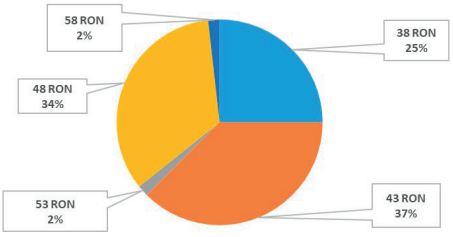

Figure 11. Most consumers are willing to pay a moderate price for one kilogram of the product

Which of the following factors influence you the most when choosing a food product?

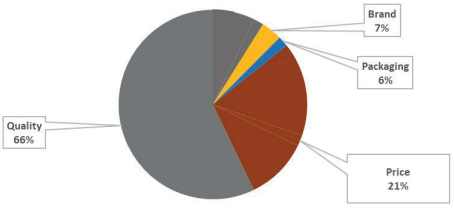

Figure 12. Quality is the dominant factor influencing consumers when choosing a food product

The recorded results show that broccoli MMS added to cow's white cheese (telemea) pie positively influence the purchasing preferences

of the final consumer. The aroma and texture of the product were perceived as being slightly influenced, without affecting overall acceptability. Colour was appreciated by the majority of respondents, being considered stable and attractive.

Taste proved to be the main sensory factor, being predominantly evaluated as satisfactory, while maintaining the characteristic salty profile of the product. Consumers prefer a low to moderate content of broccoli MMS, indicating the need for an optimal dosage.

In the purchasing decision, quality and taste are the dominant factors, and the product cow's white cheese (telemea) pie with broccoli MMS was associated with a purchase price in the range of 43-48 RON per 1 kg. In conclusion, the integration of broccoli MMS represents a viable opportunity for diversifying the product cow's white cheese (telemea) pie, with good potential for acceptance among final consumers.

CONCLUSIONS

This study presents information on the influence of broccoli MMS added to the cow's white cheese (telemea) pie on the purchasing preferences of the final consumer.

The experimental data obtained through sensory analysis and the online questionnaire indicate favourable acceptance of the product cow's white cheese (telemea) pie with broccoli MMS. The influence of broccoli MMS on aroma (38%) and texture (36%) was perceived as moderate, without a negative impact on overall product acceptability, while moderate taste (39%) and pale green colour (84%) were evaluated positively. Taste was identified as the main sensory factor in the purchasing decision, together with perceived quality and a price considered appropriate (43-48 RON per 1 kg of product).

The experimental results highlight the influence of broccoli MMS on the purchasing preferences of the final consumer and indicate that the experimental variant consisting of the product cow's white cheese (telemea) pie with 50% broccoli MMS has potential as an innovative food product.

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AI-ENHANCED ADDITIVE MANUFACTURING IN BIOTECHNOLOGY: BRIDGING THE DIGITAL GAP THROUGH 3D SCANNING AND GENERATIVE DESIGN OPTIMIZATION

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Abstract

The rise of Industry 4.0 technologies has transformed the biotechnology field by shifting competence requirements toward integrating biological sciences, digital design, and additive manufacturing systems. This study investigates the use of AI-enhanced 3D printing in higher education within the biotechnological field. The framework integrates Gemini AI for generative design optimization and predictive error compensation, enabling a transition from conventional computer-aided design workflows to an adaptive, data-driven production cycle. The ecosystem enables a continuous digital-to-physical iteration process where students design, prototype, and assess laboratory components. High-speed fabrication shortens production time, while AI-assisted optimization enhances dimensional accuracy and structural performance. The approach integrates additive manufacturing into core discipline activities and reinforces the connection between experimental practice and digital engineering. Empirical results showed a 40% decrease in prototyping failure rates and an over 90% drop in operational costs compared to traditional laboratory procurement models. Integrating affordable additive manufacturing infrastructures improves technical independence, engineering literacy, and practical problem-solving abilities. The results indicated that AI-supported fabrication systems offer a scalable approach to advancing biotechnology education and preparing graduates for technology-focused, data-driven careers.

Key words: *AI-Enhanced Manufacturing, Digital Gap in Higher Education, Gemini Multimodal AI, Generative Design, 3D Scanning & Reverse Engineering.*

INTRODUCTION

Contemporary biotechnology is undergoing a paradigm shift, moving away from isolated wet-lab procedures toward a highly integrated model known as "Biomanufacturing 4.0.". In this landscape, Additive Manufacturing (AM), or 3D printing, has emerged as a cornerstone, enabling the fabrication of microfluidic devices, customized labware, and tissue scaffolds (Wang et al., 2024; Cong & Zhang, 2025). Consequently, the competency profile of a modern biotechnologist must expand to include "digital fluency" the ability to translate biological requirements into physical objects using Computer-Aided Design (CAD).

Despite European directives emphasizing digitization, the Romanian educational system faces a structural hurdle. Observational data from USAMV Bucharest reveals a marked "digital gap" in freshman cohorts, stemming from a lack of exposure to modern technologies in the pre-university curriculum. Unlike STEAM-oriented systems, Romanian secondary education remains predominantly theoretical, leaving students deficient in 3D spatial visualization - a skill essential for understanding complex concepts such as protein folding and bioreactor geometry.

A vertically integrated curriculum can strengthen the development of digital competence in higher education. Students build foundational digital skills at the undergraduate level and deepen them during master's studies.

This approach aligns with the principles of Frugal Science. Research shows that modular,

open-source hardware reduces equipment costs and expands access to laboratory technologies. McNair et al. (2024) demonstrate that such tools democratize scientific practice and support hands-on learning. Recent advances also demonstrate the potential for fully open-source 3D-printed laboratory automation systems (Kuwahara et al., 2025).

Educational studies support this direction. Fernandez-Castane (2024) showed that integrating digital skills into technical modules increases students' adaptability. It also improves retention of abstract concepts.

In higher education, impact grows when digital training is continuous, applied, and evidence-based. Structured progression, practical implementation, and measurable learning outcomes drive stronger competence development and better preparation for evolving professional demands.

Recent developments in High-Speed FDM (Hyper-FDM) have further revolutionized educational possibilities, enabling "synchronous prototyping" in which designs are iterated within a single seminar (Aktepe & Ergün, 2025). This paper documents the methodology of setting up such a laboratory at USAMV and analyses the student response through detailed cost-benefit optimization experiments, and details the implementation of 3D printing and Artificial Intelligence (AI) as a strategic bridge to close this digital gap.

MATERIALS AND METHODS

The study was conducted within the Applied Informatics Laboratory at the Faculty of Biotechnologies, USAMV Bucharest. The infrastructure was strategically selected to cover the main pillars of digital fabrication, balancing industrial performance with open-source compatibility.

Equipment and Ecosystem. The laboratory operates a heterogeneous fleet of printers categorized by pedagogical role (Table 1):

- **High-Speed Units (Creality K1/K2)**: Used for rapid iteration (speeds up to 600mm/s), allowing students to fail fast and improve designs during class.
- **Production Units (Creality CR10-SE)**: Utilized for large-volume batch production of anatomical models.
- **Didactic Units (Ender 3 Pro)**: Open-structure printers used to teach CNC mechanics and maintenance.
- **Precision Units (Resin/SLA)**: Employed for microfluidics and high-fidelity surface details (Wężgowiec et al., 2024).
- **Digitization (Revo Scan Pro)**: Used for reverse engineering biological samples or broken parts.
- **AI-Augmented Production: Gemini-Driven Optimization**: To overcome the limitations of raw 3D scan data, we implemented a "Digital Twin" protocol optimized by the **Gemini multimodal AI** structured around two main components: **A. Slicing Configuration**: Instead of generic profiles, AI generated customized parameters for the Creality K2 printers. We validated the use of 4 wall perimeters (1.6 mm) for waterproofing and a 15% Gyroid infill; **B. Predictive Compensation**: Based on thermal expansion coefficients, Gemini calculated a +0.2 mm geometric offset for contact zones, ensuring high-precision friction fits and eliminating the need for mechanical post-processing (Zhang et al., 2026).

Table 1. Technical specifications and educational stratification of the 3D printing ecosystem

Equipment Model	Technology	Key Feature	Pedagogical Role
Creality K1/K2	FDM (CoreXY)	High-Speed (600 mm/s)	Rapid Prototyping (Synchronous)
Creality CR10-SE	FDM (Cartesian)	Large Volume	Batch Production
Ender 3 Pro	FDM (Cartesian)	Open Structure	Mechanics & Maintenance
Resin Printer	SLA/DLP	High Resolution	Precision / Microfluidics
Revo Scan Pro	Scanning	0.05 mm Precision	Reverse Engineering

The technical infrastructure documented in Table 1 reflects a strategic approach to additive manufacturing integration, prioritizing functional hierarchy over uniform performance. By deploying **Creality K2** units (Figure 1), the laboratory transitioned from asynchronous to "synchronous prototyping," significantly compressing the design-iteration cycle within single seminar sessions.

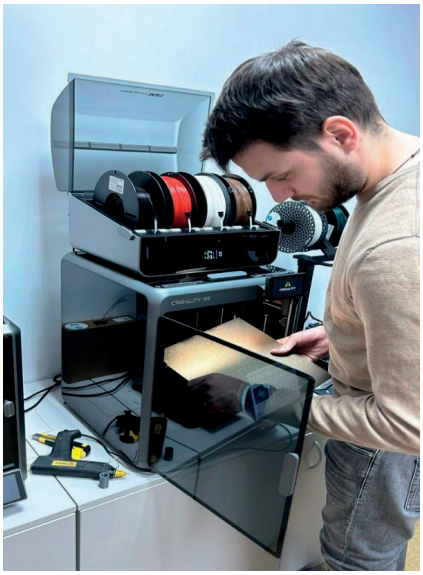

Figure 1. Creality K2 3d printer

This fail-fast approach is critical in addressing the digital gap, as it allows students to immediately visualize the consequences of CAD modelling errors. Furthermore, the inclusion of open-structure units like the **CR 10-SE** (Figure 2), serves a vital pedagogical role: demystifying the mechanical and maintenance aspects of CNC hardware, a competency that ensures the long-term sustainability and "right-to-repair" mindset within a biotechnological research unit.

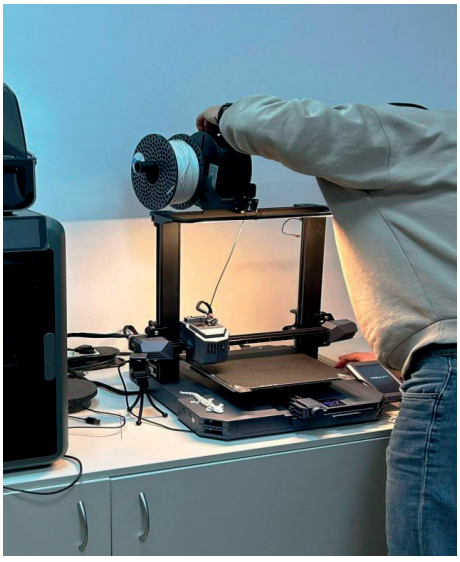

Figure 2. Creality CR 10-SE 3d printer

Optimization of Slicing Parameters. A critical component of the methodology was the optimization of slicing parameters using *Creality Print* and *Ultimaker Cura* software. Since biotechnological equipment often comes into contact with moisture or requires sterilization, standard decorative printing settings are insufficient. We established a specific "Bio-Lab Profile" for the FDM printers. For the microtube racks, the structural integrity of the mesh was prioritized over surface finish. We conducted a comparative stress test using three different wall line counts (2, 3, and 4 perimeters). Results indicated that increasing the wall line count to 4 (approx. 1.6mm thickness with a 0.4 mm nozzle) provided superior rigidity compared to increasing the infill percentage. Table 2 details the optimized parameters established for the two main material types used in the laboratory: PLA (for general organizers) and PETG (for chemically resistant parts).

Table 2. Optimized Slicing Parameters for Biotechnological Applications

Parameter	PLA (Standard)	PETG (Functional)	Reasoning for Lab Use
Nozzle Temp	210°C	240°C	Ensure layer bonding
Bed Temp	60°C	80°C	Prevent warping
Speed (K1)	300 mm/s	150 mm/s	PETG needs slower speed
Wall Count	3 lines	4 lines	Strength comes from walls
Infill Pattern	Gyroid (15%)	Grid (25%)	Gyroid allows drainage
Cooling Fan	100%	30-50%	Layer adhesion focus

The slicing parameters established in Table 2 demonstrate the adaptation of FDM technology to the specific rigors of a biological laboratory. The selection of **PETG** at 240°C with a reduced speed of 150 mm/s was technically necessary to achieve superior inter-layer adhesion, ensuring that the resulting parts are chemically resistant and mechanically isotropic. Notably, the adoption of the **Gyroid infill pattern** at 15% density represents an engineering optimum; this non-planar geometry provides exceptional multi-directional structural integrity while facilitating fluid drainage during autoclaving or chemical sterilization, thereby preventing the accumulation of contaminants within the

internal lattice - a common failure point in standard decorative 3D prints.

The Reverse Engineering Workflow. To address the issue of broken laboratory equipment parts for which no CAD files existed (e.g., a cracked centrifuge lid hinge), we implemented a digitization workflow using the **Revo Scan Pro**. The scanning process involved: (1) Coating shiny surfaces with a matting spray (AESUB); (2) Scanning in handheld mode at 0.1 mm point distance; (3) Mesh processing (STL file generation); and (4) Boolean operations in CAD to "virtually repair" the broken features (Chiriță et al., 2023).

RESULTS AND DISCUSSIONS

Optimization of Laboratory Logistics. The core experiment involved an interdisciplinary collaboration with the Microbiology Department to validate the "On-Demand Manufacturing" model. The objective was to fabricate centrifuge microtube racks (Figure 3).

Figure 3. Original PCR tube rack

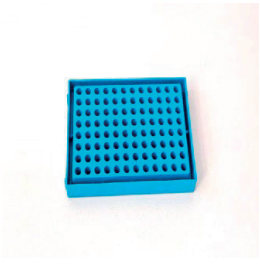

Figure 4. 3D printed PCR tube rack

Material and Efficiency Analysis. Students conducted a cost-benefit analysis by printing rack iterations with varying parameters. The analysis revealed that for static labware, "over-engineering" is unnecessary.
A rack printed at 0.28mm layer height with 15% gyroid infill offered the optimal balance, consuming 40% less material than high-density versions (Figure 4).

Detailed Economic Breakdown To rigorously validate the "Frugal Science" hypothesis, we calculated the Total Production Cost (TPC) for a batch of 10 microtube racks using the formula:
TPC = C_mat + C_energy + C_dep
Where:
- C_mat (Material Cost): 85g of filament per rack × 10 racks × 0.02 EUR/g = 17.00 EUR;
- C_energy (Energy Cost): The Creality K1 consumes approx. 350W during heating and 100W during printing. For a 3-hour batch print: 0.5 kWh × 0.30 EUR/kWh = 0.15 EUR;
- C_dep (Machine Depreciation): Calculated at 0.50 EUR per printing hour.

The final calculation revealed a TPC of roughly 1.85 EUR per unit (including amortization), whereas the catalog price for a compatible commercial rack exceeds 22.00 EUR. This represents a 91.5% cost reduction, validating the economic models proposed by McNair et al. (2024). Using AI-optimized slicing, the part was reproduced using the Creality K2, resulting in a 90% reduction in equipment downtime compared to traditional procurement.

Extending Equipment Lifespan (The "Right to Repair") was a secondary result that emerged from the reverse engineering module. Students successfully scanned, remodelled, and printed a replacement component for the laboratory. This practical success story served as a powerful proof-of-concept for the "Right to Repair" movement within academic institutions, emphasizing the role of 3D scanning in the circular economy (Chiriță et al., 2023).

Sustainability and Carbon Footprint Considerations. Beyond financial savings, the local manufacturing approach has significant environmental implications. By eliminating the shipping of plastic labware from international suppliers, the Carbon Footprint associated with transportation is negated. Furthermore, the use of PLA (polylactic acid), a bioplastic derived from corn starch, offers a more sustainable alternative to the petroleum-based polypropylene typically used in commercial racks. Recent studies confirm that optimized parameters and recycling strategies can significantly improve the mechanical properties of recycled PLA, making it a viable material for sustainable packaging and labware, reducing the carbon footprint by approximately 80% compared to petroleum-based polystyrene (PS) alternatives, largely due to renewable material origins and localized production (Tănase et al., 2024; Chien et al., 2024).

Student Perception and Skill Acquisition. The educational impact was assessed through a qualitative survey administered to 30 students enrolled in the "Applied Informatics" undergraduate course and to the 15 students enrolled in the "Digital Tools for Bio-entrepreneurs" master module.

The survey instrument consisted of a structured questionnaire comprising four items rated on a 5-point Likert scale (1 = strongly disagree, 5 = strongly agree). It was administered synchronously at the end of the semester to all enrolled students (n = 45) across both the undergraduate and master's modules (Table 3).

Table 3. Student Survey Results (n = 45)

Survey Item	Mean Score (1-5)	Interpretation
Q1: I feel confident operating a 3D printer independently	4.2	High technical adoption
Q2: 3D modeling helps me visualize biological structures better	4.6	Validates Visual Learning
Q3: I believe these skills are useful for a biotech startup	4.8	Strong links to entrepreneurship
Q4: I encountered significant frustration during design	3.5	Steep learning curve of CAD

The survey results presented in Table 3 (n=45) validate the efficacy of the implemented "Maker-Biotech" curriculum. The exceptional mean score for Q3 (4.8) indicates that students have moved beyond perceiving 3D printing as a peripheral tool and now recognize it as a core strategic asset for bio-entrepreneurial ventures. The strong correlation between high Q2 scores (4.6) and the learning objectives confirms the success of the "Dual Coding" haptic-visual approach, in which physical interaction with models compensates for pre-university deficiencies in spatial visualization. However, the moderate score in Q4 (3.5) highlights a persistent cognitive threshold in CAD software proficiency, suggesting that future curriculum iterations should incorporate more scaffolded tutorials to mitigate design-stage frustration.

Technical Challenges and Troubleshooting The implementation was not without technical hurdles (Table 4).

Table 4. Common Technical Failures and Solutions

Issue	Cause	Solution Implemented by Students
Warping	Uneven cooling (PETG)	Use of brim adhesion; Enclosure (K1)
Tolerance	Material shrinkage	Added +0.2 mm offset to CAD holes
Stringing	Wet filament	Use of filament dryer box (45°C/6 h)

Table 4 summarizes the practical challenges faced, turning technical failures into "teachable moments" about polymer thermodynamics. Combining enclosures and brim adhesion to address warping provides students with hands-on insights into thermal contraction and bed adhesion physics. Managing dimensional tolerances is especially crucial; applying a +0.2 mm offset in CAD designs marks an important step toward applied engineering. This helps students learn to predict how materials behave after processinga - key factor in successfully reverse-engineering components with the Revo Scan Pro, where precise fitment is essential for safety. Incorporating AI-driven 3D printing has greatly boosted student engagement. Moving from abstract "Digital Tools" to "Smart Manufacturing," students bridge the digital divide effectively. High-speed systems like the Creality K2 enable quick, repetitive design cycles within a single lab session.

CONCLUSIONS

This study highlights the importance of integrating additive manufacturing into higher education biotechnology programs as a strategic intervention to bridge the digital skills gap and enhance educational outcomes. The implementation of a multimodal ecosystem integrating FDM, resin-based printing, 3D scanning, and AI technologies demonstrates the pedagogical scalability and cost-efficiency of 3D printing.

The microtube rack case study illustrates how students can acquire digital fabrication skills while addressing real laboratory needs. The intervention resulted in notable reductions in operational costs and material waste, improved prototyping accuracy, and enhanced workflow efficiency. Incorporating engineering skills supports the maintenance and replication of legacy lab equipment, promoting resource efficiency and circular-economy principles in academic settings.

The findings further indicate that AI-enhanced additive manufacturing infrastructure lowers technical barriers, reduces prototyping failure rates, and fosters a maker-oriented mindset among biotechnology students. Structured digital workflows improve applied problem-solving abilities and technological autonomy.

The proposed development of a Smart Bio-Lab acts as a strategic extension of this model, with the potential to inspire a collective sense of purpose. It aims to increase innovation capacity, entrepreneurial skills, and the international competitiveness of future Romanian bio-entrepreneurs, motivating stakeholders to support this forward-looking initiative.

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RECOVERY OF BIOACTIVE COMPOUNDS FROM SELECTED INDUSTRIAL PLANT WASTES AND BY-PRODUCTS-CASE STUDIES ON: TOMATO PROCESSING RESIDUES, OLIVE WASHING WATERS AND SEA BUCKTHORN BY-PRODUCTS

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Abstract

Industrial processing of plant raw materials generates significant amounts of solid and liquid residues, which are frequently underutilized despite their high biochemical potential. By-products resulting from tomato processing, wastewater from olives and sea buckthorn residues represent heterogeneous waste streams originating from different technological processes, yet sharing a common characteristic: a substantial content of biologically active compounds. These residues contain phenolic compounds, carotenoids, organic acids and dietary fibers, exhibiting antioxidant, antimicrobial and functional properties. This review provides a comparative analysis of agro-industrial wastes derived from tomato, olive and sea buckthorn processing, with emphasis on their chemical composition, environmental impact and biotechnological valorization potential. Current extraction and recovery methods, both conventional and emerging, are discussed in terms of efficiency, sustainability and industrial-scale applicability. In addition, potential applications of recovered bioactive compounds in the field of food biotechnologies are highlighted, in accordance with sustainable production systems and circular economy principles.

Key words: *bioactive compounds, olive, sea buckthorn, tomato, waste.*

INTRODUCTION

Food production and processing in developing countries generates high levels of waste and by-products, causing negative environmental impacts and significant costs. However, the resulting by-products have a high potential to provide bioactive compounds used in the production of food additives, contributing to the correction of nutritional deficiencies and improving diet quality (Torres-León et al., 2018).

Currently, new strategies for waste reduction, use and recovery, as well as enrichment of the economic value of by-products, represent an important challenge that needs to be implemented (Silva et al., 2021).

Extracts from plant material present numerous benefits for human health, chemical compounds that have beneficial effects on human health are integral parts of plants, often included in their defense system, defined as biologically active components (Žlabur et al., 2018). Various industries have shown significant interest in the application of bioactive plant compounds, such as the pharmaceutical industry for the production of drugs and the food industry for the production of food supplements classified as "superfoods" (Žlabur et al., 2018).

Bioactive compounds include both essential nutrients, such as vitamins and minerals, and non-nutritional compounds. While some of these contribute to nutritional intake, their predominant function is related to biological activity and physiological effects exerted on the body, beyond simple nutritional value. (Riccardi, 2022) such as phytochemicals, polyphenols, carotenoids, flavonoids, probiotics, enzymes, and other naturally occurring compounds that exert beneficial physiological effects on the human body beyond basic nutritional value. Minerals are represented by macroelements (calcium, phosphorus, magnesium, chlorine, sulfur, sodium, potassium) and microelements (iron, cobalt, manganese, molybdenum, copper, zinc, vanadium, iodine, selenium, fluorine, chromium, bromine). According to the scientific

literature, the category of biologically active substances, other than minerals and vitamins, can include: flavonoids, carotenoids, allyl and diallyl sulfides, indoles, monoterpenes, phenolic acids, sterols, pectins, resveratrol from red grapes, prebiotics, etc. (Banu, 2007).

Food waste and food by-products can be nutritionally rich food ingredients with functional properties. Food waste is made up of materials intended for human consumption that are subsequently disposed of, lost, degraded or contaminated (Girotto et al., 2015). Ecologically sustainable food must reconsider by-products resulting from the food industry as a source of valuable compounds (antioxidants, fibers, etc.) that could be used as new products or raw materials in the food industry (Rodriguez-Lopez et al., 2020).

Industrial by-products from fruits and vegetables consist mainly of peel, pomace and seed fractions, which could be a good source of compounds with high nutritional value such as proteins, polysaccharides, dietary fibers, flavor compounds and phytochemicals (Galanakis, 2012).

Several examples of fruit and vegetable industry by-products and wastes that can serve as sources of bioactive ingredients for use in the food industry will be presented and discussed in this paper.

VALORIZATION POTENTIAL OF WASTEWATER FROM OLIVE PROCESSING

Wastewater produced during olive processing - such as washing water and effluents from the oil extraction stage - shows a complex and highly variable chemical composition that depends on the processing technology used (traditional, press-based, two-phase, or three-phase centrifugation), as well as the olive variety and specific operating conditions. (Paraskeva & Diamadopoulos, 2006).

One of the main characteristics of wastewater from the olive industry is the extremely high organic load. Chemical oxygen demand (COD) values reported in the literature range from 50 to 220 g/L, while biochemical oxygen demand (BOD_5) is usually between 30 and 100 g/L, indicating a high BOD_5/COD ratio, typical of effluents rich in biodegradable compounds (Roig et al., 2006; El-Abbassi et al., 2012). The BOD_5/COD ratio is frequently between 0.4 and 0.6, suggesting a moderate biodegradability potential, limited however by the presence of phenolic compounds with an inhibitory effect on microorganisms (Paraskeva & Diamadopoulos, 2006). Wastewaters from olive processing have a slightly acidic pH, with values reported between 4.0 and 6.0, due to the presence of organic acids and phenolic compounds (Niaounakis & Halvadakis, 2006). The electrical conductivity varies between 1 and 12 mS/cm, reflecting the high concentration of dissolved mineral salts (El-Abbassi et al., 2012).

Phenolic compounds represent the most problematic fraction, but also the most valuable in terms of recovery potential. The total concentration of polyphenols is reported in the range of 0.5–10 g/L, depending on the extraction method and the maturity of the olives (Cassano et al., 2018). The organic matter is composed of: carbohydrates (10-50 g/L), lipids (0.5-5 g/L), proteins and amino acids (1-10 g/L) (El Abbassi et al., 2012).

The particular chemical composition of wastewater from olive processing explains both the difficulty of treating them by conventional methods and the increased interest in biotechnological recovery methods. The high content of phenolic compounds and organic matter makes these waters suitable for antioxidant recovery processes and controlled fermentation, provided that pretreatment steps are applied (Cassano et al., 2018; Koutrotsios et al., 2023).

Polyphenols recovered from olive wash waters, especially hydroxytyrosol, tyrosol and their derivatives, show a high **antioxidant capacity**, comparable or superior to currently used synthetic antioxidants butylated hydroxytoluene (BHT) and butylated hydroxyanisole (BHA) (Gullón et al., 2023). According to studies conducted by Cassano (2018), phenolic extracts obtained from wash waters delay lipid oxidation in vegetable oils and meat products; extend the shelf life of foods rich in fat; reduce the formation of secondary oxidation compounds. (Cassano et al., 2018). These compounds can be used in products such as: margarines, sauces, bakery products.

According to EFSA (European Food Safety Authority), hydroxytyrosol has a role in protecting blood lipids against oxidative stress

when consumed in adequate amounts. Thus, olive polyphenols are associated with beneficial effects on cardiovascular and metabolic health.
Extracts obtained from washing waters can be used as bioactive ingredients for functional beverages and fermented dairy products (El-Abbassi et al., 2012)

Natural antimicrobial agents and biopreservatives

Phenolic compounds in olive washing waters exhibit antimicrobial activity against pathogenic and spoilage microorganisms, including *Listeria monocytogenes, Escherichia coli* and *Staphylococcus aureus* (Roig et al., 2006).
Yılmaz (2024) evaluated the antimicrobial effects of olive wastewater extract on microorganisms related to food spoilage and foodborne diseases. The results demonstrated that the extract exhibited significant activity against spoilage bacteria or food pathogens, suggesting its potential as a natural biopreservative in foods (Yılmaz et al., 2024).

Integration in bakery products and plant foods

Phenolic extracts can also be used in bakery products or processed plant foods, where they contribute to increasing the total antioxidant capacity without significantly affecting the sensory properties, when used in controlled concentrations (Gullón et al., 2023). Recently, the integration of olive mill wastewater (OMWW) in bakery products has become a topic of interest for the development of functional foods. Restivo et al. (2025) demonstrated that bread can be manufactured using olive mill wastewater as a partial or total replacement of water in the dough, which leads to an increase in the total phenolic content and antioxidant capacity of the final product. Research has shown that bread obtained with olive mill wastewater exhibits up to a fourfold increase in antioxidant activity (measured in Trolox equivalents) compared to the control bread.
Also, studies conducted by Galanakis et al. (2018) showed that phenols in olive mill wastewater can act as natural biopreservatives, inhibiting microbial growth and extending the shelf life of bakery products. These polyphenols can be incorporated either directly, by replacing water in the dough, or in the form of concentrated extracts, while maintaining their functional effect.
Another study conducted in Egypt (2025) used dried olive mill wastewater in bread and biscuits, demonstrating improved dough rheological properties and increased bread volume, as well as increased oxidative stability and total phenolic content (Jeba et al., 2025). In addition, Karadağ et al. (2024) integrated olive mill wastewater polyphenols into cake formulations, demonstrating that these bioactive ingredients do not negatively affect the texture of the products and improve antioxidant properties.

VALORIZATION OF WASTE AND BY-PRODUCTS FROM TOMATO PROCESSING

Tomato processing waste (tomato pomace) includes peels, seeds and residual pulp after juicing and represents approximately 10-40% of the total mass of processed tomatoes, depending on the type of product and the technology used (Knoblich et al., 2005).
Tomato processing waste is rich in dietary fiber, protein, lipids, and represents an important source of carotenoids (especially lycopene), polyphenols, and vitamins. Lycopene content in dried tomato waste can reach high levels depending on processing conditions, and significant antioxidant activity has been demonstrated using DPPH and ABTS assays (Kuvendziev et al., 2018, Szabo et al., 2021). These bioactive compounds are associated with anti-inflammatory, anticancer, and cardiovascular protective effects (Story et al., 2010)
Extracts from tomato waste, rich in carotenoids (especially lycopene) and phenolic compounds, can be used as natural antioxidants in oxidation-sensitive food systems. The study conducted by Popescu C et al. (2022) demonstrated that extracts from tomato processing waste can be applied to vegetable oils (e.g. soybean oil) in order to reduce oxidative damage during heating, suggesting a natural alternative to synthetic antioxidant additives in industrial food products (Popescu et al., 2022).
Several studies demonstrate that tomato waste (tomato pomace), rich in lycopene, β-carotene and phenolic compounds, can be utilized as a natural antioxidant in food products, contributing to the reduction of lipid oxidation

and the improvement of oxidative stability. Tomato pomace extracts have been shown to effectively reduce lipid oxidation in meat products, while extracts obtained from tomato skins and seeds exhibit high antioxidant activity, supporting their application as functional food ingredients (Ćetković et al., 2015).

Tomato pomace, as a source of fiber, carotenoids and antioxidants, is increasingly used to improve the nutritional and functional quality of food products. In a recent review, Silva et al. (2023) report that pomace can be incorporated into bakery products, sausages, dairy products or snacks as a natural functional ingredient that improves oxidative stability, increases fiber content and can extend shelf life. (Silva et al. 2023)

Studies conducted by Domínguez et al. (2020) demonstrate that partial replacement of wheat flour with 10-20% tomato pomace flour in bread allows obtaining a functional product, rich in fiber, carotenoids and antioxidant compounds, while maintaining sensory and rheological properties comparable to conventional bread, which indicates a significant potential for the valorization of tomato waste in the bakery industry (Domínguez et al., 2020).

Chabi et al. (2024) demonstrated that bioactives from tomato waste can be used to produce microcapsules of oleoresins (concentrated carotenoid extracts), which, added to juices (e.g. orange juice), confer antioxidant activity without significantly affecting the sensory quality of the product. These microcapsules can function as a natural ingredient for food fortification with antioxidants (Chabi et al., 2024).

Studies conducted by Calvo et al. (2020) showed that pomace extracts combined with vegetable oils (e.g. sunflower or rapeseed oil) improve the oxidative stability and nutritional properties of the oils, due to the high content of carotenoids and antioxidant compounds. Thus, pomace becomes a natural functional additive for edible oils that can partially replace synthetic antioxidants (Calvo et al., 2020).

Bioactive compounds, especially lycopene, β-carotene and phenols extracted from tomato pomace, exhibit antioxidant, anti-inflammatory, antimicrobial and anticancer potential. Recent scientific reviews indicate that these compounds can be used in nutraceutical formulations and dietary supplements to support cardiovascular health, the immune system, and reduce the risk of chronic diseases (Shah et al., 2020). Bioactive compounds in tomato waste reduce cell proliferation and oxidative stress, and are linked to a reduced risk of prostate and lung cancer (Tang et al., 2011; Giovannucci, 2002)

VALORIZATION OF WASTE AND BY-PRODUCTS FROM SEA BUCKTHORN PROCESSING

The waste resulting from the processing of sea buckthorn (*Hippophae rhamnoides*), also known as sea buckthorn pomace, is a rich source of bioactives: dietary fiber, polyphenols, flavonoids, minerals, fatty acids and carotenoids, which remain in the solid residues after the extraction of juice or oil. Instead of being considered a valueless residue, this pomace can be effectively utilized to obtain functional ingredients, bioactive extracts and to improve the nutritional profile of food products, in line with the principles of circular economy and sustainable production.

Sea buckthorn pomace is rich in polyphenols, flavonoids and carotenoids, compounds that confer significant antioxidant properties and can be recovered for food or nutraceutical uses. Successive extraction with food solvents or by green methods (ultrasound, supercritical CO_2) showed that this waste material can provide bioactive fractions with high antioxidant activity and free radical inhibition capacity.

Sea buckthorn by-products, including pomace, skins, and berries, are rich in bioactive compounds such as polyphenols, flavonols, carotenoids, vitamins (water- and fat-soluble), and minerals. These compounds confer multiple functional properties, making sea buckthorn waste a promising ingredient for functional foods, beverages, and nutraceutical products.

Bioactive compounds from sea buckthorn by-products, especially polyphenols, flavonols (quercetin, kaempferol, isorhamnetin), catechins, and carotenoids, exhibit strong antioxidant activity. This has been confirmed using DPPH, ABTS, and ORAC assays (Dienaitė et al., 2020; Mihalcea et al., 2021). Their antioxidant potential contributes to:

Improving oxidative stability in bakery products, beverages, and fortified foods

Preserving sensitive nutrients and bioactives

Supporting general health through free radical scavenging, Addition of 6-10% dried pomace to wheat bread increased fiber, protein, and antioxidant compounds while conferring strong antioxidant activity (Stanciu et al., 2023).

Sea buckthorn pomace incorporated into rye bread at 15-20% enhanced antioxidant content (Călin et al., 2024).

Extracts rich in polyphenols and carotenoids were used in functional beverages and fortified foods, improving oxidative stability and delivering health-promoting antioxidants (Mihalcea et al., 2021; Dienaitė et al., 2020).

Certain compounds in sea buckthorn by-products possess antimicrobial activity, which can extend the shelf life of products. Bread with 6-10% pomace showed antimicrobial effects, alongside antioxidant benefits, improving functional properties and reducing caloric energy (Stanciu et al., 2023). Sea buckthorn berry flour at 1-5% improved shelf life in wheat bread without negatively affecting sensory properties (Ghendov-Mosanu et al., 2020).

Sea buckthorn by-products are a rich source of dietary fiber, vitamins, minerals, and proteins, enhancing the nutritional value of foods and supplements.

Dried pomace at 6-10% in wheat bread increased fiber, protein, and polyphenol content; optimal sensory results were observed at ~8% addition (Stanciu et al., 2023).

Rye bread with 15–20% pomace showed increased bioactive content and antioxidant compounds, although higher additions influenced texture and moisture (Călin et al., 2024).

Cereal biscuits enriched with 5-20% sea buckthorn biomass improved oxidative stability and sensory appeal (Janotkova et al., 2021).

Extracts from sea buckthorn by-products can be incorporated into beverages and fortified foods as functional ingredients due to their high content of polyphenols, flavonols, and carotenoids. Advanced extraction techniques - supercritical CO_2, pressurized solvent extraction, ultrasound-assisted extraction - allow recovery of bioactive-rich fractions (Cosma et al., 2021).

Fortified beverages and functional foods enriched with antioxidant extracts (Dienaitė et al., 2020; Mihalcea et al., 2021)

Synbiotic beverages containing sea buckthorn bioactives with probiotics and prebiotic fibers to support digestive and immune health (Maftei et al., 2023)

Sea buckthorn processing waste (pomace-SBP) provides bioactive compounds suitable for functional supplements and nutraceuticals.

Flavonols (isorhamnetin, quercetin, kaempferol), catechins, and carotenoids with antioxidant and antiproliferative potential (Dienaitė et al., 2020)

Polyphenols that inhibit enzymes associated with oxidative stress and protect LDL-cholesterol and DNA, supporting metabolic and cardiovascular health (Renan and Fereidoon, 2024)

Carotenoids (zeaxanthin, β-carotene) with improved stability via microencapsulation technologies (Gherasim et al., 2024)

Vitamins (water and lipid-soluble, e.g., vitamin C, carotenoids) contributing to broad-spectrum antioxidant action and general health support (Dianu et al., 2024)

Potential physiological benefits in regulating glucose metabolism, lipid profiles, and inflammatory markers, relevant for metabolic syndrome and cardiovascular risk management (Shing et al., 2025)

Sea buckthorn bioactives affect color, taste, texture, and shelf life of foods. Optimal inclusion levels balance enhanced nutritional and functional properties with consumer acceptability.

Wheat bread with 8% pomace showed favorable sensory properties while improving nutritional and functional characteristics (Stanciu et al., 2023)

Sea buckthorn powders at 3-10% in bakery products modified texture, moisture, and color but maintained bioactive content (Nilova and Malyutenkova, 2018; Popovici, 2022).

The sustainable valorization strategies for tomato, sea buckthorn, and olive mill wastewater are summarized in Figure 1.

Figure 1 highlights the multiple pathways through which these by-products can be transformed into value-added products, energy sources, and other applications such as soil additives or sorbents. It illustrates how bioactive compounds, dietary fibers, proteins, and oils can be recovered and utilized, while also showing the potential for energy recovery through pyrolysis or combustion.

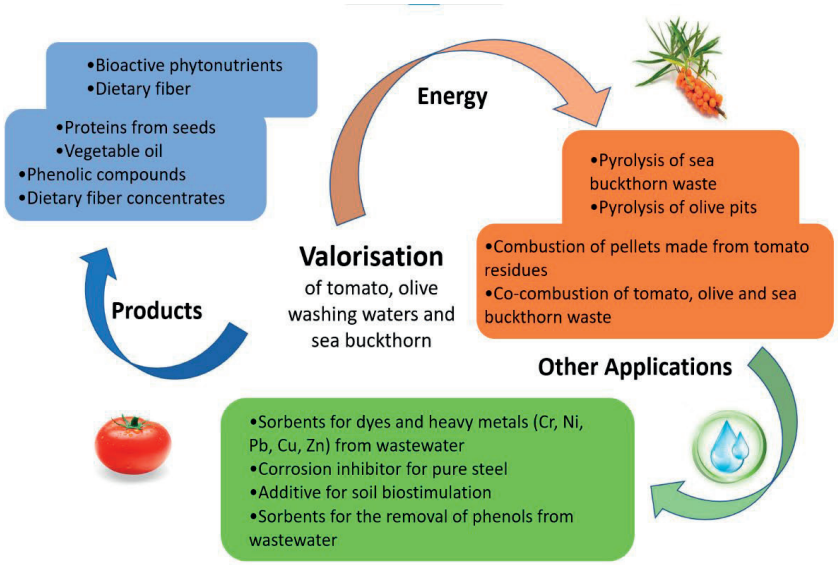

Figure 1. Integrated Valorization of Tomato, Sea Buckthorn, and Olive Mill Wastewater By-Products for High-Value Compounds Recovery

The overview emphasizes the versatility of these agri-food wastes, demonstrating that despite differences in composition and functional properties, all three streams can contribute to the development of sustainable, functional, and nutritionally enriched products. Figure 1 thus reinforces the main conclusion of this review: effective valorization of these by-products supports environmental sustainability, circular economy principles, and the creation of functional foods and nutraceuticals.

CONCLUSIONS

This paper highlights that olive mill wastewater, tomato waste, and sea buckthorn waste are valuable sources of bioactive compounds and nutrients, offering substantial potential for valorization in the food and nutraceutical industries. Olive mill wastewater is particularly rich in soluble polyphenols, including oleuropein and hydroxytyrosol, as well as flavonoids, which exhibit strong antioxidant activity. These compounds can be recovered and utilized as natural antioxidants in foods, functional ingredients, and nutraceutical products, while also helping to mitigate the environmental impact of wastewater.

Tomato pomace contains significant amounts of carotenoids, such as lycopene and β-carotene, polyphenols, dietary fiber, and minerals, maintaining considerable antioxidant activity. It can be incorporated into bakery products, snacks, beverages, and supplements to enhance nutritional value and provide natural antioxidants.

Sea buckthorn pomace is rich in polyphenols, flavonoids, carotenoids, fiber, and micronutrients, making it suitable for applications in bakery, functional beverages, fortified foods, and nutraceutical formulations. The use of these by-products results in products with enhanced antioxidant activity, improved nutritional profiles, and added functional benefits, while also supporting circular economy principles.

In conclusion, all three agri-food waste streams possess high bioactive potential, and the recovery of their antioxidant compounds for integration into functional foods represents an effective strategy to reduce environmental risks and increase the added value of secondary resources. Such practices contribute to the development of a sustainable food industry and innovative nutraceuticals, providing both health and economic benefits.

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BIOTECHNOLOGICAL BIOREMEDIATION SOLUTIONS FOR ENVIRONMENTS CONTAMINATED WITH PHARMACEUTICAL WASTE - A REVIEW

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Abstract

In recent years, increasing attention has been directed toward the issue of waste generation and the development of strategies for its mitigation. The environmental impact of pharmaceutical waste—originating from medical facilities, the pharmaceutical industry, pharmacies, and households—containing persistent and biologically active compounds capable of contaminating soils and aquatic systems, promoting ecological imbalances and the emergence of antimicrobial resistance, can be reduced through the application of biotechnological approaches as sustainable remediation solutions. The study highlights the effectiveness of organisms such as Pseudomonas spp., Trametes versicolor, and Chlorella vulgaris in decreasing the concentrations of persistent pharmaceutical compounds. The conclusions emphasize the necessity of adopting integrated management strategies and strengthening the regulatory framework, thereby positioning bioremediation as a priority direction for the protection of ecosystems and public health.

Key words*: pharmaceutical waste, bioremediation, Pseudomonas spp., Trametes versicolor, Chlorella vulgaris.*

INTRODUCTION

The ongoing rise in worldwide consumption of pharmaceutical products has resulted in a novel form of pollution: pharmaceutical waste, also referred to as medical waste. This includes expired or unused medicines, as well as residues from manufacturing processes and use in medical institutions or households. The persistent and toxic nature of these compounds, in conjunction with their capacity for bioaccumulation, poses a significant threat to the environment and public health. This contributes to phenomena such as antimicrobial resistance, ecological imbalances, and the contamination of natural resources. Inadequate management of pharmaceutical waste enables contaminants to migrate into soils, groundwater, and surface waters, thereby disturbing ecosystem stability and posing risks to public health (Sahota & Sharma, 2022). Environmental exposure to pharmaceutical compounds has been correlated with antimicrobial resistance, endocrine and behavioural effects in every kind of organism, and potential risks to human health (United Nations Environment Programme, n.d.-a). Consequently, the issue represents both an ecological and a global health challenge. Conventional disposal practices, particularly landfilling and incineration, present notable limitations related to infrastructure and economic cost, operational efficiency, and the risk of secondary emissions or leachate-related contamination (Srinivasan at al., 2025; World Health Organization, 2014a). Within this framework, emerging biotechnological approaches offer promising strategies for environmentally compatible remediation of pharmaceutical residues (Sahota et al., 2021). These methods commonly use microorganisms, microbial enzymes, and plants to facilitate degradation and detoxification processes and they are often presented as lower-impact alternatives with potential for scale-up in engineered treatment systems (Ayilara & Babalola, 2023).

The environmental implications of pharmaceutical waste constitute a complex and increasingly prominent concern in the context of globalization and expanding pharmaceutical consumption. Contamination by these

substances produces direct and indirect effects on biodiversity, natural resource quality, and ultimately human health. Furthermore, pollution generated by residual pharmaceuticals is often insufficiently addressed in conventional environmental protection policies, contributing to ongoing and difficult-to-manage environmental exposure.

A salient characteristic of pharmaceutical waste is its categorisation as a waste stream despite its inherent biologically active constituents. These constituents possess the potential to exert deleterious effects on the environment and human health if not managed in an appropriate manner. Unlike most other waste streams, pharmaceutical residues require heightened control due to associated hazards, including the development of antimicrobial resistance, endocrine disruption within ecosystems, and accumulation along trophic chains. In many situations, the absence of an efficient separate collection system results in improper disposal, such as discarding with household waste or releasing into sewage networks, leading to direct adverse environmental impacts.

Pharmaceutical waste originates from multiple sources, and its volume and composition may vary substantially depending on the sector of generation, the applied waste management policies, and the level of awareness among the personnel involved (United Nations Environment Programme, n.d.-b; World Health Organization, 2024b). These sources include medical facilities, hospital and community pharmacies, households and individual users, care centres and nursing homes, the pharmaceutical industry, veterinary practices, livestock production units, and research programs and laboratories.

The management of pharmaceutical waste is shaped by national and international legal and policy framework intended to protect public health and the environment. These frameworks allocate responsibilities for the collection, transport, treatment, and disposal of medicinal waste to manufacturers, pharmacies, healthcare facilities, waste operators, and local authorities. The legal provisions exist both at the European Union level and at the national level. The most relevant include: Directive 2008/98/EC on waste, Regulation (EC) No 1272/2008 on classification, labelling and packaging of substances and mixtures (CLP), Directive 2010/75/EU on industrial emissions, Regulation (EU) 2019/6 on veterinary medicinal products, Law No. 211/2011 on waste management, Ministry of Health Order No. 1226/2012, Law No. 95/2006 on healthcare reform, and Law No. 104/2011 on ambient air quality.

This study aims to evaluate the environmental impact of pharmaceutical waste, and identify associate risks, and examine current as well as emerging biotechnological remediation strategies. Through a multidisciplinary perspective, it seeks to provide a coherent understanding of the issue and to outline future research and implementation directions for environmental and public health protection.

BIOREMEDIATION: PRINCIPLES AND APPLICABILITY

Bioremediation is widely regarded as one of the most promising environmentally compatible approaches for reducing the impact of pharmaceutical residues in contaminated matrices. The process relies on the metabolic activity of living organisms, mainly bacteria, fungi, and microalgae, which can transform or mineralize pharmaceutical compounds into less harmful intermediates or end products. Its conceptual basis lies in the natural ability of these organisms to use complex organic molecules as carbon or energy sources, or to transform them co-metabolically through oxidative, reductive, and hydrolytic enzymatic pathways. Recent reviews emphasize that microbial remediation is particularly attractive for pharmaceutical contaminants because it can be integrated into wastewater treatment systems and may reduce the reliance on more energy-intensive physico-chemical methods (Shah et al., 2024; Bhaskaralingam et al., 2025; Srinivasan et al., 2025).

A critical factor in the practical application of bioremediation is the selection of appropriate biological agents and the optimization of environmental conditions governing their metabolic performance. Parameters such as pH, temperature, dissolved oxygen, redox state, nutrient availability, hydraulic conditions, and contaminant concentration strongly influence biodegradation kinetics and transformation

pathways. In applied systems, two main strategies are commonly discussed: biostimulation, which enhances the activity of indigenous microbial communities through nutrient or electron acceptor amendment, and bioaugmentation, which introduces specialized strains or consortia with higher degradative capacity. Current literature also stresses that the success of these strategies depends on pollutant bioavailability, competition within microbial communities, and the compatibility of inoculated strains with local environmental conditions (Shah et al., 2024; Srinivasan et al., 2025).

The applicability of bioremediation to pharmaceutical pollution spans a broad range of environmental compartments, including wastewater, surface waters, sediments, soils, and certain solid waste streams. In biological wastewater treatment plants, pharmaceuticals are often only partially removed, and removal efficiencies vary considerably according to the chemical structure of each compound, hydraulic retention time, sludge age, and operational conditions. For contaminated soils, both *in situ* and *ex situ* approaches have been investigated, including nutrient amendment, aeration, engineered biopiles, and bioreactor-based treatment. Although these systems can substantially reduce the concentration of some pharmaceuticals, the literature consistently notes that highly persistent compounds such as carbamazepine and some antibiotics remain difficult to eliminate completely through biological treatment alone (Singh et al., 2024; Mansour et al., 2025; Srinivasan et al., 2025).

Figure 1 presents specific techniques of removal efficiency (in average) for some compounds.

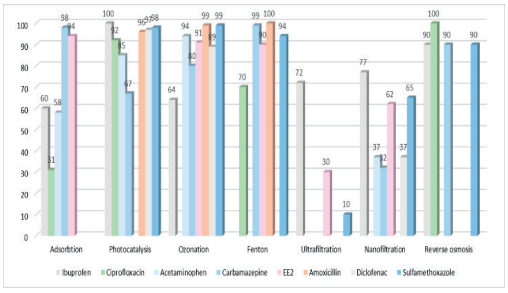

Figure 1. Removal efficiency (%) of selected compounds (Source: Mansour et al., 2025)

The major advantages of bioremediation include relatively low energy demand, lower chemical input, operational flexibility, and the possibility of targeting complex mixtures of organic contaminants under environmentally mild conditions. However, the method also presents important limitations, including long treatment times, incomplete mineralization, formation of transformation products, and limited predictability under variable field conditions. For this reason, the recent literature increasingly frames bioremediation not as a universal stand-alone solution, but as a core component of integrated treatment systems coupled with enzymatic, photocatalytic, electrochemical, or adsorption-based technologies (Bhaskaralingam et al., 2025; Mansour et al., 2025; Singh et al., 2024).

MICROORGANISMS USED IN PHARMACEUTICAL DEGRADATION

Microorganisms constitute the central biological drivers of pharmaceutical bioremediation because of their metabolic diversity and their ability to transform structurally complex xenobiotics. In the context of pharmaceutical removal, bacteria, filamentous fungi, and microalgae have received the greatest attention. Their contribution may involve direct biodegradation, co-metabolic transformation, biosorption, intracellular accumulation, or the secretion of extracellular enzymes capable of attacking recalcitrant molecular structures. Recent reviews indicate that no single microbial group is universally effective against all classes of pharmaceuticals, which explains the growing interest in mixed cultures and engineered consortia (Shah et al., 2024; Srinivasan et al., 2025; Pantha et al., 2025).

Among bacteria, genera such as *Pseudomonas*, *Bacillus*, *Sphingomonas*, and *Rhodococcus* are frequently highlighted because of their broad catabolic versatility and tolerance to contaminated environments. These taxa have been associated with the degradation or transformation of pharmaceuticals including analgesics, antibiotics, beta-blockers, and antiepileptic compounds. Their performance is generally linked to oxygenase-mediated oxidation, hydrolysis, and co-metabolic

pathways, as well as to their capacity to participate in biofilm-based treatment systems. Nevertheless, the literature also emphasizes that observed removal efficiencies are strongly compound-specific and often depend on the presence of auxiliary substrates and acclimatized microbial communities (Shah et al., 2024; Srinivasan et al., 2025).

Fungi, particularly white-rot fungi, are increasingly recognized as effective agents for the transformation of pharmaceutically active compounds because they produce extracellular oxidative enzymes with relatively broad substrate specificity. Species such as *Trametes versicolor*, *Phanerochaete chrysosporium*, and *Pleurotus ostreatus* have been widely investigated for the removal of anti-inflammatory drugs, antibiotics, endocrine-active compounds, and other persistent micropollutants. Their remediation potential is typically attributed to laccases, manganese peroxidases, lignin peroxidases, and cytochrome P450-related systems, which allow the oxidation of aromatic and otherwise recalcitrant molecules. The fungal approach is especially promising for compounds that are poorly removed in conventional activated sludge systems (Chmelová et al., 2024).

Microalgae have also emerged as relevant biological tools for the treatment of pharmaceutical contaminants, especially in aquatic systems and polishing stages of wastewater treatment. Species such as *Chlorella vulgaris* and *Scenedesmus obliquus* are frequently cited for their capacity to remove pharmaceuticals through a combination of bioadsorption, bioaccumulation, photobio-transformation, and metabolism-linked degradation. Although their removal efficiencies can vary widely with light, nutrient balance, biomass productivity, and contaminant concentration, recent reviews identify algal systems as promising low-impact platforms for integrated remediation strategies (OECD, 2022).

At the mechanistic level, pharmaceutical biodegradation is usually discussed in terms of oxidation-reduction reactions, hydrolysis, dealkylation, dehalogenation, ring cleavage, sorption-mediated uptake, and co-metabolic conversion. Oxygenases and peroxidases are especially relevant for aromatic and hydrophobic compounds, whereas hydrolases, esterases, and amidases contribute to the cleavage of ester and amide bonds present in several drug classes. In many cases, partial transformation rather than complete mineralization is observed, which makes the identification of intermediates and toxicity assessment of degradation products an important research priority (Chmelová et al., 2024; Shah et al., 2024; Srinivasan et al., 2025).

PHYTOREMEDIATION

Phytoremediation is increasingly described in the literature as a sustainable, low-input technology for the remediation of soils, sediments, and waters contaminated with pharmaceutical residues and other emerging pollutants. The approach exploits the capacity of plants and their associated rhizosphere microbiota to absorb, accumulate, transform, immobilize, or stimulate the degradation of contaminants. In contrast with more aggressive treatment methods, phytoremediation is generally valued for its lower energy requirements, landscape compatibility, and potential integration into nature-based systems such as constructed wetlands and buffer zones (Singh et al., 2024; Di Stasio et al., 2025; Pantha et al., 2025).

The principal phytoremediation mechanisms discussed in the literature include phytoextraction, phytodegradation, phytostabilization, and rhizodegradation. In the context of pharmaceutical pollution, rhizosphere-driven processes are often particularly important because plant roots release exudates that stimulate microbial communities capable of transforming micropollutants. Direct plant uptake may also occur, followed by translocation, sequestration, or enzymatic transformation inside plant tissues. However, the relative contribution of plant metabolism versus microbially mediated rhizosphere degradation remains system-dependent and is still being actively investigated, especially for complex mixtures of pharmaceuticals (Singh et al., 2024; Pantha et al., 2025).

Several plant taxa have been examined for their remediation potential, including woody species

such as *Populus* spp. and *Salix* spp., as well as wetland and crop species used in engineered treatment systems. Woody plants are often considered advantageous because of their rapid growth, deep root systems, and strong interaction with rhizosphere microbes, while aquatic and semi-aquatic species are frequently incorporated into constructed wetlands for continuous treatment of contaminated water. By contrast, the use of model plants such as *Arabidopsis thaliana* is mainly relevant for mechanistic research rather than for direct large-scale remediation. The literature therefore supports the use of plant-based systems, but usually in the context of carefully selected species, site-specific design, and combined plant-microbe interactions (Pantha et al., 2025).

Phytoremediation also benefits from integration with other biological approaches, especially rhizosphere bioaugmentation, plant growth-promoting microorganisms, and enzyme-assisted treatment concepts. More exploratory research has extended this framework toward engineered plants, synthetic biology, and hybrid phytoremediation platforms, although these approaches remain largely at the experimental or pilot stage. Current reviews consistently point out that phytoremediation is constrained by slow kinetics, seasonal variability, contaminant bioavailability, and limited effectiveness under very high contamination loads. As a result, it is best interpreted as a sustainable complementary technology rather than a universally sufficient stand-alone remediation option (Singh et al., 2024; Wang et al., 2024; Pantha et al., 2025).

INNOVATIVE BIOLOGICAL TECHNOLOGIES

Beyond classical bioremediation and phytoremediation, recent research has increasingly focused on innovative biological technologies that attempt to improve selectivity, stability, and process efficiency in the removal of pharmaceutical contaminants. These technologies include free and immobilized enzymes, microbe-material interfaces, nanobiotechnology-based systems, engineered microbial consortia, and hybrid bio-physico-chemical platforms. Although many of these solutions remain under development, they reflect a broader shift in remediation research toward modular and multifunctional treatment systems designed for complex contaminant mixtures (Mansour et al., 2025; Srinivasan et al., 2025; Salehpour et al., 2026).

Enzyme-based remediation has attracted substantial attention because enzymes can catalyze highly specific transformation reactions under mild operating conditions. Among the most studied biocatalysts are laccases, peroxidases, monooxygenases, dioxygenases, esterases, and lipases. Laccases derived from fungal systems, particularly *Trametes versicolor*, are widely reported to oxidize a broad spectrum of pharmaceuticals, including anti-inflammatory compounds, antibiotics, hormones, and other aromatic pollutants. Immobilization on solid supports can improve enzyme stability, facilitate reuse, and support integration into membranes, packed-bed reactors, and advanced filtration units, although cost, enzyme deactivation, and mediator requirements remain important practical challenges (Chmelová et al., 2024; Thathola et al., 2024; Singh et al., 2024).

Nanobiotechnology has introduced new possibilities for pharmaceutical remediation by combining biological catalysts or cells with functional nanomaterials that enhance adsorption, catalysis, immobilization, sensing, or electron transfer. Reported approaches include enzyme-coated nanoparticles, nanostructured supports for immobilized biocatalysts, reactive metal oxides, graphene-based composites, and hybrid biopolymer nanocomposites. These systems may improve catalytic efficiency and contaminant capture, but the current literature also highlights concerns regarding material recovery, life-cycle sustainability, ecotoxicological assessment, and large-scale deployment. Consequently, nanobiotechnology is best described at present as a rapidly developing research direction with significant potential, rather than a mature universal solution (Salehpour et al., 2026).

Another emerging direction involves the use of synthetic biology and engineered microbial consortia to enhance the transformation of difficult pharmaceutical compounds. In principle, genetically tailored microorganisms may be designed to express specific catabolic

enzymes or optimized pathways, while synthetic communities may distribute degradation functions across multiple compatible strains. This concept is scientifically compelling because it addresses metabolic bottlenecks and broadens substrate coverage; however, in environmental remediation it is still constrained by biosafety, regulatory acceptance, ecological stability, and deployment challenges outside controlled reactors. For that reason, most current discussions remain prospective rather than implementation-driven (European Commission, 2024).

A significant trend within this field is the development of hybrid systems that combine biological processes with photocatalytic, electrochemical, adsorption-based, or membrane-assisted treatment. Examples discussed in recent reviews include photo-biocatalytic systems, bioelectrochemical reactors such as microbial fuel cells, microalgae-electrocatalytic configurations, and bioactive porous filters. These integrated platforms are attractive because they can improve removal of recalcitrant pharmaceuticals while reducing some of the limitations of individual technologies. At the same time, the literature repeatedly notes that scale-up, economics, by-product control, and treatment of real wastewater matrices remain major barriers to routine application (Corona-Martínez et al., 2025; Mansour et al., 2025).

CLASSICAL AND MODERN BIOREMEDIATION METHODS IN THE LITERATURE

Over the past two decades, pharmaceutical residues have been increasingly recognized as a distinct class of emerging contaminants because they occur in surface waters, groundwater, drinking-water sources, soils, and sediments, while many conventional wastewater treatment systems were not designed to remove them efficiently (OECD, 2019; Srinivasan et al., 2025). This growing concern is also linked to evidence that some pharmaceutical compounds can affect aquatic organisms at very low concentrations and may contribute to broader ecological risks, including endocrine disruption, behavioural changes, and the selection of antimicrobial resistance (OECD, 2019).

Conventional remediation and disposal methods, including standard biological wastewater treatment, incineration, and controlled landfilling, remain important components of pharmaceutical waste management, but the literature consistently highlights their limitations. Wastewater treatment plants frequently achieve only partial removal of pharmaceutical residues, particularly for structurally persistent compounds, while disposal-based strategies may generate secondary environmental burdens if emissions, ash, or leachate are not properly controlled (OECD, 2019; Mahmud et al., 2025). Consequently, conventional systems are increasingly regarded as necessary but insufficient for addressing diffuse and chronic pharmaceutical contamination.

Among modern approaches, microbial bioremediation is one of the most extensively investigated strategies because it relies on the metabolic and co-metabolic capacity of bacteria, fungi, and mixed microbial consortia to transform pharmaceutical contaminants under aerobic or anaerobic conditions (Srinivasan et al., 2025). Recent reviews identify bacterial genera such as *Pseudomonas*, *Bacillus*, and *Rhodococcus* as particularly relevant due to their catabolic versatility, whereas fungal systems are valued for extracellular oxidative enzyme production and their capacity to attack more recalcitrant molecules (Flórez-Restrepo et al., 2025; Srinivasan et al., 2025). However, degradation efficiency remains highly dependent on environmental conditions, contaminant bioavailability, and compound-specific physicochemical properties (Srinivasan et al., 2025).

Phytoremediation occupies an important place in the recent literature as a low-input and environmentally compatible option for soils, wetlands, and polishing systems exposed to pharmaceutical contamination. Its effectiveness is generally associated with plant uptake, rhizosphere-assisted degradation, sequestration, and plant-microbe interactions rather than with rapid contaminant destruction alone. At the same time, recent meta-analytical work emphasizes that phytoremediation is limited by

slow kinetics, seasonal variability, and reduced effectiveness against highly persistent compounds, which is why it is usually presented as a complementary technology rather than a stand-alone solution (Pantha et al., 2025).

More advanced developments in the field include enzyme-based and hybrid remediation systems. In particular, laccases and other oxidative enzymes, especially those derived from ligninolytic fungi, have been widely studied for their ability to degrade a broad range of pharmaceutically active compounds under relatively mild conditions (Chmelová et al., 2024; Singh et al., 2024). Immobilization strategies have further increased interest in enzymatic systems by improving catalyst stability and reusability, although practical implementation is still constrained by enzyme cost, deactivation, and process optimization challenges (Chmelová et al., 2024; Ezra et al., 2025).

In parallel, nanobiotechnology and other hybrid platforms have expanded the scope of modern bioremediation by combining biological catalysts with functional materials for adsorption, photocatalysis, and enhanced transformation. These systems show substantial promise in controlled studies, but the literature also stresses unresolved concerns related to scale-up, catalyst recovery, ecotoxicological safety, and long-term environmental performance (Srinivasan et al., 2025; Salehpour et al., 2026). Consequently, the dominant conclusion in recent reviews is that the future of pharmaceutical remediation lies in integrated treatment trains that combine classical biodegradation principles with enzymatic engineering, materials-based enhancement, and system-level process design (Pantha et al., 2025; Salehpour et al., 2026).

COMPLEMENTARY PLANT-BASED, ENZYMATIC, AND HYBRID STRATEGIES FOR PHARMACEUTICAL WASTE REMEDIATION

Phytoremediation is increasingly described as a complementary remediation strategy for pharmaceutical pollution because it combines plant uptake, rhizosphere-assisted transformation, and microbially mediated detoxification under relatively low-input conditions. In this framework, the rhizosphere is particularly important, since root-associated microbial communities can enhance the degradation of pharmaceutical residues through co-metabolic and enzyme-driven processes. Recent reviews therefore present plant-microbe systems not as isolated biological tools, but as interactive remediation platforms in which plants improve contaminant access and microbial communities accelerate transformation and detoxification (OECD, 2019; Pantha et al., 2025).

A consistent theme in the recent literature is that rhizosphere engineering can improve phytoremediation performance. Reviews on microbe-assisted phytoremediation show that inoculation with plant growth-promoting and pollutant-degrading rhizobacteria can stimulate root development, increase biomass, and enhance contaminant removal, especially in wetland and wastewater-treatment settings. At the same time, authors emphasize that the outcome remains species- and site-dependent, because plant physiology, microbial compatibility, pollutant class, and environmental conditions jointly determine remediation efficiency. For this reason, plant–microbe consortia are increasingly presented as promising but still optimization-dependent systems rather than universally transferable solutions (Oubohssaine & Dahmani, 2024).

Another expanding branch of the literature concerns enzyme-based remediation, particularly the use of purified oxidoreductases such as laccases and peroxidases for the transformation of structurally complex pharmaceutical molecules. These enzymes are attractive because they offer relatively high substrate specificity, operate under mild reaction conditions, and avoid the process-control challenges associated with maintaining viable microbial cultures in industrial applications. However, reviews consistently note that commercial deployment is still constrained by enzyme instability, mediator requirements, production cost, and limited operational lifetime. As a result, enzyme immobilization on solid supports has become a major research direction, since it can improve catalyst stability, facilitate recovery, and increase reuse potential in continuous treatment

systems (Chmelová et al., 2024; Thathola et al., 2024; Wang et al., 2024; Singh et al., 2024).

Nanotechnology is also increasingly discussed as an enabling platform for pharmaceutical remediation, especially in hybrid systems that combine photocatalysis, adsorption, and biologically assisted degradation. Functional nanomaterials can accelerate contaminant transformation, improve catalyst immobilization, and support compact treatment configurations, but the literature remains cautious about large-scale implementation. Reviews repeatedly identify unresolved concerns related to nanoparticle recovery, long-term environmental fate, ecotoxicological safety, and the possibility that the remediation material itself could introduce secondary risks. Consequently, recent scholarship tends to frame nanotechnology not as a stand-alone answer, but as part of a broader class of hybrid remediation systems that require life-cycle and risk assessment before full-scale adoption (OECD, 2019; Salehpour et al., 2026).

From a management perspective, the literature increasingly argues that effective control of pharmaceutical waste requires more than end-of-pipe treatment. OECD and European policy documents stress that source reduction, separate collection of unused medicines, public awareness, and integration with existing waste and wastewater systems are essential complements to biotechnological remediation. In practice, this means that the environmental value of phytoremediation, enzymatic treatment, and hybrid technologies depends not only on technical performance but also on regulatory design, collection infrastructure, and stakeholder coordination. The dominant conclusion in current reviews is therefore that sustainable pharmaceutical-waste management must combine biological innovation with prevention, governance, and public participation (European Commission, 2019; OECD, 2019; OECD, 2022).

CONCLUSIONS

The issue of pharmaceutical waste has gained significant prominence as a major environmental and public health concern, primarily due to its persistence, biological activity, and pervasive presence in both aquatic and terrestrial ecosystems. As shown throughout this review, the inadequate management of expired, unused, and residual pharmaceuticals facilitates their release into soils, surface waters, groundwater, and wastewater systems, where they may contribute to antimicrobial resistance, ecotoxicological effects, endocrine disruption, and long-term ecosystem imbalance. The problem is further intensified by the diversity of waste sources, the incomplete effectiveness of conventional treatment systems, and the persistent gap between existing regulatory frameworks and practical implementation.

The literature reviewed indicates that conventional disposal and treatment practices, although still necessary, are not sufficient to address the complexity of pharmaceutical contamination. Wastewater treatment plants frequently achieve only partial removal, while disposal options such as landfilling and incineration may create secondary environmental burdens when not adequately controlled. For this reason, biotechnological remediation has gained increasing attention as a lower-impact and potentially more sustainable alternative.

Among the available strategies, microbial bioremediation remains one of the most extensively investigated approaches because of the metabolic versatility of bacteria, fungi, and microalgae in transforming pharmaceutical compounds. At the same time, the evidence clearly shows that no single organism or process is universally effective, and that removal performance depends strongly on environmental conditions, contaminant properties, and system design. Phytoremediation also represents a valuable complementary option, especially in low-input and nature-based treatment systems, although its application is constrained by relatively slow kinetics and strong site dependence. Likewise, enzyme-based technologies, nanobiotechnology, and hybrid treatment platforms offer promising advances in selectivity and efficiency, yet they still face important barriers related to cost, stability, scale-up, ecological safety, and operational feasibility.

A central conclusion of this study is that the remediation of pharmaceutical waste cannot

rely on a single technology or on end-of-pipe treatment alone. The most promising direction is the development of integrated management systems that combine source reduction, separate collection, public awareness, regulatory enforcement, and advanced biological or hybrid remediation technologies. In this context, bioremediation should be viewed as a key component of broader, multi-barrier treatment strategies.

Finally, future progress in this field will depend on closer integration between environmental science, biotechnology, public policy, and waste-management practice. Further research is needed on transformation products, long-term ecotoxicological effects, full-scale process optimization, and the adaptation of remediation systems to local environmental and institutional conditions. Sustainable pharmaceutical-waste management will therefore require both technological innovation and coordinated governance in order to reduce environmental exposure and better protect ecosystem integrity and human health.

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MICROBIAL BIOSURFACTANTS AS ANTIMICROBIAL AND THERAPEUTIC AGENTS: A MINI-REVIEW OF RECENT ADVANCES

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Abstract

Microbial biosurfactants are amphiphilic molecules produced by diverse microorganisms and are increasingly investigated as alternative antimicrobial agents in the context of increasing antimicrobial resistance (AMR). This mini-review summarizes current knowledge on the antimicrobial and antibiofilm activities of major biosurfactant classes, including rhamnolipids, lipopeptides, sophorolipids, and mannosylerythritol lipids (MELs), with emphasis on their mechanisms of action and structure-activity relationships.
Rhamnolipids and lipopeptides exhibit the highest antimicrobial potency, primarily through membrane disruption, whereas sophorolipids and MELs show reduced antimicrobial activity but improved biocompatibility. All classes act mainly through physico-chemical interactions with microbial membranes, resulting in membrane disruption and inhibition of biofilm formation. In addition, several studies report enhanced efficacy of conventional antibiotics in combination with biosurfactants against multidrug-resistant pathogens.
Despite these advantages, their clinical application remains limited by production costs, process variability, and regulatory constraints. Nevertheless, the use of renewable and agro-industrial waste substrates for biosurfactant production aligns with circular economy principles and improves their sustainability profile. Overall, microbial biosurfactants represent promising candidates for next-generation antimicrobial strategies, although further optimization and translational studies are required to support their practical implementation.

Key words*: microbial surfactants, antimicrobial activity, therapeutic applications.*

INTRODUCTION

Microbial biosurfactants are structurally and functionally diverse amphiphilic molecules produced by a wide range of microorganisms, including species belonging to the genera *Pseudomonas*, *Bacillus*, *Candida*, and *Moesziomyces*.
Their amphipathic nature enables strong interfacial activity and interaction with biological membranes, which supports their broad antimicrobial and antibiofilm properties.
Among the main biosurfactant classes, significant differences in antimicrobial potency and biomedical applicability have been reported.

Rhamnolipids, primarily produced by *Pseudomonas* spp., generally exhibit the strongest antibacterial activity, particularly against Gram-negative pathogens, due to their ability to insert into cell membranes, disrupt membrane integrity, and induce leakage of intracellular components (Soberón-Chávez et al., 2021). In comparative studies, rhamnolipids often exhibit lower minimum inhibitory concentrations (MICs) than sophorolipids against several Gram-positive and Gram-negative bacteria, including *Staphylococcus aureus* and *Escherichia coli*, although activity is strongly dependent on molecular structure and biosurfactant form. In contrast, lipopeptides produced by *Bacillus* species, including

surfactin, iturin, and fengycin, demonstrate superior antifungal activity. Their mechanism involves pore formation and membrane permeabilization, with surfactin additionally affecting membrane-associated signaling processes, which contributes to both antimicrobial and antibiofilm effects (Ongena & Jacques, 2008). This makes lipopeptides particularly effective against fungal pathogens, such as *Candida albicans* and *Aspergillus* spp.

Yeast-derived glycolipids, including sophorolipids and mannosylerythritol lipids (MELs), generally exhibit lower direct antimicrobial potency compared to rhamnolipids and lipopeptides; however, they are characterized by significantly lower cytotoxicity and improved biocompatibility. Sophorolipids produced by *Candida* spp. display moderate antibacterial activity, mainly against Gram-positive bacteria, while MELs show promising antifungal and anti-inflammatory properties, which make them particularly suitable for dermal and cosmetic applications (Cho et al., 2022; Holguín-Salas et al., 2025).

Biosurfactants act mainly on microbial membranes through physico-chemical interactions, leading to increased membrane fluidity, permeability changes, and protein destabilization. This non-specific mode of action reduces selective pressure and the risk of resistance development compared to conventional antibiotics (Marchant & Banat, 2012; Luong et al., 2026).

Despite these properties, clinical translation remains limited. Major constraints include high production costs, and challenges in downstream purification. Recent advances in the metabolic and genetic engineering of *Pseudomonas* and *Bacillus* strains have led to improved production yields and modified congener profiles, indicating a promising route toward industrial scalability and functional optimization (Soberón-Chávez et al., 2021; Banat et al., 2021). Importantly, biosurfactants produced using renewable substrates and agro-industrial waste streams align with circular economy principles by enabling waste valorisation into high-value bioproducts. This dual functionality-biomedical relevance combined with environmental sustainability-highlights microbial biosurfactants as key candidates for the development of next-generation antimicrobial and eco-friendly technologies.

MATERIALS AND METHODS

A literature review was conducted using PubMed, ScienceDirect, and Web of Science databases to identify publications on microbial biosurfactants. The search was performed using keywords such as “microbial biosurfactant”, “rhamnolipid”, “lipopeptides”, “antimicrobial activity”, “sophorolipids”, or “biomedical applications”. Studies reporting *in vitro*, *in vivo*, or clinically relevant data on biosurfactant production and biological activity were considered. Representative biosurfactant-producing genera, including *Pseudomonas*, *Bacillus*, *Candida*, and *Moesziomyces*, were included. Selected papers were further evaluated based on their focus on antimicrobial mechanisms, antibiofilm properties, structure-activity relationships, and biomedical applications of microbial biosurfactants.

RESULTS AND DISCUSSIONS

Antimicrobial activity of biosurfactants

Microbial biosurfactants exhibit a broad antimicrobial spectrum against Gram-positive and Gram-negative bacteria, as well as pathogenic yeasts and filamentous fungi. Their antimicrobial activity is largely determined by the chemical structure of the biosurfactant, the producing microorganism, and environmental conditions such as pH, temperature, and ionic strength (Banat et al., 2010; Rodrigues et al., 2006). Structural properties, such as lipid chain length, degree of saturation, and glycosylation patterns, are key determinants of antimicrobial potency and selectivity, enabling selective activity against specific pathogens (Fracchia et al., 2015). The main characteristics, antimicrobial targets, mechanisms of action, advantages, and limitations of the major microbial biosurfactant classes are summarized in Table 1.

Table 1. Comparison of major biosurfactant classes

Biosurfactant type	Main producer organisms	Target pathogens	Main antimicrobial mechanism	Advantages	Limitations	References
Rhamnolipids	*Pseudomonas aeruginosa*, *Pseudomonas fluorescens*	*Staphylococcus aureus*, *Escherichia coli*	Membrane disruption and release of intracellular constituents	Antibacterial activity against Gram-positive and Gram-negative bacteria, antibiofilm properties	Potential cytotoxicity, production associated with pathogenic strains, high purification cost	Alyousif et al., 2023; Zhao et al., 2022; Wongsirichot et Winterburn, 2025
Lipopeptides (surfactin, iturin, fengycin)	*Bacillus subtilis*, *Bacillus licheniformis*	*Candida albicans*, *Aspergillus* spp., *Penicillium* spp., *Staphylococcus epidermidis*	Pore formation and membrane permeabilization	Strong antifungal activity, synergistic effects, potent antibiofilm action	Production variability, sensitivity to environmental conditions	Markelova et Chumak, 2025
Sophorolipids	*Candida bombicola*	*Staphylococcus aureus*, *Bacillus subtilis*, *Candida albicans*	Interaction with membrane lipids and alteration of membrane integrity	Low cytotoxicity, high biodegradability and good biocompatibility	Moderate antimicrobial potency compared with rhamnolipids and lipopeptides	Lourenço et al., 2024; Dengle-Pulate et al., 2014; Cho et al., 2022
Mannosylerythritol lipids (MELs)	*Moesziomyces* spp.	*Candida* spp., *S. aureus*	Membrane interaction and inhibition of microbial adhesion	Antifungal and antibacterial activity, low toxicity, moisturizing and skin-protective properties, favorable dermatological tolerance	Limited large-scale production data	Coelho et al., 2020; Ceresa et al., 2020; Holguín-Salas et al., 2025

Rhamnolipids, primarily produced by *Pseudomonas aeruginosa* and *Pseudomonas fluorescens*, have demonstrated antibacterial activity against clinically relevant pathogens, including *Staphylococcus aureus*, *Listeria monocytogenes*, *Bacillus cereus*, and *Escherichia coli* (Das et al., 2008; Sivasankar et al., 2010). Several *in vitro* studies have reported inhibition zones and low minimum inhibitory concentrations (MICs), highlighting the efficacy of rhamnolipids against both Gram-positive and Gram-negative bacteria. In addition to antibacterial effects, rhamnolipids exhibit antifungal activity against species such as *Fusarium solani* and *Penicillium funiculosum*, further expanding their biomedical relevance (Das et al., 2008).

Lipopeptides synthesized by *Bacillus* species, including surfactin, iturin, and fengycin, represent one of the most highly active classes of antimicrobial biosurfactants (Ongena & Jacques, 2008). These compounds display strong antibacterial activity against *Staphylococcus epidermidis*, *Listeria monocytogenes*, and *Salmonella enterica*, as well as pronounced antifungal effects against *Candida albicans*, *Aspergillus niger*, and *Fusarium oxysporum* (Sivasankar et al., 2010; Van Bogaert et al., 2009). Notably, lipopeptides often act synergistically, with mixed lipopeptide fractions exhibiting enhanced antimicrobial efficacy compared to individual components (Ongena & Jacques, 2008).

Sophorolipids, produced mainly by *Candida bombicola*, exhibit moderate antibacterial activity, particularly against Gram-positive bacteria such as *Staphylococcus aureus* and *Bacillus subtilis*, as well as antifungal effects against *Candida albicans* (Singh et al., 2004; Van Bogaert et al., 2009). Although their antimicrobial potency is generally lower than that of lipopeptides or rhamnolipids, sophorolipids are characterized by low cytotoxicity and excellent biodegradability, which are critical attributes for biomedical applications.

MELs synthesized by yeasts such as *Moesziomyces* and *Candida* species demonstrate antifungal activity against *Candida*

albicans and *Aspergillus fumigatus*, as well as antibacterial effects against Gram-positive bacteria (Singh et al., 2004; Van Bogaert et al., 2009).

Their unique molecular structure confers additional functional properties, including moisturizing and skin-protective effects, supporting their use in pharmaceutical and cosmetic formulations.

Antibiofilm and synergistic effects

Beyond their activity against various microorganisms, biosurfactants have demonstrated significant antibiofilm properties.

Biofilm-associated infections represent a significant challenge for eradication due to increased microbial resistance and protection from host immune responses. Several studies have shown that biosurfactants inhibit microbial adhesion to surfaces and disrupt established biofilms by altering surface hydrophobicity and interfering with extracellular polymeric substances (Rodrigues et al., 2006).

Several studies have shown that combining biosurfactants with enzymatic treatments or nanomaterials enhances biofilm disruption, providing innovative strategies to control persistent infections (Fracchia et al., 2015). Biosurfactants have also demonstrated potential in reducing biofilms on industrial surfaces, including food processing equipment and water systems, demonstrating their applicability beyond clinical contexts. Rhamnolipids and lipopeptides, in particular, have been shown to reduce biofilm formation by *Staphylococcus aureus* and *Escherichia coli* on medical-grade materials, highlighting their potential for application in medical device coatings (Das et al., 2008; Sivasankar et al., 2010). In addition, synergistic interactions between biosurfactants and conventional antimicrobial agents have been reported, leading to reduced antibiotic dosages and enhanced antimicrobial efficacy against multidrug-resistant (MDR) strains (Singh et al., 2004; Abdel-Mawgoud et al., 2014).

Recent studies suggest that microbial biosurfactants can enhance the efficacy of antibiotics (e.g., ciprofloxacin, vancomycin) against MDR pathogens by lowering minimum inhibitory concentrations (MICs) and disrupting resistance mechanisms, representing a promising combined approach for persistent infections (Dias-Souza et al., 2025). Such synergistic approaches may represent a promising strategy to extend the clinical utility of existing antibiotics.

Biomedical and therapeutic applications

The multifunctional properties of microbial biosurfactants have enabled their application across diverse biomedical fields.

In wound care, biosurfactant-based formulations have demonstrated both antimicrobial activity and the ability to promote tissue regeneration, contributing to improved healing outcomes (Sivasankar et al., 2010; Singh et al., 2004). Biosurfactant-coated surfaces have been explored for catheters, implants, and other medical devices to prevent microbial colonization and biofilm formation (Rodrigues et al., 2006).

Emerging research indicates that microbial biosurfactants can serve as functional components in advanced drug delivery systems, including biosurfactant-coated nanoparticles and stimuli-responsive hydrogels, improving drug stability, targeted release, and bioavailability of hydrophobic compounds (Bjerk et al., 2021; Rodrigues et al., 2006).

Recent studies further highlight the integration of biosurfactants into smart and stimuli-responsive biomaterials to enhance targeted antimicrobial performance and therapeutic efficiency (Wang et al., 2024).

Additionally, topical formulations containing sophorolipids and mannosylerythritol lipids (MELs) have demonstrated promising antifungal activity combined with favorable dermatological tolerance, supporting their application in medical and cosmetic formulations (Van Bogaert et al., 2011; Lourenço et al., 2024). Their combination with nanomaterials or polymeric carriers can further enhance antimicrobial efficacy and formulation stability, opening novel opportunities for advanced therapeutic and drug delivery systems (Abbot et al., 2022). From a sustainability perspective, the production of biosurfactants using renewable substrates, including agro-industrial waste and food industry by-products, aligns with circular economy principles and contributes to reduced environmental impact (Banat et al., 2010; Sivasankar et al., 2010). The

use of agro-industrial residues not only reduces production costs, but also minimizes environmental impact, promoting sustainable biotechnology practices. This aspect further enhances the attractiveness of biosurfactants as next-generation antimicrobial agents.

Limitations and future perspectives

While numerous studies highlight the biomedical potential of microbial biosurfactants, their translation from experimental models to practical applications remains challenging (Marchant & Banat, 2012). Despite promising laboratory-scale results, several obstacles limit their widespread use, including low production yields, high downstream processing costs, and variability in biosurfactant composition (Banat et al., 2014).

From an economic and technological perspective, biosurfactant production is not yet cost-competitive with conventional antimicrobial agents due to high costs associated with substrates, fermentation processes, and purification steps, which limits large-scale industrial adoption. Scaling up microbial biosurfactant production requires precise control of fermentation parameters, such as pH, temperature, and aeration, to ensure consistent yield, composition, and bioactivity.

Genetic and metabolic engineering of biosurfactant-producing strains offers promising strategies to enhance yields, optimize molecular structures, and tailor functional properties for specific biomedical applications (Soberon-Chavez et al., 2021; Banat et al., 2010; Abdel-Mawgoud et al., 2010). In addition, formulation stability and the absence of standardized regulatory frameworks further constrain their clinical translation. Future research should focus on optimizing fermentation processes, identifying novel high-yield producers, and exploring structure-activity relationships to enhance antimicrobial potency. Comprehensive preclinical and clinical studies will be essential to validate safety, efficacy, and long-term performance in biomedical applications (Fracchia et al., 2015).

CONCLUSIONS

Microbial biosurfactants, including rhamnolipids, lipopeptides, sophorolipids, and mannosylerythritol lipids, represent a versatile and sustainable class of bioactive compounds with significant antimicrobial and therapeutic potential. Their effectiveness against a broad range of pathogenic microorganisms, including MDR bacteria and fungi, combined with antibiofilm activity and favorable biocompatibility, highlights their relevance for biomedical applications.

Although challenges related to production scalability, formulation stability, and regulatory approval remain barriers, ongoing advances in microbial biotechnology and process optimization are expected to facilitate their transition from laboratory research to clinical and pharmaceutical use.

Furthermore, their production from renewable and agro-industrial waste substrates aligns with circular economy principles, offering environmentally friendly alternatives to synthetic surfactants.

Continued research into innovative formulations, combination therapies, and strain engineering will expand their application scope and reinforce their role as next-generation antimicrobial agents. In this context, microbial biosurfactants may play a key role in the development of future antimicrobial strategies that integrate efficacy, safety, and sustainability within an integrated biomedical framework.

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CONTRIBUTION TO THE STUDY OF CONSERVATION BY THE METHOD OF LYOPHILIZATION OF FUNGI AND BACTERIA

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Abstract

Microorganisms, especially filamentous fungi and bacteria, are a safe and inexhaustible source of potential producers of bioactive substances for agricultural use. The preservation and storage of these microorganisms plays an important role in biotechnology. There are different protective media for lyophilization of microorganisms, but this parameter is necessary to optimize in dependence of microorganism subjected to lyophilization. This study aims to elucidate the action of various protective media used in the process of lyophilization, strains of filamentous fungi and bacteria, isolated from aquatic basins, with high antimicrobial potential over a wide range of phytopathogens, on the viability and stability of antimicrobial properties after lyophilization. Four protective media were developed based on skimmed milk supplemented with glucose, sucrose and antioxidants in different concentrations. As a result of the research, the optimal protective medium for the lyophilization of bacteria and filamentous fungi was selected. After lyophilization in optimized media, the viability of bacteria varied within 88-92%, and of fungi 92-98%, compared to the initial titre, which ensures the preservation and long-term preservation of biotechnological interest strains.

Key words*: fungi, bacteria, protective medium, lyophilization.*

INTRODUCTION

Microorganisms are present in the endosphere, phyllosphere and rhizosphere of plants, in water and air, function effectively in a wide range of temperatures and represent a reliable and inexhaustible source of potential producers of biologically active substances for various areas (Brazhnikova et al., 2025; Harirchi et al., 2023; Kamal et al., 2018; Ratnam et al., 2014). Bacteria(Ayaz et al., 2023; Iqbal et al., 2024; Sahgal et al., 2024) and filamentous fungi (Armenova, 2025; Boughalleb-M'Hamdi et al., 2018; Al-Obaidy, 2019; Guzmán-Guzmán et al., 2025; Kumar et al., 2023) are widely used in the production of biopreparations for agricultural use. Plants are constantly exposed to various phytopathogens, which can significantly reduce crop yields. The use of chemical pesticides to control numerous crop diseases has negative impacts on the environment and human health. An alternative method of controlling phytopathogens, based on microorganisms, is now widely used. It is safer, less toxic, and reduces the severity of various crop diseases. These microorganisms, used as biocontrol agents for phytopathogens, also possess various capabilities, including the potential to stimulate plant development and growth, induce immune resistance in host plants, reduce pathogen infestation, degrade various types of hydrocarbons, and fertilize the soil (Ayaz et al., 2023; Mourou et al., 2022)**.**

The preservation and storage of microorganisms of industrial interest plays a vital role in biotechnology. Due to the widespread use of microorganisms in various industries, it is necessary to develop appropriate preservation and storage procedures to maintain their genotypic and phenotypic characteristics unchanged over time. Lyophilization is the most suitable method for preserving, storing, and transporting large collections of microbial cultures. During the lyophilization process, microorganisms are subject to various damages that lead to a loss of viability and cellular stability. This loss of viability is primarily caused by freezing, shock arising from dehydration, osmotic stress, low pressure leading to membrane rupture, fluctuations in cell membrane fluidity, loss of intracellular enzymatic activity, oxidation of fatty acids, and

loss of gene and protein function. In addition, the viability and stability of microorganisms during and after lyophilization and storage depend on factors such as cell density, freezing duration, residual moisture content after lyophilization, and the rehydration procedure (Li et al., 2011; Liu et al., 2019; Li et al., 2024; Morgan et al., 2006; Mputu Kanyinda, 2013).

To prevent harmful processes during lyophilization, a wide range of protective excipients has been developed. These can be classified as: colloids of animal, plant, or mineral origin; soluble substances such as sugars, peptones, and amino acids; and antioxidants. These components can be included in protective media individually or in a wide variety of combinations and serve as a medium for microbial cells, as well as ensuring osmotic balance and stress protection during freezing and sublimation (Okhapkina, 2009; Kaito et al., 2018; Rockingera et al., 2021).

Disaccharides are most often used as lyoprotectants in combination with protein products. Commonly used sugars include glucose (1% and 5%), and trehalose (4.74%, 5%, 7.5%, and 10%) (Li et al., 2024; Peiren et al., 2016; Wang et al., 2019), xylitol 1.45%, cellobiose 5%, D-galactose 5%, 7% and 10% sucrose (Bellali et al., 2020; Zhan et al., 2012), fructose 5% and lactose 7% (Wang et al., 2020).

The individual stages of the lyophilization process (freezing, primary drying, and secondary drying) expose proteins to various stresses. The use of appropriate excipients and control of the combined freezing and sublimation process can protect proteins during lyophilizetion (Izutsu, 2018). Trehalose is often used as an effective protectant due to its unique ability to displace water molecules tightly bound to carbonyl groups (Luzardo et al., 2000). Furthermore, the high viscosity and low mobility of trehalose may contribute to maintaining the integrity of microbial protein functions during lyophilization (Ohtake & Wang, 2011).

Dry skimmed milk is considered an ideal lyoprotectant, as it forms a viscous layer that increases the viscosity of the solution and prevents the growth of ice crystals during freezing (Castro et al., 1995; Suo et al., 2021). Skimmed milk powder is often used both alone and in combination with disaccharides, antioxidants, etc. (Valdez-Tenezaca et al., 2025). Thus, the combination of lactose 5.0% (w/v), mannitol 5.0% (w/v), trehalose 5.0% (w/v), skimmed milk powder 10.0% (w/v), ascorbic acid 0.5% (w/v) and gelatin 0.5% (w/v) was found to be the optimal choice for lyophilization of 18 marine strains belonging to the genera *Pseudoalteromonas*, *Vibrio*, *Photobacterium*, *Planomicrobium*, *Edwardsiella*, *Enterococcus*, *Bacillus* and *Saccharomyces*, with the survival rate after lyophilization ranging from 2.3% to 95.1%. (Zhang et al., 2020).

Crowe & Crowe (2003) hypothesized that sugar combined with glass-forming excipients such as macromolecules would yield optimal results in the lyophilization of microorganisms. Polyols such as sorbitol and glycerol have also shown promise as cryopreservation excipients, but this success has not been translated to cell drying.

Lyophilization is considered a safe method for long-term preservation and storage of microorganisms, but to increase their survival and stability, it is necessary to use appropriate protective measures in this process.

The aim of this study is to determine the effect of various protective media used in the process of lyophilization of strains of filamentous fungi and bacteria isolated from water reservoirs and possessing high antimicrobial potential against a wide range of phytopathogens on the viability and stability of antimicrobial properties after lyophilization.

MATERIALS AND METHODS

The objects of the study were 10 strains of filamentous fungi (5 strains of *Trichoderma* spp. and 5 strains of *Penicillium* spp.) and 6 strains of bacteria represented by the genera: *Bacillus* sp. (4 strains), *Pseudomonas* sp. (1 strain) and *Micrococcus* sp. (1 strain), isolated from water ponds in the Chisinau Municipality, Republic of Moldova.

Lyophilization of microorganisms

For lyophilization of fungi and bacteria, four protective media were developed: 1 - skimmed milk + 5% sucrose + 5% glucose + 0.01% ascorbic acid; 2 - skimmed milk + 7% glucose + 0.1% KI; 3 - skimmed milk + 10% glucose +

0.1% ammonium molybdate; 4 - skimmed milk + 5% glucose + 5% glycerol + 0.01% ascorbic acid. Skimmed milk + 7% glucose was used as a control protective medium for fungi, and skimmed milk + 7% sucrose was used for bacteria.

The lyophilization process consists of freezing and vacuum sublimation. Freezing was performed in the Ult Freezer 80 refrigerator at a temperature of -50ºC. Sublimation of the samples was performed in the sublimation system "LABCONCO 6 plus" (temperature - 92ºC, pressure 6 Pa, duration 8 hours).

The titer of the spore suspensions of the studied strains was determined before lyophilization. The following phytopathogens were used as test strains: *Alternaria alternata*; *Botrytis cinerea*; *Fusarium solani*; *Fusarium oxysporum*; *Agrobacterium tumefaciens*; *Corynebacterium michiganensis*; *Erwinia carotovora* and *Xanthomonas campestris*.

After lyophilization in various protective media, the strains were activated with sterile distilled water and subcultured in Petri dishes on agar media, after which their viability was assessed. The antimicrobial activity of *Penicillium* sp. 1, *Penicillium* sp. 6, *Trichoderma* sp. 3, *Trichoderma* sp. 14, and *Bacillus* sp. 13, which demonstrated high antagonism to the reference strains before lyophilization, was assessed after lyophilization.

The viability of the strains before and after lyophilization (expressed in colony-forming units (CFU) per mL^{-1}) was determined by counting colonies on agar medium after serial dilutions. The number of viable cells was expressed as the *log*10 of colony-forming units (CFU) in 1.0 mL of suspension. Viability was calculated using the formula BSR = (*log*AL/*logB*L) × 100, where BSR is the strain survive rate in %, *log*BL is the logarithm of the CFU count before lyophilization, and *log*AL is the logarithm of the CFU count after lyophilization or storage (Muñoz-Rojas et al., 2006).

The antimicrobial activity of microorganisms was determined using the disc-diffusion method (Egorov, 2004; Ratnam et al., 2014).

The tested cultures were subcultured in Petri dishes. The 8 mm agar blocks were cut with a sterile cork borer from the nutrient substrate where the strains of tested microorganisms grew abundantly. The agar blocks were then transferred to prepared cavities in agar nutrient medium with instantly subcultured tests. Petri dishes were kept in a cool place for 1 hour before incubation to allow the diffusion of biocidal substances. The diameter of the growth inhibition zones was measured after incubation at 37°C for 24 h for bacteria, and at 28°C for 72 h for fungi, respectively.

There were three replications for each test (was applied to the significance threshold p=0.05) and the biocidal assessment was performed twice. Statistical calculations were performed using MS Excel 2010 software.

RESULTS AND DISCUSSIONS

Viability of microorganisms before and after lyophilization

Protective media used in the lyophilization process have different effects on microorganisms, and a universal protective medium has not yet been developed.

In the process of lyophilization, dehydration of microorganisms occurs, and as a result, the oxidative state of the cells increases and the production of reactive oxygen species (ROS), free radicals. An imbalance between ROS and antioxidants or enzymes, which capture peroxide, can lead to peroxidation of lipids, proteins and nucleic acids (França et al., 2007; Len et al., 2019; Van Engeland et al., 2025). Furthermore, the osmotic imbalance resulting from lyophilization (freezing and sublimation) leads to water loss, which seriously impacts cell membranes by altering the physico-chemical properties of the lipid bilayer. Although organisms employ various adaptive strategies under dehydration conditions, immediate adaptation to osmolytes (OMA), such as sugars and amino acids, is critical for maintaining membrane stability (Van Engeland et al., 2025).

The following substances are often used as lyoprotectants for lyophilization of microorganisms: skimmed milk, trehalose, and monosodium glutamate, while water and skimmed milk are used as rehydration media. Carbohydrates, glycerol, and antioxidants (ascorbic acid, thiourea, potassium iodide, and ammonium molybdate) are used to enhance the growth effect and stabilize the protective

medium (Okhapkina, 2009; Pajuelo et al., 2023; Rockingera et al., 2021; Van Engeland et al., 2025).

When assessing the viability of strains of *Penicillium* fungi (Figure 1) after lyophilization in the developed protective media, it was found that the viability of 5 strains varies significantly depending on the composition of the protective media used.

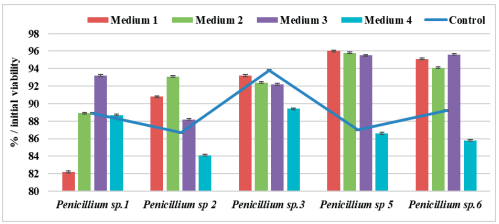

Figure 1. Viability of *Penicillium* strains after lyophilization in different protective media compared to the initial titre

The viability of the lyophilized strains in the control medium varied within the limits of 86-92%, compared to the initial viability, of the strains that were lyophilized in Medium 1 was 90.8-96%, of the strains in Medium 2 - 88.9-95.5%, of those that were lyophilized in Medium 3 - 88.2-95.6%, and of those lyophilized in Medium 4 - 84.1-89.4%, compared to the initial titre before lyophilization. Thus, we can see that the strains lyophilized in 3 elaborated protection media (Medium 1; Medium 2 and Medium 3) demonstrated a higher viability than the initial one, compared to the control variant, with the exception of the strains lyophilized in protection Medium 4, in which the viability was lower than the control. In most strains, the highest viability was obtained in the variant in which protection Medium 1 was used, which contains: SM + 5% S + 5% G + 0.01% Aa.

The evaluation of the viability of *Trichoderma* strains after lyophilization in the elaborated protection media demonstrated a higher viability, compared to *Penicillium* strains (Figure 2).

The viability of 5 *Trichoderma* strains after lyophilization in the control protective medium was 91.6-95.6% compared to the initial titer before lyophilization. After lyophilization in protective Medium 1 (SM + 5% S + 5% G + 0.01% Aa), the viability of *Trichoderma* strains ranged from 93.8% to 97.3%, and for lyophilized strains in protective Medium 2, the viability was 92.1-96.6% compared to the initial viability.

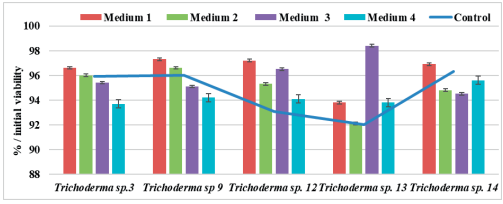

Figure 2. Viability of *Trichoderma*strains after lyophilization in different protective media compared to the initial titre

High viability was recorded for *Trichoderma* strains lyophilized in Medium 3. Thus, for 4 strains, viability was in the range of 94.5-96.5%, and for the strain *Trichoderma* sp. 13, viability was 98.0% compared to the initial viability before lyophilization. In the variant in which the strains were lyophilized in protective Medium 4, viability was 93.7-95.6% compared to the original. The highest viability after lyophilization of *Trichoderma* strains was obtained in the variant in which medium 1 containing (SM + 5% S + 5% G + 0.01% Aa) was used as a protective medium.

According to the obtained results, the viability of fungal strains after lyophilization in the protective medium M1 is higher compared to the viability of lyophilized strains in the control protective medium (SM +7% G).

After lyophilization in various protective media, bacterial strains demonstrated high viability in all tested variants (Figure 3).

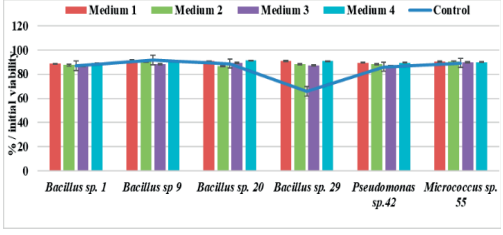

Figure 3. Viability of bacteria strains after lyophilization in different protective media compared to the initial titre

The viability of 6 bacterial strains after lyophilization in the control medium (SM + 7% S) varied within 65.5-89% compared to the

viability before lyophilization. When lyophilized in the developed protective media, their viability increased compared to the control variant. Thus, in the variant with protective Medium 1, the viability was 88.5-91.2% compared to the initial. In the variant that used protective Medium 2, the recorded viability varied within 86.5-90.1%. After lyophilization in protective Medium 3, the recorded viability values were within 86.6-89.6%, and in the variant with protective medium 4, the bacterial strains demonstrated the highest level of viability - 89.1-91.5% compared to the initial viability before lyophilization.

The results demonstrated higher viability of fungal and bacterial strains after lyophilization in variants M1 and M4, in which the protective medium was supplemented with ascorbic acid, a powerful antioxidant essential for reducing oxidative stress. These results are consistent with literature data showing that certain compounds, such as glucose, trehalose, and ascorbic acid, can reduce lipid peroxidation and improve cell survival (Pajuelo et al., 2023; Rodklongtan et al., 2022; Van Engeland et al., 2025).

Rodklongtan et al. (2022) demonstrated that the addition of a small amount of ascorbic acid to lactose-based medium could promote cell viability during lyophilization, but the effect on viability became negative with increasing concentration.

Antimicrobial activity of some strains before and after lyophilization

As a result of studying the antimicrobial properties of the strains before lyophilization, 4 fungal strains (*Penicillium* sp. 1, *Penicillium* sp. 6; *Trichoderma* sp. 3 and *Trichoderma* sp. 14) and 1 bacterial strain (*Bacillus* sp. 13) with high antimicrobial potential againsttest strains were identified.

Filamentous fungi of the genera *Tichoderma* and *Penicillium* are successfully used in agriculture as biological control agents, phytostimulants for agricultural plants, in soil bioremediation, etc. (Mirsam et al., 2025; Sun et al., 2025; Woo et al., 2014).

To assess the stability of the antimicrobial properties of the specified strains after lyophilization, the antimicrobial activity of the lyophilized strains was determined in 4 developed protective media, as well as in a control protective medium.

The antimicrobial activity of *Penicillium* strains before lyophilization revealed zones of inhibition of test fungi ranging from 18 mm to 31 mm, while the zones of inhibition of phytopathogenic bacteria ranged from 16.0 to 18.7 mm. Evaluation of antimicrobial activity after lyophilization revealed a decrease in the zones of inhibition of phytopathogens by 1-3 mm compared to the initial zone before lyophilization. Thus, under the influence of *Penicillium* spp. 1 (Figure 4) and *Penicillium* spp. 6 (Figure 5), lyophilized in a control medium, the inhibition zone of test fungi varied from 16 to 29 mm. In the variant where *Penicillium* strains were used after lyophilization in protective Medium 1, the inhibition zones of phytopathogenic fungi ranged from 17 mm (*F. solani*, *F. oxysporum*) to 30 mm (*A. alternata*), and for strains lyophilized in protective Medium 2, protective Medium 3 and protective Medium 4, the inhibition zones of control fungi ranged from 15 mm to 27 mm.

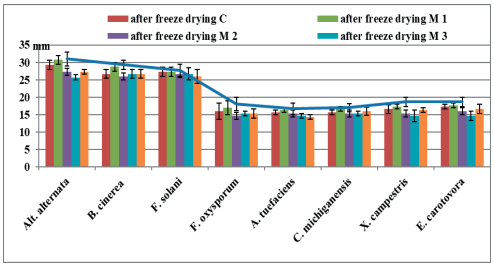

Figure 4. Antimicrobial activity of *Penicillium* sp. 1 strain before and after lyophilization in different protection media

Testing of *Penicillium* strains after lyophilization in the developed protective medium on test bacteria showed inhibition zones from 15 to 17 mm, regardless of the variant tested.

Compared with the variant in which the control protective medium was used, the highest antimicrobial activity against the test strains was recorded for the strains *Penicillium* sp. 1 and *Penicillium* sp. 6 in the variant in which the strains were lyophilized in protective medium 1 (SM + 5% S + 5% G + 0.01% Aa), and the lowest in the variant with protective medium M 3.

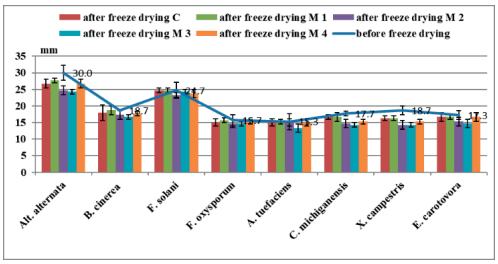

Figure 5. Antimicrobial activity of *Penicillium* sp. 6 strain before and after lyophilization in different protection media

Testing of *Trichoderma* sp. 3 (Figure 6) and *Trichoderma* sp. 14 (Figure 7) strains on reference fungal strains before lyophilization showed high activity: inhibition zones from 20.7 mm (*F. oxysporum*) to 39 mm (*A. alternata*), and against phytopathogenic bacteria, the inhibition zones ranged from 12 to 17 mm.

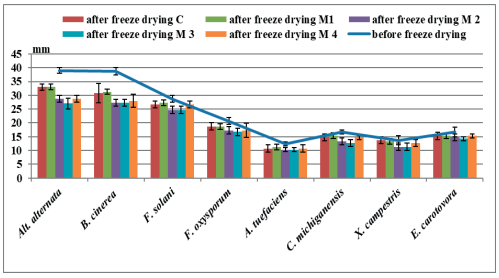

Figure 6. Antimicrobial activity of *Trichoderma* sp. 3 strain before and after lyophilization in different protection media

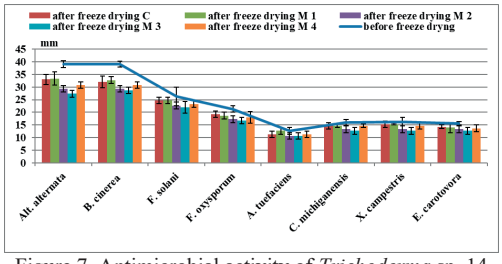

Figure 7. Antimicrobial activity of *Trichoderma* sp. 14 strain before and after lyophilization in different protection media

After lyophilization in various protective media, the inhibition zones of phytopathogens decreased. Thus, under the influence of strains in the control variant, the zones ranged from 15 mm (*F. oxysporum*) to 31 mm (*A. alternata*), under the influence of strains lyophilized in protective medium 1, the zones were 18–32 mm, for strains lyophilized in protective medium 2, the zones ranged from 17 to 29 mm. Lyophilized strains on medium 3 demonstrated zones of inhibition of phytopathogenic fungi ranging from 17 to 27 mm, while lyophilized strains on medium 4 demonstrated zones of inhibition of phytopathogenic fungi ranging from 17 to 30 mm. Compared to the reference bacteria, the zones of inhibition were small and did not exceed 15 mm, regardless of the *Trichoderma* strain variant tested.

The highest antimicrobial activity compared to the control variant was recorded under the action of exometabolites of the mentioned strains, lyophilized in protection Medium 1.

According to the obtained results, it was established that the inhibition zones of reference strains (phytopathogenic fungi and bacteria) are larger under the influence of exometabolites of *Trichoderma* strains lyophilized in protective medium 1, but still 2-3 mm smaller compared to the data obtained before lyophilization of the indicated strains.

Bacteria of the genus *Bacillus* play a crucial role in the prevention and control of plant diseases, producing a wide range of compounds with antifungal properties, such as isocoumarin and amino acids, bacillomycin, bacteriocins, iturin, surfactin, cyclic lipopeptides, polyketides, lantibiotics, phospholipids, polyketide microlectins (Iqbal et al., 2024; Zhang et al., 2008).

The *Bacillus* sp. 13 strain before lyophilization, demonstrated antagonism against 3 phytopathogenic fungi with inhibition zones of 20-22 mm and against 2 phytopathogenic bacteria with inhibition zones of 17.7-21.7 mm (Figure 8).

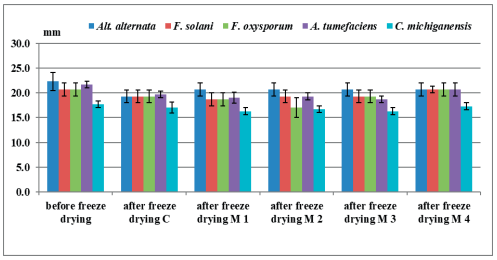

Figure 8. Antimicrobial activity of *Bacillus* sp. 13 strain before and after lyophilization in different protection media

Under the influence of exometabolites of the *Bacillus* sp. 13 strain (Figure 8), lyophilized in

the control medium (SM + 7% S), the inhibition zones of phytopathogens: *A. alternata*, *F. solani* and *F. oxysporum* were 19 mm, and the inhibition zones under the influence of the lyophilized strain in protective Medium 1, Medium 2 and Medium 3 varied from 17 mm to 20 mm. The larger inhibition zones were obtained under the influence of the lyophilized strain in protective medium 4, which were 20-22 mm.

Compared with the test bacteria *A. tumefaciens* and C. *michiganensis*, regardless of the medium in which lyophilization was carried out, the *Bacillus* sp. 13 strain demonstrated inhibition zones of 17-20 mm.

CONCLUSIONS

Lyophilization is a widely used method of long-term preservation, in which the survival of microorganisms depends significantly on the protective medium used.

Testing of five protective media on fungal and bacterial strains during lyophilization revealed minor but significant differences depending on the type of protective medium used for lyophilized cultures ($p < 0.001$).

Among the tested media, the highest viability and antimicrobial activity after lyophilization of filamentous fungal strains was obtained in a protective medium containing skimmed milk + 5% sucrose + 5% glucose + 0.01% ascorbic acid, while for bacterial strains, the highest viability was achieved in a medium containing skimmed milk + 5% glucose + 5% glycerol + 0.01% ascorbic acid.

Cell viability of mycelial fungi after lyophilization ranged within 92-98%, and of bacteria within 88-91.5%

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DEVELOPMENT AND CHARACTERISATION OF POLYVINYL ALCOHOL BASED POLYMERIC SYSTEMS AS POTENTIAL PLATFORMS FOR BIOMEDICAL AND AGRICULTURAL APPLICATIONS

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Abstract

In the context of developing biocompatible materials for medical and agricultural applications, polyvinyl alcohol (PVA) has emerged as a versatile polymer, serving as the core component of the formulations investigated in this study. The work focused on the development of PVA-based gel and film systems for topical drug delivery systems and wound dressings, as well as for soil mulching and controlled-release fertilizer carriers. Preliminary studies were conducted to select appropriate concentrations of polymer, plasticizer and crosslinking agent. The resulting gels underwent rheological characterization, and were subsequently processed into films by casting. The films were evaluated in terms of visual appearance, water absorption capacity, and surface wettability. Based on the gels flow properties and the films hydrophilicity, two formulations were selected for further investigation: 10 and 15% PVA gels containing 15% citric acid relative to dry substance. To improve their physicochemical properties, the gels were then blended with a 3% methylcellulose (MC) gel in various ratios. The newly obtained gels exhibited enhanced pseudoplastic behavior, which facilitates the film-forming process, resulting in films with significant potential for future biomedical and agricultural applications.

Key words*: polyvinyl alcohol, hydrogels, films, methylcellulose, flow behavior, hydrophilicity.*

INTRODUCTION

Polymers are crucial in various industries as the current trend in biomaterials focuses on developing biocompatible polymeric materials, often incorporating natural excipients to improve systemic properties (Kalirajan et al., 2021).
Polyvinyl alcohol (PVA) is a synthetic polymer with thermoplastic behaviour, high biodegradability, and biocompatibility. Unlike most polymers, PVA is not obtained through the polymerization of its component monomers but through the hydrolysis of polyvinyl acetate (Liang et al., 2024).
The first synthesis of PVA dates back to 1924 and is attributed to Dr. Herman Staudinger (Li et al., 2025), who described it as an odourless polymer, soluble in water, acids and polar solvents. The polymer solubility in aqueous solutions is highly dependent on its degree of hydrolysis and molecular weight.
PVA can be processed into various forms, including hydrogels and polymeric films, which are widely used in applications ranging from

medical contact lenses and wound dressings to food packaging, detergent doses, clothing, and agricultural mulch materials (Teodorescu et al., 2018; Yang et al., 2024).

PVA-based hydrogels and films have attracted increasing attention due to their favourable properties, such as soft and elastic structure, superior mechanical features, ease of handling, better barrier properties, stability, loading capacity, and controlled release of various active ingredients (Rahman Khan & Rumon, 2025).

Both forms exhibit adjustable physical, chemical, and biological properties, which can be modified according to the intended application through the incorporation of specific additives (Feldman, 2020).The main methods for adjusting the physico-chemical properties of polymers are crosslinking and plasticizer addition (Liang et al., 2024; Yang et al., 2024).

Crosslinking can be achieved through both physical and chemical methods. Physical crosslinking techniques include freeze-thaw cycles, directional freezing, UV or γ irradiation, and thermal treatments (Rahman Khan & Rumon, 2025). Chemical crosslinking is carried out using various agents, among which the most commonly used are glutaraldehyde, citric acid (Lodi et al., 2024) and boric acid (Lambertini et al., 2024). In the context of applications in biomedical and agricultural fields, the use of a biocompatible and environmentally friendly crosslinking agent, such as citric acid, is preferred (Salihu et al., 2021; Rodrigo & Munaweera, 2025).

Plasticizers are low molecular weight substances that intercalate between polymer chains, reducing intermolecular forces, and increasing molecular mobility, thereby improving the mechanical properties of the material (Hacker & Mikos, 2011). Examples of plasticizers reported in the literature include glycerine (Han et al., 2023), propylene glycol, polyethylene glycol (Shojaee Kang Sofla et al., 2020), caprolactam, and sorbitol (Xu et al., 2020).

To improve the physicochemical properties of the formulations, beyond plasticizers and crosslinking agents, PVA could be blended with various polymers, such as cellulose derivatives (Kida et al., 2020; Abdullah et al., 2021), chitosan (Pugar et al., 2024), sodium alginate (Kumar et al., 2022), starch (Iskalieva et al., 2023), and gelatine (Rivera Hernández et al., 2024) allowing for tailored film properties across a wide range of applications.

Among the cellulose derivatives, methylcellulose was selected for this study. A mixture of PVA and methylcellulose, each at an approximate concentration of 4%, has previously been reported, with radiation used as the crosslinking agent, designed as an oral drug delivery platform (Kenessova et al., 2025). In an another study, MC/PVA composite hydrogels with tunable thermoresponsive properties were obtained through a mild physical preparation method, for potential use as injectable carriers (Geng & Xiao, 2009).

In agriculture, PVA hydrogels and films can be used to minimize resource loss associated with the use of conventional fertilizers, mitigate the risks related to non-biodegradable polymer-coated urea (Wan Anuar et al., 2025) and also contributing to the stabilization and controlled release of nutrients and biocides, thereby supporting sustainable and economically efficient agricultural practices (Malka & Margel, 2023; Jayanudin et al., 2024).

In the biomedical domain, PVA-based hydrogels and films are suitable for delivering drugs to the wound site, exhibit self-adhesion to the injury, and reduce the risk of wound reopening upon dressing removal due to their biodegradable nature (Huang et al., 2023). These properties can also be extended to the development of transdermal patches for drug delivery, where PVA is combined with specific excipients or permeation enhancers to facilitate drug transdermal absorption (Solís et al., 2021).

PVA is also widely used in tissue engineering for repair and replacement of damaged tissues (Nathan et al., 2023; Thakare & Hazarika, 2025). Although PVA hydrogels exhibit a high water retention capacity, they lack the needed mechanical strength and flexibility under physiological (homeostatic) conditions, which limits their standalone applicability in such contexts, also requiring additives to fine-tune their characteristics (Han et al., 2023).

Another application in the field of tissue engineering involves the use of polyvinyl alcohol and calcium silicate as a matrix for bone regeneration, as currently, bone fractures are frequently treated using metal implants, which present significant drawbacks, including the risk

of incompatibility, high cost, and the need for multiple surgical interventions (Spaniol et al., 2019; Shendage et al., 2025).

Taking into account the aforementioned aspects, the main objective of this work was the selection and optimization of PVA-based formulations to improve the film-casting process and the physicochemical properties of the resulting polymeric systems. Specifically, this study aimed to: (i) determine the effect of citric acid concentration on the rheological behaviour of the gels; (ii) evaluate the influence of the crosslinking agent and plasticizer on the water absorption capacity and surface wettability of PVA films; (iii) identify the optimal formulation parameters for an adequate pseudoplasticity of the gels, and a balanced hydrophilicity and swelling of the films; (iv) assess the influence of methylcellulose blending on pseudoplasticity to facilitate the film-casting.

The selection of biocompatible and environmentally sustainable components, PVA, glycerine, citric acid, and methylcellulose, minimizes ecological impact while enhancing the safety profile for biomedical applications.

MATERIALS AND METHODS

For the preparation of polyvinyl alcohol-based gels, the reagents consisted of: polyvinyl alcohol with a molecular weight of 70,000-100,000 g/mol (Sigma), anhydrous glycerine 92.10 g/mol (Chemical Company), citric acid 102.13 g/mol (Loba Chemie), methylcellulose 4000 cP (Janssen Chimica) and distilled water.

The equipment used in the preparation stage consisted of: analytical balance (Sartorius), high-precision laboratory electric stirrer 3000RPM, 220V/50Hz 200W (Velp Scientifica), magnetic stirrer with heating DLAB MS-H380 Pro, 200-1500 RPM (DLAB Scientific Co.), water bath with immersion thermostat, and recirculation pump 230V/50-60 Hz 1300W (Falc).

The composition of the prepared gels is presented in Table 1.

In this paper, samples A0, A1, A2 and A3 will be referred to as series A and samples B0, B1, B2, B3 will be referred to as series B.

The preparation of gels for both series was carried out according to the protocol presented in Table 2.

Table 1. Composition of gels based on PVA, anhydrous glycerine and citric acid

Sample code	PVA (%)	Anhydrous glycerine (%)	Citric acid (%)*
A0	10	10	-
A1	10	10	10
A2	10	10	15
A3	10	10	20
B0	15	5	-
B1	15	5	10
B2	15	5	15
B3	15	5	20

*expressed relative to dry PVA weight

Table 2. Gels preparation protocol

Step	Instruction
1	The water bath was heated to a temperature of 90°C. A support was installed to hold the vessel used for preparation, ensuring that the vessel walls remained immersed in water up to the level of the solution inside.
2	The required amount of PVA was weighed, taking into account the desired final concentration.
3	The necessary amount of distilled water for the preparation was placed in a Berzelius beaker.
4	Using a magnetic stirrer, PVA was gradually added to the distilled water at room temperature to ensure proper dispersion and minimize material loss.
5	The Berzelius beaker was then secured in the support and placed in the water bath previously heated to 90°C.
6	The contents of beaker, consisting of PVA dispersed in water, were subjected to mechanical stirring until the PVA was completely dissolved and a homogenous solution was obtained.
7	Subsequently, after cooling the mixture to 50°C, the required amount of anhydrous glycerine was weighed and added to the PVA colloidal solution under continuous mechanical stirring to ensure optimal incorporation.
8	Finally, the pre-weighed citric acid was gradually added under continuous mechanical stirring to obtain the corresponding gels.
9	The mixture was maintained under mechanical stirring at 200 rpm until complete homogenization was achieved, after 35 minutes.

The films were prepared by casting the gels onto glass slides and dried at room temperature for 72 hours. To ensure uniform thickness, 1.5 g of gel was applied to each slide.

The rheological analysis of the gels was performed using a rotational viscometer, model RM 100 PLUS (Lamy Rheology). In this context, a coaxial cylindrical system was used, consisting of a set of accessories, namely the MK-DIN1 spindle with a diameter of Ø = 30 mm, together with an attachment composed of a DIN1-Cap and a DIN1-Tube. The measurements were conducted at room temperature (23°C), and the flow behavior of the gels was evaluated over a rotational speed range between

0.3 and 60 rpm, corresponding to shear rates between 0.4 and 78 s^{-1}.
The Ostwald-de Waele model (Eq. 1) was applied to the experimental data:

$$\tau = K \cdot \dot{\gamma}^n \quad (1)$$

Where: τ represents the shear stress (Pa); $\dot{\gamma}^n$ - shear rate (s^{-1}); K - consistency index ($Pa \cdot s^n$); n - flow behaviour index (dimensionless).
The goniometric analysis of the films was performed using a KSV Cam 101 Scientific Instrument goniometer (Helsinki, Finland), equipped with a digital camera. The contact angle method was applied, using distilled water as the testing liquid. Briefly, a small droplet of water (~3 µL) was deposited onto the surface of the film using a Hamilton microsyringe. The digital camera, positioned perpendicular to the polymeric film surface, captured the image of the droplet in contact with the film (Huang et al., 2023).
The droplet image was analysed to determine its shape, and the contact point between the water and the polymeric film surface. Both the left and right contact angles were measured to obtain an average value.
All measurements were carried out in triplicate, and the reported values represent the mean of three determinations.
This parameter provides insight into the hydrophilic behavior of the film in different applications, and its equilibrium value is determined using Young equation (Eq. 2):

$$\gamma_{SG} = \gamma_{SL} + \gamma_{LG} \cdot \cos\theta \quad (2)$$

Where: θ defines the contact angle, and γSG, γSL, γLG - the interfacial surface tensions at the solid-gas, solid-liquid, and liquid-gas interfaces, respectively.
The water absorption capacity of the films was evaluated using a gravimetric method, with distilled water as the swelling medium. The analysis was performed at room temperature (23°C). Initially, pieces of approximately 0.25cm^2 were cut from the polymeric films and weighed to record their initial dry mass. The weighed samples were then placed in 2.5 mL of distilled water (Tudoroiu et al., 2023).
At predetermined time intervals, the samples were removed from the water and weighed to determine their hydrated mass. The water absorption capacity (g/g) was calculated using the equation found below (Eq. 3):

$$\text{Absorption capacity (g/g)} = \frac{W_t - W_i}{W_i} \quad (3)$$

Where: W_t represents the mass of the films after hydration at different time intervals, and W_i - the initial mass of the films in their dry form.
Taking into consideration the results obtained during the evaluation stage of the previously prepared polymeric systems, correlated with the desired properties of the final products – moderate water absorption capacity and an adequate degree of hydrophilicity – two representative compositions were selected, one from each developed series, the compositions corresponding to samples A2 and B2.
To improve the rheological characteristics of the gels, and film-casting process, a 3% methylcellulose (MC) gel was prepared (Tudoroiu et al., 2023). The composition of the PVA-MC gels is presented in Table 3.

Table 3. Percentage composition of polymeric systems based on PVA, anhydrous glycerine, citric acid and methylcellulose

Sample code	A2 gel (%)*	B2 gel (%)*	3% MC gel (%)*
MC1	80	-	20
MC2	70	-	30
MC3	-	80	20
MC4	-	70	30

*expressed to 100 g of final gel

Further, the rheological behaviour of the MC1-MC4 gels was evaluated as previously described.

RESULTS AND DISCUSSIONS

The rheological behaviour of the gels based on polyvinyl alcohol, anhydrous glycerine, and citric acid was evaluated from rheograms of shear stress versus shear rate, presented in Figure 1 for the series A, and Figure 2 for the series B.
Analysis of Figures 1 and 2 reveals similar profiles, showing an increase in shear stress with increasing shear rate for all samples in series A (10% PVA) and series B (15% PVA). For both series, the smallest increase in shear stress was observed for uncrosslinked samples A0 and B0,

while the most pronounced increase was noticed for samples A2 and B2, containing 15% concentration of citric acid reported to dry mass of PVA.

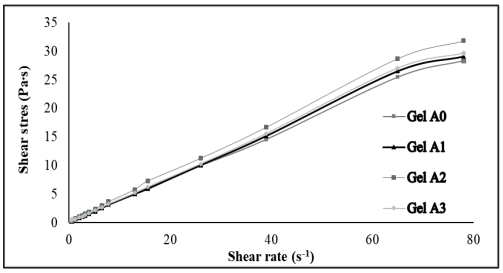

Figure 1. Shear stress vs. shear rate rheograms for series A gels (10% PVA) containing 0-20% citric acid

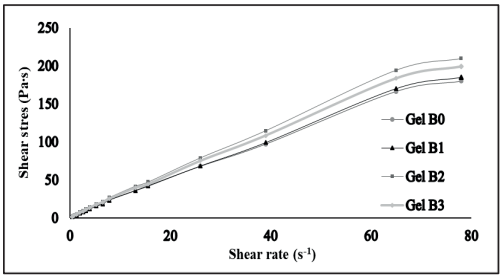

Figure 2 Shear stress vs. shear rate rheograms for series B gels (15% PVA) containing 0-20% citric acid

To quantify the rheological behaviour of the designed polymeric systems, the Ostwald-de Waele model (Eq. 1) was applied, describing the variation of shear stress as a function of shear rate. This allowed the assessment of the specific rheological parameters for this model, namely the flow behaviour index and the consistency index, presented in Table 4, along with determination coefficient (R^2) values.

Table 4. Rheological parameters (K, n) and determination coefficient (R^2) according to Ostwald-de Waele model for the gels of series A and B

Gel	Consistency index (K-Pa·s^n)	Flow behavior index (n)	Determination coefficient (R^2)
A0	0.400	0.99	0.9984
A1	0.420	0.98	0.9976
A2	0.610	0.89	0.9978
A3	0.560	0.85	0.9930
B0	3.530	0.95	0.9976
B1	3.640	0.92	0.9977
B2	4.250	0.89	0.9975
B3	3.970	0.88	0.9977

Values obtained for determination coefficient higher than 0.9900 indicate that the data for all gels fit well the Ostwald-de Waele model.

The flow index (n) suggested rheological behaviour ranging from near-Newtonian to weakly pseudoplastic.

As the crosslinker concentration increased, a progressive decrease in n values was observed, with A3, respectively B3 showing the most noticeable pseudoplastic behaviour in their corresponding series.

Regarding the consistency index, similar values were observed for sample A0 (uncrosslinked) and A1 (crosslinked with 10% concentration of citric acid). Maximum value was registered for sample A2 (15% concentration of citric acid), with an increase of consistency index of about 1.45 times relative to samples A0 and A1.

A similar trend is noticed for series B, noting that the initial values of K parameter were higher for all samples compared to those obtained for series A, which may be attributed to a higher PVA concentration.

For this series, an increase in the citric acid content to 15%, corresponding to sample B2, led to a 1.20-fold increase in the consistency index compared to B0 and B1.

In summary, for both series a 10% addition of citric acid did not significantly change the rheological behavior, while the maximum viscosity is reached for a 15% concentration of citric acid. A further increase in the crosslinking agent concentration to 20% did not result in an additional increase in this parameter, suggesting a limit in the crosslinking efficiency was reached at 15%.

Regarding the PVA-to-glycerine ratio, polymer concentration was found to play a determining role in the consistency of gels across for both series. Notably, gels formulated with 15% PVA (series B) exhibited values of the consistency parameter *K* approximately 7-9 times higher than those recorded for 10% PVA gels (series A), for the same concentration of citric acid relative to PVA dry mass, demonstrating that increasing polymer content leads to a considerably denser and more robust gel network.

Regarding other studies on PVA-based gels, Teodorescu et al. (2016) reported gels containing 15% PVA/PVP at various ratios,

crosslinked through freeze-thaw cycles, which exhibited viscoelastic behaviour.

Another approach, described by Hapipi et al. (2020), involved blending PVA with magneto-rheological plastomers. These materials exhibited distinctive rheological behaviour, characterized by shear thickening at low shear rates and shear thinning at high shear rates. The content of carbonyl iron particles was found to directly correlate with rheological performance (Hapipi et al., 2020).

The rheological behaviour of PVA and hyaluronic acid (HA) mixture was investigated by Lewandowska (2020). Pure PVA solutions exhibited shear-thinning behaviour regardless of solvent type, with no significant solvent-dependent differences in apparent viscosity. For HA/PVA gels the flow behaviour was governed by composition and solvent. A synergistic viscosity enhancement was observed in acidic blends at low shear rates, resulting from attractive intermolecular forces between polymeric components, though this effect diminished at higher shear rates due to hydrogen bond disruption. The consistency index (k) values also decreased with increasing temperature, consistent with the expected thermal behaviour of solution viscosity (Lewandowska, 2020).

Following formation by casting method, films obtained from the corresponding gels were clear, homogeneous, exhibiting transparency, absence of air bubbles, ease of peeling, and good elasticity.

The surface hydrophilicity of films was assessed by contact angle measurements. Several factors influence this assessment, including surface tension (higher surface tension leads to a larger contact angle due to reduced interaction with the film surface), film roughness (a rough surface provides more contact points for water, resulting in a lower contact angle, while a smooth surface increases the contact angle), physicochemical properties of the film and the liquid (molecular interactions between the two phases determine whether the liquid spreads over the film), and ambient temperature and pressure (variations in temperature and pressure can modify the contact angle, thereby affecting the equilibrium between the three phases) (Quéré, 2008; Drelich, 2019; Owusu et al., 2025).

A low contact angle (< 90°) indicates strong interaction between the film and the liquid (Giovambattista et al., 2007). All samples exhibited contact angles lower than 40°, indicating a pronounced hydrophilic character, adequate for water absorption (Figure 3).

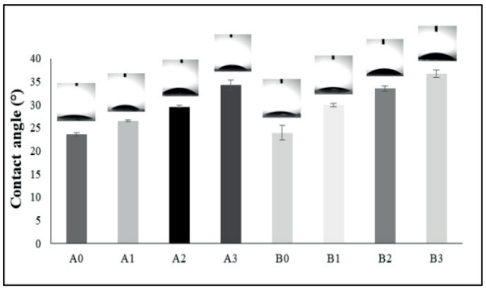

Figure 3. Images of the water droplets in contact with PVA-based films for series A and B, corresponding to the contact angles; values are mean ± SD (n = 3)

For series A, the average contact angle values ranged from 23.61 ± 0.31 to 34.30 ± 1.05°. The lowest contact angle was observed for sample A0, which did not contain a crosslinking agent. It appears that increasing the concentration of the citric acid led to a progressive increase in the contact angle values, with the highest value recorded for sample A3, containing the maximum crosslinker concentration. These results can be explained by the increase in polymer network density associated with higher concentrations of the crosslinking agent in the samples, leading to an increase in contact angle of 1.45 times.

Regarding the samples from series B, a similar trend was observed, with an increase in contact angle values as the concentration of the crosslinking agent in the samples varies from 0 to 20%. Within this series, the average contact angle values ranged from 23.94 ± 1.39 (B0) to 36.73 ± 0.76° (B3), translating into an increase by a factor of 1.53.

Based on our knowledge there is no previous study regarding contact angle measurement for a formulation of PVA/MC films. However, there is a study measuring the contact angle using another cellulose derivate, namely carboxymethyl cellulose blended with PVA and *Calendula officinalis* extract. The study reported an average angle of 14.6°, indicating a very hydrophilic film was obtained (Huang et al., 2023). Simple MC films are less hydrophilic,

with a contact angle of 45° being reported (Tudoroiu et al., 2023).
Overall, for our study a compositional balance between 10% PVA/10% glycerine and 15% PVA/5% glycerine resulted in a similar surface hydrophilicity of the films.
To further characterize the films, the absorption capacity was evaluated. This represents a critical parameter in designing systems that achieve an optimal balance between mechanical stability, water retention, and controlled permeability (Priya et al., 2024).

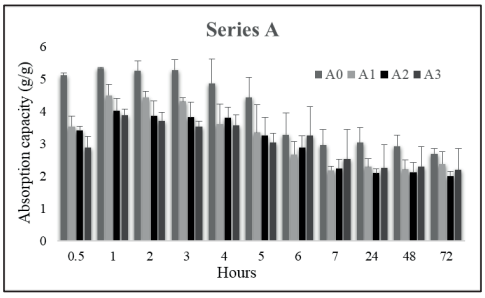

Figure 4. Absorption capacity for the film series A

It is observable that for all the samples from series A, the maximum absorption capacity was reached one hour after the start of the experiment (Figure 4). This behaviour may be explained by the hygroscopic nature of glycerine, which is present in higher concentration in this series. The maximum absorption capacity values for the samples in series A ranged between 3.88 ± 0.20 (A3) and 5.34 ± 0.02 g/g (A0), indicating 1.37 times difference between the two samples. The obtained results may be explained by the effect of the crosslinking agent, which promotes the formation of a denser polymeric network, resulting in a reduced absorption capacity.

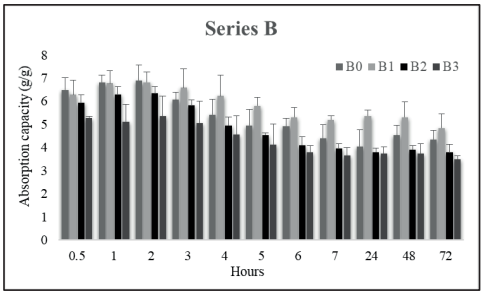

Figure 5. Absorption capacity for the film series B

Regarding the samples from series B (Figure 5), a similar trend was observed, with a decrease in absorption capacity as the concentration of the crosslinking agent increased in the samples. Within this series, the maximum absorption capacity was reached two hours after the start of the experiment. The maximum absorption capacity values for series B ranged between 5.34 ± 0.75 (B3) and 6.90 ± 0.56 g/g (B0), showing a 1.30 times difference between the uncrosslinked sample and the one crosslinked with the maximum amount of citric acid.
An excessive crosslinking may impede solvent penetration and potentially result in the delayed or incomplete release of incorporated bioactive agents.
Comparing series A and series B, it appears that the samples containing a higher PVA concentration exhibited a superior absorption capacity, which may be attributed both to the increased availability of hydrophilic groups and to the higher amount of polymer capable of swelling.
When comparing PVA/MC gel to other systems found in literature, a similar absorption profile was observed, with the highest concentration of PVA corresponding to the highest swelling degree observed between samples (Kenessova et al., 2025).
Taking into account the results of all the previous analysis, two representative compositions, namely A2 and B2, were selected. To further improve their physicochemical properties and to optimize the film-forming process as well, these formulations were mixed with a 3% methylcellulose gel in different proportions, as described in the Materials and Methods section.
All gels of the series bellow are based on polyvinyl alcohol, anhydrous glycerine, citric acid, and methylcellulose. Shear stress - shear rate rheograms were plotted (Figure 6). An increase in shear stress with increasing shear rate was observed.
To assess the effect of incorporating 3% methylcellulose gel at varying ratios into PVA-based gels A2 and B2 on their flow behaviour, the rheological parameters of the Ostwald-de Waele model were determined (Table 5).

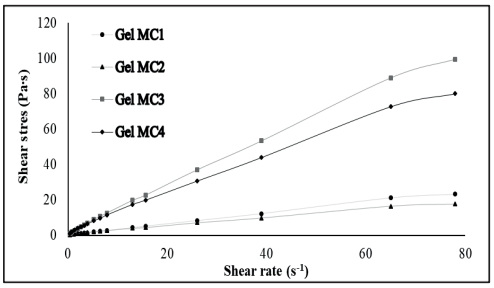

Figure 6. Comparative representation of the shear stress-shear rate rheograms for MC1-MC4 gels

Table 5. Rheological parameters (K, n) and determination coefficient (R^2) according to Ostwald-de Waele model for the gels A2, B2, MC, and MC1-MC4

Gel	Consistency index (K-Pa·s^n)	Flow behavior index (n)	Determination coefficient (R^2)
A2	0.610	0.89	0.9978
B2	4.250	0.89	0.9975
MC	53.160	0.48	0.9938
MC1	0.580	0.79	0.9925
MC2	0.670	0.72	0.9907
MC3	2.220	0.86	0.9985
MC4	2.250	0.81	0.9979

It can be observed that the n value obtained for the 3% methylcellulose gel was 0.48, indicating a pronounced pseudoplastic character. For gels MC1 and MC2, consisting of A2 and methylcellulose gels combined in different proportions (Table 3), lower values of the flow behaviour index were observed compared to that of the A2 gel, indicating an increase in pseudoplasticity related to the incorporation of methylcellulose gel. The lowest n value within this group (0.72) was recorded for the gel MC2, which contained a higher proportion of methylcellulose gel compared to MC1.

For gels MC3 and MC4, consisting of B2 and methylcellulose gels, a decrease in the flow behaviour index was also observed compared to the value obtained for B2 gel, showing an increase in pseudoplasticity within this group as well, with the minimum value recorded of 0.81, corresponding to MC 4, the gel with the higher concentration of methylcellulose from its series. To the best of our knowledge, the flow behaviour index of PVA-MC gel formulations has not been specifically investigated in previous studies. Regarding simple MC gels, they present a documented strong pseudoplastic behaviour (Tudoroiu et al., 2023)

Shi et al. described the rheological behavior of PVA, gelatine and carboxymethyl celllulose mixture gels using the Ostwald-de Waele model, confirming its non-Newtonian character. These findings were consistent with the pseudoplastic nature reported for PVA-based gels containing cellulose derivatives (Shi et al., 2024).

Analysis of the consistency index across both groups (MC1-MC2 and MC3-MC4) revealed a slight decrease in consistency index, correlated with increasing proportions of methylcellulose gel within the samples. Additionally, it was observed that in A2, MC1 and MC2 series, the consistency index values were relatively similar, whereas for the B2, MC3 and MC4 series, K values decreased by almost 2 times. This may be explained by the methylcellulose interaction with hydrogen bonds of PVA chains, also resulting in spatial separation of those chains (Kopač, Ručigaj, & Krajnc, 2022).

Overall, the incorporation of methylcellulose did not significantly alter the general rheological behaviour in terms of viscosity, with the polyvinyl alcohol gel remaining the main component. This is favourable, as a large increase in viscosity could hinder the film casting process. However, an improvement in pseudoplastic behaviour was observed, which will have a positive impact on the film-casting process of newly obtained gels.

CONCLUSIONS

The present work reports the design and optimization of polyvinyl alcohol-based polymeric system, using citric acid as a crosslinking agent, glycerine as a plasticizer, and methylcellulose as a rheological modulator. Rheological characterization demonstrated that the consistency index of the PVA-glycerine-citric acid-based gels was primarily increased by higher polymer concentration and, to a lesser extent, by crosslinker content. An optimal crosslinking was achieved at 15% citric acid (reported to dry PVA), beyond which no further improvement in flow resistance was observed.

Hydrophilic properties of the resulting films were evaluated through wettability and water absorption studies. The results indicate that an increase in the crosslinking degree inversely correlates with the hydrophilic character of the film surface, evidenced by higher contact angle values. Similarly, water absorption capacity decreased with advanced crosslinking for both

series. Concerning the polymer and plasticizer concentration, their ratio influences the water swelling behaviour of the films, whereas the surface hydrophilicity remains similar.

Comparative analysis showed that Series B performed superiorly to Series A, exhibiting higher water uptake and reaching swelling equilibrium after two hours, compared to one hour.

To refine the processing characteristics, methylcellulose was incorporated as a rheology enhancer before the casting stage. This addition modulated the flow behaviour by increasing pseudoplasticity, ensuring the formation of uniform and structurally stable films.

A limitation of this study was that the PVA/methylcellulose mixed gels were not cast and analysed in film form. Future studies will address this by casting the gels into films and conducting additional analyses to evaluate the mechanical properties (tensile strength, elongation at break) and incorporating active compounds to develop advanced delivery systems for wound healing and the controlled release of agrochemicals, extending the functional scope of these materials across biomedical and agricultural applications.

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IMMUNOLOGICAL ASPECTS REGARDING THE EVALUATION OF CELLULAR INDICES IN CATTLE

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Abstract

The paper represents a study conducted on cattle regarding the evaluation of cellular indices, the main ones in triggering cellular immunity depending on different age periods. Aspects of the behavior of the immune cell system against antigens represented by bacteria, parasites, fungi, etc. are presented. The objective of this research is to present an evaluation of some cellular indices in cattle depending on age and to follow the behavior of immune resistance. The results of the study generate possibilities for assessing the importance of immune cellular effectors in determining the resistance of the animal organism and provide valuable information about the reactivity of the animal organism according to the levels of some immune cellular indicators.

Key words*: cell, immunological indices, immune system, lymphocytes.*

INTRODUCTION

The body's immunobiological defense to cell surfaces, where they can be recognized by lymphocytes bearing antigen receptors. Each antigen receptor is highly specific, and each lymphocyte expresses only one form of antigen receptor. Thus, millions of cells have the potential to recognize millions of antigens (Găjăilă, 2003; Golban, 2016).

For these reasons, the main objectives of this research are to study the dynamics of immunobiological indices in cattle depending on age constitute a powerful weapon system whose use must be controlled to minimize external damage. For these reasons, a large part of the immune system is dedicated to the production of regulatory cells, whose function is to ensure that immune responses occur only under appropriate circumstances (Rosen, 2008; Golban, 2024).

At present, the immune system is a term increasingly used in biological sciences to define the defense mechanisms of the animal body against pathogens. This defense role is important for maintaining the health of the body, due to the multiple interaction with the environment, where it is continuously subject to the influence of external factors. For these reasons, living organisms have a phagocytic defense system, and the immune system is all the more complex as the organism considered is located on the scale of biological evolution and development. Acting more differentiated and more effective, in animals this system has matured, becoming an important complexity (Brocaw, 2013)

The immune responses to defend the body cannot always guarantee protection. In some situations, they lack the flexibility to respond optimally to a diverse set of microorganisms, which can cause substantial tissue damage (Siloși, 2013; Golban, 2019).

The most important defense mechanism is the immune system with its key characteristics, which include its ability to act automatically in response to microbial invasion, to generate resistance and to improve the general condition of the newborn and adult body (Andrieș, 2014; Siloși, 2014; Tașbac, 2014; Golban, 2015).

At the same time, normally, the immune system, through its mechanisms, contributes to the removal or elimination of microorganisms, viruses and fungal agents that have entered the body (pathogens), contributing to the destruction of harmful internal structures, providing protection and resistance to various infections of the animal body.

Bibliographic studies confirm that a strong immune system stops the millions of microbes with which the body constantly comes into contact. For these reasons, some infections

occur in the animal body given that the immune system can be weakened by the lack of nutrients, an unbalanced diet, environmental pollution, and aging. Stress hormones secreted in excess unbalance the immune system and predispose the body to infections and other diseases (Carp-Carare, 2014).

It is important to note that immune-based defenses are not the only forms of defense. The stages of manifestation of the establishment of immunity are of interest by involving the mechanisms of synthesis of some classes of immunoglobulins. The initial period constitutes the processing of external factors by transferring them to some internal environments of the organism, these being represented by the elements of the immune system. These reports confirm the importance of foreign elements with certain peculiarities characteristic of the antigen-antibody interaction. However, the elements of the immune system present characteristics of identifying numerous groups of foreign elements with action on the immune system (Golban, 2007).

According to studies in the field of immunology, the immunological profile or immune status results from the process of complex analysis of cellular immunobiological elements, which are part of the immune system. It is made up of the sum of qualitative and quantitative data, which characterize the morphological - structural aspects and the mode of functioning of the immune system in animals in various physiological and pathological states. The values, indices and qualifiers through which the immunological profile is expressed (cellular elements, phagocytic indices) represent an aspect, which includes both the components and the set of immune effectors and mechanisms. Ultimately, through the immune status, a synthesis of the defining elements of immunological competence is achieved, of the immune system's ability to recognize disturbances of exo- or endogenous origin, to integrate or eliminate them, to develop the most appropriate replica and to maintain the state of immunostasis (Cristea, 2011).

Through immunological laboratory tests, which allow a true exploratory dissection, therefore knowledge of the immune system's response capacity, it becomes possible to assign a "grade" that appreciates these important immunological mechanisms, or a qualification to all or most of the mechanisms involved in this system. Going beyond the initial stage of analyzing isolated properties of each effector, the methodology by which the immunological profile is defined ensures the characterization of the essential pathways through which the path from the aggression of any foreign factor to its recognition and then to the response is achieved.

These processes are important through mechanisms of capture, processing and presentation of foreign antigens, cellular cooperation, achieving the balance between stimulation and suppression, cooperation at the molecular level between antigen, receptor and histocompatibility antigen and modeling the activity of the system through molecular messages. Therefore, the constitutive data of the immunological profile provide, not perfectly, a concrete image of the network of cells – with their molecular membrane structures – messages, humoral effectors and mechanisms that articulate in the immune system (Golban, 2015).

For these reasons, the main objectives of this research are to study the dynamics of immunobiological indices in cattle depending on age.

MATERIALS AND METHODS

Immunological scientific investigations were carried out in a didactic farm of cattle of different ages in different seasons. Blood samples from the investigated cattle were used for the investigations.

Blood samples were collected from the jugular vein with heparin based on the calculation of 0.3 ml of heparin per 10 ml of blood for the purpose of anticoagulation. The samples were investigated with the aim of identifying the dynamics of the number of leukocytes, erythrocytes, lymphocytes, opsonophagocytic indices.

To perform the opsonophagocytic test, 1.0 ml of blood stabilized with heparin and 0.1 ml of E. coli microbial culture suspension were used from the calculation of 1.0 ml of physiological solution per 500 ml microbial cells.

The tubes were shaken, then incubated in a thermostat for 30 minutes at T-37ºC and centrifuged at 1500 rpm.

The supernatant was removed using a Pasteur pipette. Stained smears were made by special microbiological staining methods, which were exposed with chemicals regarding the rule of performing bacterioscopic preparations. Smears made from the samples investigated concurrently were microscopically examined using a microscope.

As a result of performing immunological laboratory evaluations, the amount of bacteria involved in the mechanism of incorporation was assessed.

Simultaneously, the indicators regarding the degree of incorporation characteristic of the phagocytic process were also assessed at the same time and it was assessed after evaluating the amount of microbial germs digested by lymphocyte cells. The final determination was made taking into account the calculation of the total number of incorporated bacteria and the amount of lymphocytes present in the opsonphagocytic reaction.

RESULTS AND DISCUSSIONS

The results obtained regarding the immunelogical investigations on the immune system regarding the study of immunological aspects on the activity of some immunobiological indices in cattle of physiological groups in different periods denote important immunelogical indices.

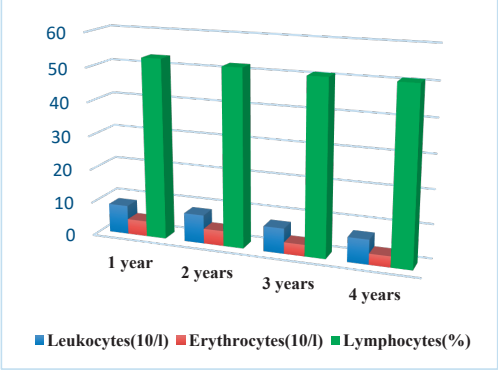

Figure 1. Evaluation of immunological indices in cattle during the winter period depending on age

The data obtained shown in Figure 1 denote variations in the level of leukocytes, erythrocytes, lymphocytes at different ages in cattle investigated in the winter period. Significant values were recorded in cattle aged 1; 2 years where the values constituted the number of leukocytes, erythrocytes and lymphocytes higher than 8.56±1.53; 4.50±0.14; 53.81±4.3 and 8.45±0.03; 4.45±0.12; 52.24±4.1 compared to the age of 3; 4 years with the values of 7.43±1.42; 3.45±0.12; 51.21±4.2 and 7.22±1.51; 3.22±0.11; 51.14±4.2.

Figure 2 reflects the dynamics of immunelogical indices in cattle during the winter period depending on age.

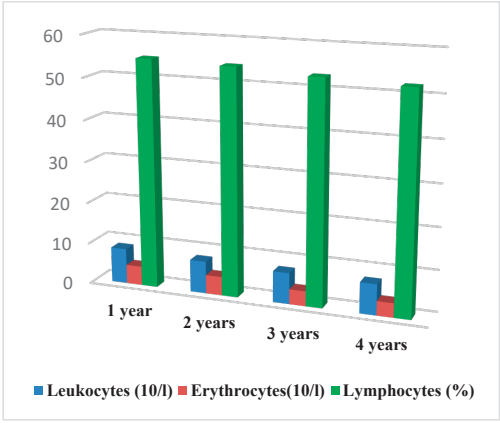

Figure 2. Evaluation of immunological indices in cattle during the spring period depending on age

It can be seen from the data of Figure 2 that leukocyte, erythrocyte and lymphocyte indices in cattle aged 1; 3 years are higher compared to cattle aged 2; 4 years, where the indices constituted values of 8.54±1.53; 4.60±0.14; 54.81±4.2/cattle aged 1 year and 7.51±0.13; 3.61±0.11 and 53.11±4.3/cattle aged 3 years compared to the immunological indices of cattle aged 2; 4 years where the indices constituted values of 7.81±0.02; 4.52±0.12; 54.21±4.1/2-year-old cattle and 7.42±0.03; 3.45±0.13 and 52.21±4.4/4-year-old cattle.

Investigations of the evaluation of immunological indices in cattle in the summer period depending on age according to Figure 3 regarding the dynamics of immunological indices in cattle in the spring period depending on age recorded higher indices of 7.92±0.05; 4.63±0.16; 54.84±4.6 and 7.81±0.02; 4.53±0.13; 55.24±4.3 at cattle aged 1 and 2, compared to cattle aged 3 and 4 where the

recorded indices confirmed values of 7.52±0.04; 3.63±0.16; 54.84±4.6 and 7.33±0.03; 3.53±0.13 and 52.24±4.3.

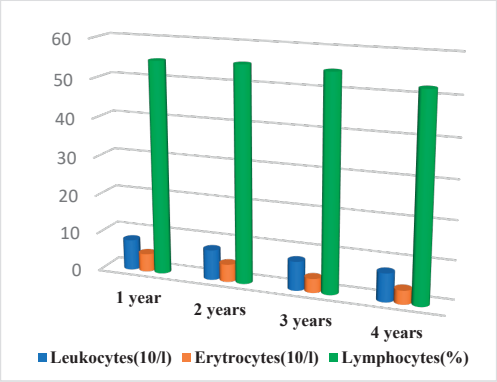

Figure 3. Evaluation of immunological indices in cattle during the summer period depending on age

The results of the investigations shown in Figure 4 regarding the evaluation of immunelogical indices in cattle in the autumn period depending on age reveal higher indices of the number of lymphocytes and leukocytes with values of 8.28±0.02; 5.12±0.15; 56.24±4.0 in 1-year-old cattle, compared to the indices in 2; 3; 4-year-old cattle, which constituted lower values.

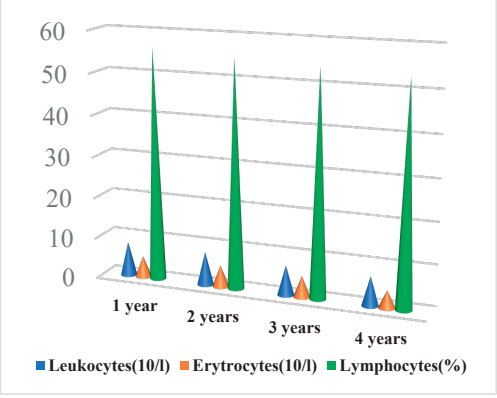

Figure 4. Evaluation of immunological indices in cattle during the autumn period depending on age

The results indicate indices of 8.12±0.01; 5.25±0.12; 55.14±3.2 in 2-year-old cattle compared to 3-year-old cattle, where the values constituted leukocyte, erythrocyte and lymphocyte indices of 7.21±0.02; 5.11±0.13 and 54.22±3.0 and a level of 7.11±0.01; 4.25±0.12; 53.24±3.1 of these indices in 4-year-old cattle. These recorded indices reveal some increases in their level in the cattle studied in different age periods, especially the concentration of leukocytes and lymphocytes, which justifies the importance of cellular and humoral resistance specific to the animal immune status.

The dynamics of phagocytic indices in cattle during the winter period depending on age regarding phagocytic activity and intensity denotes the values shown in Figure 5, where the aspects of these indicators regarding phagocytic activity constitute in animals of different ages values of 53.45±1.21 in cattle aged 1 year, being the highest, compared to the ages of 2; 3 and 4 years, where the values constituted 51.40±1.71; 50.32±1.19 and 48.40. Regarding the values of phagocytic intensity, the highest index also constituted in younger cattle aged 1 year, being 5.04±0.13; compared to cattle of other ages, the values being 4.00±0.11; 4.46±0.12; 3.20±0.11. These results demonstrate that the body's resistance gradually decreases with increasing age.

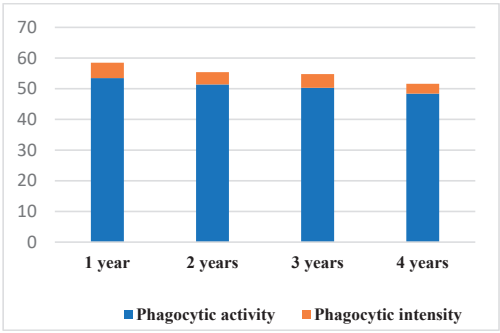

Figure 5. Dynamics of phagocytic indices in cattle during the winter period depending on age

In the spring period, the dynamics of phagocytic indices in cattle depending on age according to Figure 6, indicates that in young animals aged 1-2 years, phagocytic activity and intensity revealed indices of 54.25±1.11; 52.12±1.62 and 5.25±0.12; 5.62±0.11 compared to 3-year-old animals where the indices obtained demonstrated values of 55.25±1.11; 48.11±1.62 regarding phagocytic activity and 4.35±0.12 and 3.62±0.11 regarding phagocytic intensity values.

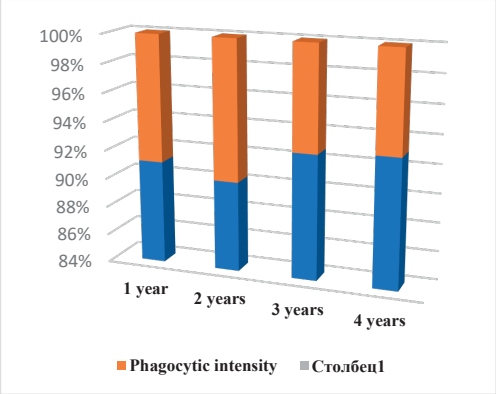

Figure 6. Dynamics of phagocytic indices in cattle during the spring period in dependence on age

The characteristic of the dynamics of phagocytic indices in cattle during the summer period depending on age is demonstrated in Figure 7, values in animals of different ages where the index of phagocytic activity and intensity confirms important characteristics of the phagocytic mechanisms that we determined by obtaining the following indices: 51.25±1.12; 49.12±1.72; 47.22±1.12; 45.32±1.72 which confirms phagocytic activity and 5.01±0.13; 4.64±0.12; 3.01±0.13; 3.64±0.12, which confirm the phagocytic intensity in animals investigated at different ages of research on the phagocytic index.

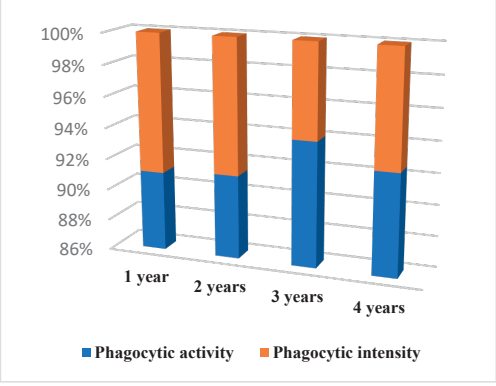

Figure 7. Dynamics of phagocytic indices in cattle during the summer period depending on age

The results of the investigations shown in Figure 8 indicate the dynamics of phagocytic indices in cattle in the autumn period depending on age, which denotes a mixing of the body's resistance in all animals studied in different age periods. The values in the animals studied regarding phagocytic activity and intensity constituted phagocytic activity indices of 48.13±1.11; 46.12±1.61; 44.11±1.11; 42.12±1.61 and phagocytic intensity indices of 3.42±0.04; 4.22±0.03; 3.40±0.04; 4.22±0.03.

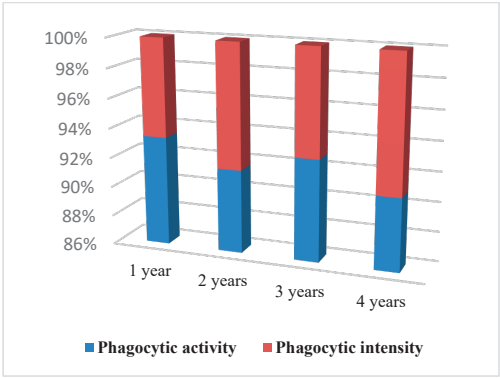

Figure 8. Dynamics of phagocytic indices in cattle during the autumn period depending on age

Therefore, as a result of the investigations carried out, immune mechanisms are essential biological mechanisms that protect living organisms from foreign invaders, distinguishing between self and non-self tissues. In this process, two categories of mechanisms with protective functions are involved: nonspecific and specific. According to some bibliographic reports, nonspecific defense is present even in primitive animals, includes multiple physical barriers (such as skin), chemical responses (such as mucus and gastric acid) and presents mechanisms of action of phagocytic cells that engulf foreign germs. Specific defense, characteristic of vertebrates, is based on the ability of the immune system to generate characteristic immune responses regarding antigenic mechanisms due to the particularities of the mechanisms of lymphocytes in the T and B categories.

T cells are involved in cell-mediated immunity, while B cells produce antibodies for humoral immunity. The adaptive nature of these immune responses allows for rapid responses upon re-encounter with pathogens, demonstrating the importance of memory cells. The immune system also plays an important role in preventing autoimmune diseases, in which the body mistakenly attacks its own cells. Research on animal immune systems has

provided insights that inform human health and therapies, particularly autoimmune infections. Understanding these mechanisms highlights the complexity and adaptability of immune responses across animal species.

Previous reports confirm that the immunological aspects of the animal body's resistance against various pathogenic germs are due to cellular immune elements. These, being involved in autoimmune reactions, present defense functions and, respectively, mechanisms for strengthening the animal body from foreign aggressors.

The immune system is important through its forms of defense determined by the elements of the immune system. The immune protection of the body constitutes the development of tolerance by imposing regulatory mechanisms.

These bibliographic assessments in recent years have become an extremely dynamic field in which research and discoveries follow each other very quickly. The qualitative and quantitative accumulations increasingly require the study of the defense mechanisms of organisms against the aggression of various agents from the external environment, recognized as antigens and/or allergens, as well as against their own modified or altered structures, and the understanding of immunological mechanisms starting with the recognition, processing and analysis of antigens, the transmission of antigenic information, the humoral and cellular immune response, as well as the implications in the production of diseases or lesions with an autoimmune mechanism are increasingly becoming elements that must be known by all those who are trained in the field of biology, especially in the biomedical field, both in human and veterinary medicine.

For these reasons, the immune response to antigen aggression can favor unfavorable consequences for the survival of animals in different age periods. In this context, the investigations carried out are important through some aspects of the activity of cellular immunity in cattle in different age periods.

CONCLUSIONS

Phagocytic cells, lymphocytes, other cellular elements constitute the essential elements of immunity in animals, which determine regulatory effects in different physiological periods.

Immunobiological indices constitute important values in different age periods in the investigated cattle, which justifies the importance of cellular resistance by establishing the body's immunity.

The study of phagocytic activity and intensity indices in different periods determined significant values, demonstrating the increase in the animal's resistance.

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MECHANISMS FOR STRENGTHENING THE ECONOMIC SECURITY OF ENTERPRISES IN THE REPUBLIC OF MOLDOVA

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Abstract

Economic security is essential for the sustainable development and competitiveness of enterprises, especially in economies facing instability and rapid digital change. In the Republic of Moldova, small and medium-sized enterprises (SMEs) encounter significant challenges such as limited access to finance, legislative uncertainty, digital risks, and weak managerial capacity. This study analyzes the main mechanisms that can strengthen the economic security of Moldovan SMEs. Using a qualitative research approach based on literature review, policy analysis, case studies, and interviews with SME managers, the study identifies key vulnerabilities and evaluates existing security mechanisms. The findings show that economic security can be enhanced through effective risk management, diversification of financial sources, adoption of digital and cybersecurity solutions, improved corporate governance and legal compliance, investment in human capital, and cooperation with public institutions. The study concludes that economic security cannot be achieved through isolated measures, but requires an integrated and systematic approach. Implementing coordinated mechanisms increases enterprise resilience to economic shocks and supports sustainable economic growth in the Republic of Moldova.

Key words: *economic security, enterprises, financing, governance, innovation, Republic of Moldova, regulation, risk management.*

INTRODUCTION

Economic security of enterprise operations largely depends on their capacity to respond in a timely and adequate manner to the economic needs and requirements of various stakeholder groups. Private interests can be considered justified only when they simultaneously contribute to achieving state objectives and maintaining economic stability (North, 1990). An analysis of enterprise economic security reveals its capacity to generate goods and services aligned with market demand through the balanced and efficient utilization of available resources, even amid conditions characterized by economic instability and uncertainty (Aghion & Howitt, 2009). Cumulative performance outcomes of reliable enterprises serve as a pivotal driver of economic development, while simultaneously advancing the material and cultural standards of living within society (Arrow, 1972). Originating from the Greek *mechanic*, denoting an "instrument" or "machine", the concept of a "mechanism" has long been associated with the totality of means through which economic processes are structured and regulated (Hodgson, 2006). In the context of contemporary economic scholarship, the term has acquired a more expansive and nuanced interpretation, encompassing the diverse methods, instruments, and regulatory levers employed by both enterprises and the state to shape and direct economic activity. Within this analytical framework, an economic mechanism represents a coherent system of coordination and governance, reflecting the capacity to organize and harmonize economic processes in a manner that sustains efficiency, resilience, and stability amid conditions of continuous change and uncertainty (Samuelson & Nordhaus, 2010). During the planned economy period, the economic mechanism was understood as a complex system of interrelated elements, including the organizational form of economic activity, management structure, as well as the totality of methods and levers of influence used in the management process. Their role was to ensure the efficient achievement of production objectives and to contribute to satisfying social, collective, and individual interests. Under contemporary economic conditions, this approach remains highly relevant, as the economic mechanism

integrates a comprehensive set of organizational, managerial, and economic instruments, indispensable for the efficient functioning and advancement of production processes and economic activities.In the planned economy system, the economic mechanism was conceived as an interdependent system of elements, including the organizational form of economic activity, management structure, and the totality of methods and instruments of influence used in the management process (Balabanov, 2008). They ensured the efficient realization of production objectives while actively advancing social, collective, and individual interests. In contemporary economies, this interpretation retains its relevance, as the economic mechanism continues to encompass the essential elements required for the efficient organization and advancement of production activities (Abalkin, 2014). Limited approaches describe the economic framework as the collection of methods, policies, and regulatory measures used by society and, in some cases, by the state to influence and guide economic processes. In contrast, broad approaches emphasize that a proper understanding of economic regulation requires consideration of the objective laws that shape economic development, thereby linking it to the core processes and structural dynamics of the economy (Kleiner, 2012). From a functional perspective, any mechanism is goal-oriented, as its operation is determined by the existence of an objective. In the absence of a clearly defined goal, a function loses its rationale. Therefore, a mechanism can be understood as a mode of action through which the necessary means are mobilized and coordinated to achieve a specific result. Consequently, contrivance and goal are interdependent, since achieving objectives requires a system of instruments and procedures through which they can be realized (Bulgakov, 2005). From a pragmatic perspective, the concept of an economic machinery cannot be equated with the economy itself. Financial objectives are set by economic agents, but pecuniary processes also include components that develop objectively, independent of individual will. At the same time, the evolution of these processes can sometimes diverge from the initially intended direction. In this context, the economy represents not only a means for achieving set objectives but also becomes the object upon which the fiscal acts, with the role of organizing and coordinating economic processes in accordance with the objectives formulated by society at a given point in time (Popescu, 2018).

MATERIALS AND METHODS

Market-based systems are often associated with a system of material incentives and management methods designed to ensure high labour productivity among employees, specialists, and other staff. However, this interpretation cannot be considered exhaustive. At the same time, an economic mechanism is defined as an “integrated, multi-layered system of forms and methods for economic governance” (Smith, 2007). From this perspective, the structure of an enterprise’s strategic approaches includes “the system of internal socioeconomic interactions, which establishes production and operational linkages between structural subdivisions, methods for assessing the impact of these linkages on overall performance, as well as subsystems of stimulation, planning, control, standardization, accounting, and analysis of economic activity” (Popescu, 2016).

Considering these perspectives, the mechanism for ensuring the economic security of enterprise operations can be understood as an integrated system composed of relatively autonomous, yet interdependent and interacting elements. The main components of this arrangement include the organizational form of production, business-related and operational relationships, the incentive system, management, planning, financing, taxation, and pricing policies. The fundamental elements of the management systems are the economic agents and the relationships they establish in the process of organizing production and economic linkages.

Material interrelations take various forms: direct and indirect, immediate or mediated, productive and unproductive, legally formalized or informal, spontaneously emerging or institutionally established. Such relationships exist both within the organizational form of production and outside

it, and they can also be classified according to where they occur: in production, exchange, distribution, or consumption. Within a specific organizational form of production, economic relations among participants are generally realized through the exchange of activities, while relations among economic agents occur through the exchange of goods and services (Ionescu, 2012).

The methodology for ensuring the developmental security of the enterprise aims to create conditions that motivate the efficient functioning of all enterprise components, while ensuring a high degree of alignment between societal, corporate, and individual interests. It is intended to guarantee economic safety both at the input and output levels of the system, creating reliable conditions for the simultaneous functioning of managerial and managed subsystems (Rusu, 2018).

The enterprise economic protection mechanism can be structured around four principal components:

- The philosophy of the concept within the enterprise;
- The functions that ensure the process of economic encryption management;
- The resources required by the machinery;
- The goal-oriented component, including the main organizational forms and policy tools necessary to ensure the enterprise's operational economic security (Popescu, 2018).

A theoretical-philosophical component constitutes the foundational pillar of the structure. The philosophy of organizational safeguarding embodies a coherent and structured system of organizational beliefs, reflecting both the collective mindset and the individual commitment of employees to carry out their responsibilities in full compliance with established security requirements. It is imperative that this philosophy be rigorously articulated, formally validated, and comprehensively communicated across all hierarchical levels of the enterprise to ensure uniform understanding and consistent implementation (Smith, 2007).

The Goal-oriented component of the financial structure reflects its structure in relation to the specific elements of the enterprise and represents an integrated set of interconnected organizational forms and economic instruments.

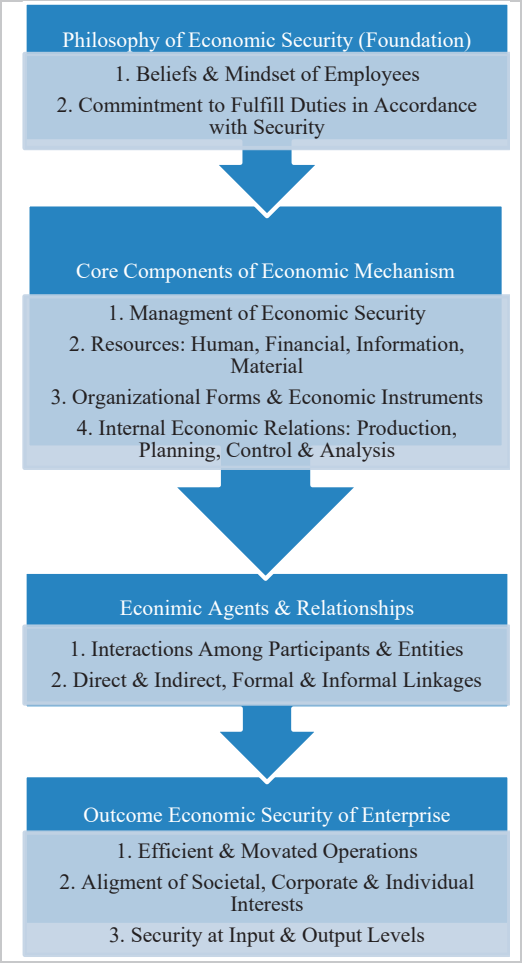

Figure1. Mechanism of enterprise economic security

These elements enable the continuous operation of the enterprise in a dynamic budgetary environment, ensuring the achievement and maintenance of planned parameters through the implementation of economic firewall procedures regulated by internal and external norms (Popescu, 2018).

The actions of the framework are aimed at:

- ensuring economic defence at the system's entry point;
- protecting the managerial subsystem;
- protecting the managed subsystem;
- guaranteeing economic security at the system's exit point.

A comprehensive support component encompasses scientific and methodological

foundations, informational resources, legal and organizational frameworks, material and technical infrastructure, human capital, and financial provisions. At its core lies a system of administrative-economic instruments through which the underlying principles of the model are translated into practical action. From an operational perspective, these administrative-economic instruments may be structured across three interconnected levels: national, regional, and enterprise-specific. At the macro and meso levels, their manifestation includes legislative frameworks, licensing regimes, quota systems, standardization requirements, regulatory limits, and supervisory controls. Direct economic instruments are represented by public procurement mechanisms, subsidy schemes, and targeted financing programs, whereas indirect instruments encompass taxation policies, fiscal incentives, deferred payment arrangements, and monetary-credit tools-such as central bank policy rates, mandatory reserve requirements, leasing arrangements, and preferential lending. Complementary to these are currency-based instruments, including exchange rate policies; customs-related measures, such as duties, tariffs, and exemptions; as well as investment-oriented instruments, including depreciation policies, reinvestment of profits, and the provision of government guarantees (Rusu, 2018). At the enterprise level, instruments include diagnostics, controlling, planning, standardization, training, sanctions, and material incentives. Controlling serves as a self-regulation tool, collecting data for decision-making, assessing deviations from planned indicators, and developing managerial recommendations. It integrates planning, managerial accounting, control, diagnostics, and information flow management, providing feedback for corrective action (Ionescu, 2012). Administrative instruments, such as regulations, norms, and authorizations, must be applied with caution in a market economy. The necessity for state intervention should be assessed based on operational efficiency, the impact on capital-related processes, and the effectiveness of fiscal policy. Interventions must be market-compatible and support efficient interaction among economic actors (Smith, 2007).

Priority in the configuration of financial instruments should be accorded to subsidies, fiscal incentives, and public investment in infrastructure, given that large-scale financial support exerts a decisive influence on the performance and resilience of the production system. Functional disruptions within the enterprise are addressed through an integrated system comprising four principal structure: reservation, protection, regulation, and compensation. The reservation process is activated in situations where deviations from the normative level of business-related preservation exceed permissible thresholds, enabling the utilization of previously unengaged resources to recalibrate operational standards and support the implementation of investment initiatives. The protection arrangement is designed to respond to minor disturbances, ensuring the conservation of resources while maintaining the enterprise within stable and acceptable operational parameters. The regulation approach entails the mobilization of supplementary resources to mitigate critical disturbances and remains operative until disrupted functional interdependencies are restored. Under such conditions, the level of trade-related cover is deemed diminished, necessitating decisive intervention by the managerial subsystem through structural adjustments and the attraction of additional resources to re-establish equilibrium (Popescu, 2018). The compensatory methodology is employed in circumstances where the existing organizational structure and its inherent homeostasis prove insufficient to withstand internal or external shocks. In such cases, substantive modifications to both structure and systemic equilibrium become imperative, achieved through the deployment of internal and external resources, including unconventional solutions. These interventions are indicative of managerial initiative, which itself constitutes a strategic resource capable of transforming vulnerable activities into more stable and secure ones (Popescu, 2018). Under such circumstances, enterprises typically require comprehensive structural reorganization to restore stability and ensure sustainable functioning.

Situational management is implemented within the regulatory operational scheme, whereby each known state corresponds to an action that restores the enterprise's financial and capitalist activities to a normal level. Within the compensatory governance structure, initiative and creativity are utilized as resources to generate efficient combinations of inputs aimed at satisfying previously unmet needs that caused the initial problem (Rusu, 2018).

Information and advisory support measures should be applied more broadly. For instance, in certain cases, public opinion needs to be mobilized via mass media, and objectives must be aligned with the means of achieving them through economic-political set of measures. A qualitatively new form of financial intermediation, which reduces risk and uncertainty, is the provision of information to investors. This is based on consulting and marketing services, investor participation in asset management, or the creation of collective investment systems (Smith, 2007).

Enterprise economic stability cannot be ensured without the extensive reproduction of all components of fixed capital. This process should be reflected in the continuous enhancement of technical, energy, and informational capabilities of production, the modernization of equipment across the entire industrial sector, and the establishment of a stable basis for intensive extended reproduction (Popescu, 2018).

The extended reproduction of fixed capital and its proportions exerts a long-term effect on all aspects of market enterprise operations, forming the foundation for future development. A critical condition for extended reproduction of fixed capital is the implementation of substantial capital investments, alongside the optimization of existing equipment utilization. A key quantitative indicator of extended reproduction is the value of capital investments relative to accumulated depreciation, since depreciation alone permits only simple reproduction (Rusu, 2018).

The main characteristics of the coordination system defining directions for enterprise improvement, aimed at satisfying the interests of all social strata, include: creating a favourable environment for enterprise and entrepreneurial development through stable legislation, monitoring key economic indicators, ensuring equality among forms of ownership, prioritizing those that enhance production efficiency and living standards, and developing production oriented toward processing agricultural products and local raw materials (Popescu, 2018). Accordingly, the procedure for strengthening enterprise financial stability is oriented toward attaining optimal operational parameters, safeguarding production capacities and human resource potential, and fostering a market-oriented enterprise that effectively integrates entrepreneurial initiative with state regulatory frameworks, while ensuring the conditions required for secure and stable functioning. A systematic analysis of this pattern, grounded in rigorous scientific principles and applied theoretical approaches, underscores its inherent complexity and the imperative of selecting and calibrating specific instruments in accordance with prevailing operational conditions, the stage of financial and cost-efficient development, and the existing level of economic assurance within the enterprise (Ionescu, 2012 & Smith, 2007).

RESULTS AND DISCUSSIONS

The research findings demonstrate that enterprise economic protection in the Republic of Moldova is influenced by a complex interaction of internal and external factors, including financial stability, managerial capacity, institutional support, technological adaptation, and the overall practical environment. The study confirms that economic resilience represents not only a defensive configuration against risks, but also an essential strategic component for ensuring sustainable enterprise development and competitiveness.

One of the main results of the research is the identification of the multidimensional structure of the economic safeguarding network. The analysis revealed that enterprises with integrated management systems combining financial planning, operational control, strategic risk management, and digital reliability instruments exhibit a higher capacity to adapt to economic uncertainty and market instability. This finding is consistent with

previous studies emphasizing the role of systemic governance and organizational flexibility in strengthening enterprise resilience (North, 1990).

The study highlights that the effectiveness of financial defence platform depends significantly on the quality of managerial decision-making. Enterprises characterized by proactive leadership, strategic planning, and continuous monitoring of economic indicators demonstrated greater operational stability and a reduced vulnerability to external shocks. These results support the theoretical approaches proposed by Stiglitz and Samuelson & Nordhaus, according to which institutional efficiency and adaptive management are fundamental determinants of economic sustainability.

An important trend identified through the research concerns the increasing role of digital transformation in strengthening enterprise economic security. The implementation of digital technologies, cybersecurity systems, enterprise resource planning platforms, and information management tools contributes substantially to operational efficiency, financial transparency, and risk mitigation. At the same time, the findings indicate that digitalization also generates new categories of risks, including cyber threats, informational vulnerabilities, and technological dependency. Consequently, enterprises are required to integrate cybersecurity strategies into their overall economic permanence frameworks.

The analysis further revealed that enterprises with diversified financing sources demonstrate a significantly higher degree of economic resilience. Organizations relying exclusively on limited financial resources or unstable credit toolkit are considerably more vulnerable to market fluctuations and wealth crises. In contrast, enterprises benefiting from investment diversification, public support programs, and strategic partnerships showed improved capacity to maintain operational continuity and financial stability.

The research also confirms the strategic importance of human capital in ensuring economic security. Enterprises investing in continuous employee training, managerial development, and organizational culture exhibited higher adaptability and improved crisis-management capacity. These findings correspond to modern theories of organizational resilience, which emphasize the role of knowledge, innovation, and human competencies in maintaining sustainable economic performance.

Another relevant result concerns the role of state regulation and institutional support configuration. The study demonstrates that stable legislation, transparent fiscal policies, investment incentives, and effective public-private cooperation contribute significantly to strengthening enterprise income stability. However, the research also identified several structural challenges specific to the Republic of Moldova, including bureaucratic inefficiency, legislative instability, limited access to financing, and insufficient technological infrastructure. These constraints negatively affect the capacity of enterprises-particularly SMEs-to implement advanced stable security process.

The comparative analysis with international studies indicates that Moldovan enterprises face vulnerabilities similar to those observed in other transition economies, especially regarding access to capital, digital adaptation, and institutional support. Nevertheless, the research suggests that enterprises capable of integrating innovation, strategic planning, and flexible organizational structures are more likely to achieve long-term stability and competitiveness.

The study additionally revealed that the method of protection, regulation, reserve management, and compensation function as interconnected components within enterprise precaution systems. The protection apparatus ensures the preservation of operational stability under moderate disturbances, while the regulation model facilitates adaptive intervention during periods of increased instability. The reserve mechanism allows the mobilization of additional resources under crisis conditions, whereas the compensatory strategy supports organizational restructuring and strategic transformation when traditional structures become ineffective.

The results further emphasize that economic solidity should not be interpreted exclusively through financial indicators. Instead, it represents a broader organizational capacity

involving institutional stability, technological modernization, strategic governance, informational security, innovation potential, and social adaptability. This multidimensional perspective reflects contemporary approaches to enterprise management under conditions of globalization and digital transformation.

Overall, the findings demonstrate that strengthening enterprise economic security in the Republic of Moldova requires the implementation of integrated managerial, financial, technological, and institutional mechanisms capable of ensuring organizational resilience and sustainable profit-oriented development in a continuously changing environment.

CONCLUSIONS

The in-depth analysis of the engine for ensuring and strengthening the prosperity of enterprises in the Republic of Moldova highlights that it represents a complex, multidimensional, and continuous process, which cannot be reduced to the application of isolated instruments or to ad hoc responses to risks. Under current conditions, characterized by macroeconomic instability, accelerated globalization, and digital transformation, material surveillance becomes not merely a component of organizational management, but a fundamental strategic pillar of sustainable enterprise development, particularly for small and medium-sized enterprises (SMEs).

The research findings confirm that the mechanism of social certainty should be conceptualized as an integrated system composed of interdependent components-organizational, financial, informational, legal, and managerial-which operate in a complementary relationship. This systemic approach enables enterprises to anticipate, prevent, and effectively manage risks, thereby ensuring operational stability and long-term competitiveness. In this context, the role of management becomes essential, as decision-making capacity, organizational flexibility, and institutional culture directly influence the level of sustainable prosperity.

An important aspect highlighted by the research is the significance of functional mechanisms-protection, regulation, compensation, and reserve-which contribute to maintaining the internal balance of the enterprise. These tools allow for rapid adaptation to internal and external disturbances, reduction of vulnerabilities, and capitalization on emerging opportunities. In particular, the compensatory system underscores the role of innovation and managerial initiative as strategic resources for transforming critical situations into competitive advantages.

Furthermore, the study emphasizes the decisive role of economic-financial and administrative instruments applied at the macro-, meso-, and microeconomic levels. Fiscal policies, investment incentives, access to finance, government support programs, and a stable legislative framework represent essential external factors influencing enterprise resource steadiness. In the Republic of Moldova, these instruments require improved coordination and better alignment with the real needs of the business environment, especially for SMEs, which are more vulnerable to revenue-generating shocks and have limited resources.

Another relevant outcome of the study is the identification of the role of digital transformation in strengthening economic self-sufficiency. The digitalization of processes, the use of advanced information systems, the implementation of cybersecurity solutions, and the development of digital competencies significantly contribute to reducing operational risks and increasing efficiency. At the same time, digitalization generates new challenges, such as cyber risks, technological dependency, and the need for data protection, which necessitate the integration of information policing into the overall strong financial foundation.

An essential element highlighted is the need for an integrated approach between the private and public sectors. Cooperation between enterprises and state institutions, access to information, decision-making transparency, and public-private partnerships contribute to the creation of a stable and predictable economic environment. In the Republic of Moldova, strengthening these relationships represents a strategic direction for enhancing financial balance at the national level.

Based on the obtained results, several major directions for future research can be outlined:

First, there is a need to deepen the analysis of the impact of digitalization on the economic screening of SMEs. Future research could focus

on developing econometric models to quantify the relationship between the level of digitalization and economic resilience, as well as identifying the most effective technologies for risk mitigation.

Second, it is necessary to study setups for managing emerging risks, particularly those associated with climate change, geopolitical instability, and global economic crises. Integrating these factors into economic trust methods would contribute to the development of more robust and adaptive strategies.

Another important direction involves the international comparative analysis of economic security operations. Examining best practices from other countries could provide applicable solutions for the Republic of Moldova, contributing to the improvement of public policies and organizational strategies.

Additionally, future research should focus on developing synthetic indicators for assessing economic security at the enterprise level. The creation of standardized measurement tools would facilitate performance monitoring and strategic decision-making.

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THE CHALLENGE OF COMPARING TART CHERRY POLYPHENOLS FROM THE USA AND EUROPE

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Abstract

Tart cherries (Prunus cerasus L.) are globally recognized as a substantial source of polyphenolic compounds with associated bioactive effects. However, direct comparison of the published data is challenging due to differences in cultivar (e.g., US Montmorency vs. European Oblačinska, Łutówka, Újfehértói Fürtös, etc.), environmental conditions, processing methods, storage conditions, and analytical methods. Our recent study investigated five different forms of a single cultivar, Montmorency, using a TSQ triple quadrupole LC-MS/MS with authentic external standards, with the results were reported as ppm dry weight (DW) for precise cross-comparison. The employment of a single cultivar and advanced triple quadrupole LC-MS/MS techniques facilitated the sensitive detection and precise quantification of both major and minor polyphenols. European studies typically report polyphenolic data on a fresh weight (FW) basis and use less selective HPLC-UV/DAD, UHPLC-PDA, and spectroscopic assays. European data are highly variable and intended only for contextual comparison, as they reflect results from multiple cultivars and studies. For example, our work indicated that the Montmorency variety contained a balanced profile of cyanidin-3-rutinoside and cyanidin-3-glucoside, whereas European cultivars, such as Oblačinska, are typically characterized by a predominance of cyanidin-3-glucosylrutinoside. These findings will underscore the recommendation for standardized analytical methods and reporting units to enhance cross-regional comparisons to support future nutritional and clinical research.

Key words*: anthocyanins, polyphenols, Prunus cerasus, sour cherry, tart cherry.*

INTRODUCTION

Tart cherries, aka sour cherries, are rich in polyphenolic compounds, mainly anthocyanins, flavonols, hydroxycinnamic acids, and flavan-3-ols, which have been associated with numerous health benefits, such as antioxidative effects, anti-inflammatory effects, and modulation of cardiovascular and metabolic biomarkers (Jawad et al., 2025a; Kelley et al., 2018). Despite these promising health benefits, cross-study comparisons of polyphenolic composition are complicated by several factors.

Cultivar selection is a crucial determinant of polyphenolic composition, with US Montmorency cherries demonstrating a balanced profile of cyanidin-3-rutinoside and cyanidin-3-glucoside, while European cultivars such as Oblačinska, Łutówka, or Újfehértói Fürtös often show predominance of cyanidin-3-glucosylrutinoside and more variable minor phenolic content (Borowy et al., 2018; Nemes et al., 2018; Tešovic et al., 1996).

Environmental factors such as soil composition, sunlight exposure, rainfall, and cultivation practices significantly influence polyphenol biosynthesis (Zagoskina et al., 2023). Processing and storage methods, such as cold pressing, drying, thermal processing, or long-term storage, can significantly impact polyphenol stability (Sejbuk et al., 2024). Furthermore, there are differences in analytical methods and the reporting units between studies from the USA and Europe.

In the USA, a study reported the tart cherry polyphenol composition in dry weight (DW) values, combined with highly sensitive LC-MS/MS (Jawad et al., 2025b), while numerous European studies reported the tart cherry polyphenolic composition in fresh weight (FW) values using HPLC-UV/DAD, UPLC-PDA, or spectrophotometric assays (Mitic et al., 2012; Repajić et al., 2015; Sokół-Łętowska et al., 2020; Wojdyło et al., 2014), which may under-represent minor compounds or may produce variable results.

These differences are not superficial; they directly influence the estimation of polyphenolic doses in tart cherry clinical trials and the reproducibility of the research findings.

Tart cherry supplementation is renowned for its clinical applications, particularly in reducing inflammation, mitigating oxidative stress, enhancing sleep quality, and exhibiting anti-cancer and neuroprotective effects (Jawad et al., 2025a). However, the published data are equivocal. One possible reason behind the inconsistent outcomes is the variability in the reported polyphenol composition of tart cherry interventions. Numerous published trials do not report the complete phenolic profile of the products used, making it difficult to determine whether negative or inconsistent outcomes result from insufficient polyphenol dosing. For example, studies that utilize juice concentrates often operate under the assumption that different cultivars or products contain equivalent doses of anthocyanins. However, the anthocyanin content can vary significantly by more than ten times, depending on factors such as methodology, cultivar, and the basis for reporting. As clinical trial designs increasingly depend on precise polyphenolic dosing, the need for standardized reporting practices has become critical.

The main objective of this conference paper is to recommend the standardization of reporting units, analytical techniques, methodologies, and cultivar selection to improve reproducibility, reliability, and clinical relevance. It is crucial to standardize reporting units, use sensitive and comprehensive analytical methods, and clearly document cultivar and processing conditions.

MATERIALS AND METHODS

This conference paper is based on our previously published analyses of five forms of Montmorency tart cherry products: freeze-dried powder, frozen whole fruit, sweet-dried fruit, unsweetened dried fruit, and juice concentrate. The results were reported on a ppm DW basis to account for variations in water content and to facilitate accurate comparisons between different products (Jawad et al., 2025b).

Different European studies were reviewed to provide a comparison of polyphenol concentration across different cultivars. The differences in reporting units and analytical methods employed are highlighted.

RESULTS AND DISCUSSIONS

Detailed information on the polyphenolic profiles of US Montmorency tart cherries (DW) compared with European tart cherry cultivars (FW, converted to DW assuming 88% moisture) is presented in Table 1. This conversion serves as a contextual approximation to facilitate side-by-side comparison with US Montmorency tart cherry data. The differences observed highlight the challenges involved in comparing results across different studies. Variability can arise from several factors, including the genetics of the cultivars, growing environment conditions, processing and storage techniques, as well as analytical and data reporting units.

Table 1. Polyphenolic Profile Comparison; Montmorency Tart Cherry (MTC) vs European Tart Cherry Cultivars

Class	Specific Polyphenol	[1]MTC (Raw Fruit ppm, DW)	[2]European Cultivars (Raw Fruit ppm, DW)	% Difference (European Cultivars & MTC)
Antho-cyanins	Cyanidin-3-rutinoside	1667	10032.5	502%
	Cyanidin-3-glucoside	981	585.8	- 40%
	Cyanidin-3-glucosylrutinoside	ND	7994.14	
	Cyanidin-3-sophoroside	9930	870	- 91%
	Peonidin-3-rutinoside	2630	ND	
Flavonol	Kaempferol-3-rutinoside	149	661.7	344%
	Rutin (quercetin-3-O-rutinoside)	15,940	635.8	-96%
	Quercetin-3-glucoside	299	16.7	-94%
Hydroxycin-namic acid	Chlorogenic acid	29,392	5027.5	-83%
	Feruloquin-ic acid	8087	ND	
	Ferulic acid	3067	1666.7	-46%
	p-Coumaric acid	1750	4224.2	141%
	o-Coumaric acid	1759	ND	
	Caffeic acid	169	ND	
	Caffeic acid glycoside	453	ND	
Flavanols	(-)-Epicatechin	2614	2351.7	-10%
	Catechin	2157	ND	
	Procyanidin B2	1291	2629.2	104%
	Taxifolin	43	ND	
Flavanones	Naringenin	255	ND	
Hydroxyben-zoic acid	Protocatec-huic acid	102	ND	

[1](Jawad et al., 2025b)

[2](Sokół-Łętowska et al., 2020) (Głowacka et al., 2020)

NOTES: MTC = Montmorency tart cherries; ND = Not detected; This data should be considered contextual, not equivalent, because mg/100 g FW was converted into DW ppm by considering 88% moisture for the sake of concentration comparison; MTC study used TSQ triple quadrupole LC-MS/MS; European studies used HPLC-PDA analytical techniques.

In our study, Montmorency tart cherries exhibited a diverse and balanced polyphenolic profile. Major anthocyanins included cyanidin-3-rutinoside and cyanidin-3-glucoside (Jawad et al., 2025b), whereas European cultivars frequently showed a predominance of cyanidin-3-glucosylrutinoside (Repajić et al., 2015). Flavonols, such as quercetin and kaempferol derivatives, were consistently detected in Montmorency tart cherry products (Jawad et al., 2025b). In contrast, European studies revealed high variability, likely due to both cultivar differences and analytical sensitivity limitations (Głowacka et al., 2020; Sokół-Łętowska et al., 2020). Hydroxycinnamic acids, including chlorogenic and caffeic acids, etc., were quantified in Montmorency samples (Jawad et al., 2025b); however, European Fruit-Based literature often reported lower or variable levels. Differences in reporting units and analytical sensitivity have significant implications for clinical research, as they can lead to misleading estimates of the actual polyphenol dose administered in intervention studies. For example, two studies that report the same volume of tart cherry juice may yield significantly different amounts of polyphenol compounds, depending on whether the measurements are presented in DW or FW. Addressing these methodological issues is essential. Reporting polyphenol content on a dry weight basis eliminates variability caused by water content, enabling more precise comparisons across studies.

Using an advanced LC-MS/MS analytical approach with authentic standards enables sensitive detection of both major and minor polyphenols, capturing biologically relevant compounds that might otherwise be overlooked. Transparent reporting of cultivar, processing, and storage conditions further strengthens reproducibility and allows researchers to accurately interpret the relationship between polyphenol intake and clinical outcomes. Finally, coordination of the sample extraction process, analytical quantification, and reporting protocols across research groups, including cross-regional collaboration, which is essential to improve comparability and translational relevance.

By implementing standardized practices, future research can more effectively connect the polyphenolic profiles of tart cherries to health outcomes in humans. This will ensure that interventions provide consistent and biologically relevant doses. These improvements are especially crucial for clinical trials focused on inflammation, oxidative stress.

CONCLUSIONS

Differences in tart cherry cultivars, environmental conditions, processing methods, storage techniques, analytical methods, and reporting units make it challenging to compare studies on tart cherry polyphenols. Montmorency tart cherries offer a consistent and reproducible polyphenolic profile, which includes anthocyanins, flavonols, hydroxycinnamic acids, flavan-3-ols, and minor phenolics. In contrast, studies conducted in Europe often report limited or variable profiles due to differences in methodology and cherry cultivars used. To enhance reproducibility, reliability, and the relevance of findings, it is essential to standardize the reporting units (DW), use sensitive and comprehensive analytical methods (LC-MS/MS or UHPLC-MS), and clearly document the cultivars and processing conditions. By addressing these issues, researchers can improve the interpretation of clinical outcomes and develop evidence-based dietary recommendations for tart cherry consumption.

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AUTHENTICATION OF FOOD PRODUCTS IN ROMANIA: PUBLIC CROSS-BORDER VISIBILITY, DOCUMENTED CAPABILITIES AND EUROPEAN CONTROL CONTEXT

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Abstract

Food authentication is a core component of official controls, consumer protection and fair trading in the European Union. This paper reassesses food authentication in Romania through a documentary case-study design and a comparative European perspective, using only public cross-border visibility signals, publicly documented institutional capabilities and peer-reviewed analytical studies. The analysis is explicitly not a prevalence assessment and does not rank national control performance. European evidence was structured through the Alert and Cooperation Network, monthly agri-food fraud-suspicion reports, EU legal instruments and Joint Research Centre materials, while Romanian evidence was interpreted as public visibility in cross-border reports and capabilities indicated by public documents. The results separate European patterns from Romanian cases and show that honey, meat, fish and dairy products remain analytically relevant matrices because they combine vulnerability to misdescription, substitution, adulteration or traceability failure with a need for validated laboratory and documentary evidence. The revised framework highlights the importance of Regulation (EU) 2017/625, Implementing Regulation (EU) 2019/1715, Directive 2001/110/EC, EN 17972:2024 and PRISMA-based reporting transparency for a publishable assessment of Romanian food authentication within the EU control architecture.

Key words*: Alert and Cooperation Network, food authentication, food fraud, Romania, traceability.*

INTRODUCTION

Food authenticity concerns the agreement between the characteristics of a food product and the explicit or implicit claims attached to it. In the European regulatory environment, authenticity problems are linked to official controls, traceability, consumer information and the prevention of deceptive practices. EN 17972:2024 is especially relevant because it standardises concepts, terms and definitions used for food authenticity and food fraud, thereby reducing ambiguity in the description of non-authentic products and fraudulent practices (CEN, 2024).

At EU level, the legal and operational framework is anchored in Regulation (EU) 2017/625 on official controls and other official activities, and in Commission Implementing Regulation (EU) 2019/1715, which establishes the functioning of the Information Management System for Official Controls and its components, including iRASFF, RASFF, AAC, the Food Fraud Network and TRACES (European Parliament and Council, 2017; European Commission, 2019). For honey, Directive 2001/110/EC remains a central compositional and labelling reference because it defines honey and composition criteria, including the absence of added ingredients (Council of the European Union, 2001). In practice, honey adulteration may involve the addition of sugar syrups and other undeclared substances intended to increase volume or modify composition. Common adulterants include glucose syrup, invert syrup, high-fructose corn syrup, rice syrup, beet syrup, and cane sugar syrup. Besides the direct addition of foreign substances, indirect adulteration methods may also occur, such as overfeeding bees with industrial sugar syrups during nectar flow periods, which can alter the natural composition of the honey while making adulteration more difficult to detect. Other indirect practices include excessive heating or improper storage conditions that modify

enzymatic activity, hydroxymethylfurfural (HMF) levels, and other quality parameters associated with authentic honey (Zhang et al., 2023).

Romania is assessed in this article only through two cautious categories: public cross-border visibility and capabilities indicated by public documents. Therefore, references to Romanian cases do not represent estimates of national fraud prevalence, market incidence, enforcement performance or exhaustive system capacity. They indicate only that Romanian operators, products, destinations or authorities were visible in selected public EU cross-border information flows, or that public institutional documents describe analytical and control capabilities.

The objective of the review paper (Figure 1) is to identify how Romanian food authentication can be positioned within the EU control architecture without over-interpreting public datasets. The paper therefore distinguishes: European-level patterns of agri-food fraud suspicion; Romanian public cross-border visibility; analytical matrices and methods relevant to authentication; and the governance link between laboratory evidence, documentary controls and digital traceability.

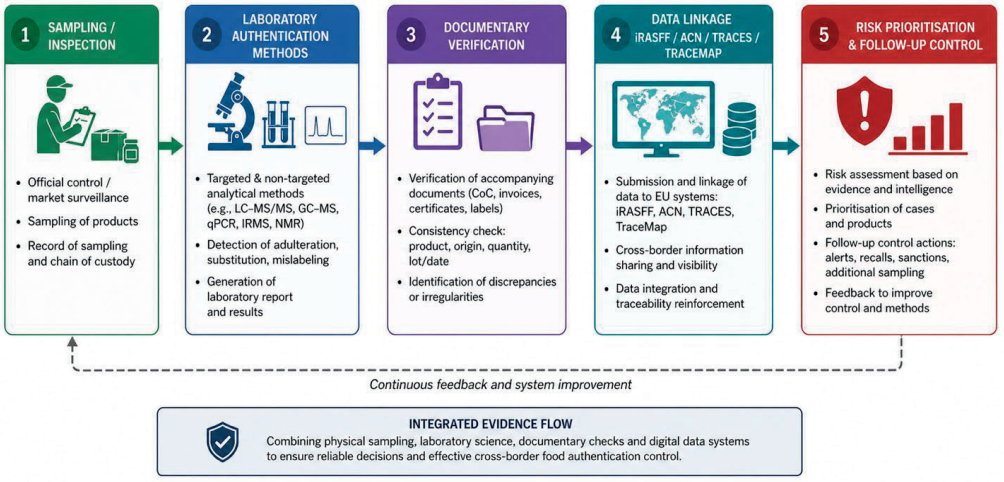

Figure 1. Objective and interpretive limits of the study

MATERIALS AND METHODS

The study used a documentary case-study and structured review design. The evidence base consisted of EU legislation and standards, European Commission and Joint Research Centre documents, ACN/FFN public reports, EFSA materials on traceability-data standardisation, Romanian public institutional documents and peer-reviewed papers on analytical food authentication. The method was revised to follow PRISMA 2020 and PRISMA-S reporting principles for transparency of search strategy, screening and inclusion (Page et al., 2021; Rethlefsen et al., 2021).

The last search was conducted on 26 April 2026. Searches were performed in EUR-Lex, European Commission Food Safety webpages, the Publications Office of the European Union, JRC publications, EFSA webpages, ANSVSA public materials, Scopus, Web of Science, PubMed and Google Scholar. The exact search strings, eligibility criteria and PRISMA counts are PRISMA counts used for the revised documentary corpus: records identified from databases and public sources, n = 86; duplicate records removed, n = 12; records screened, n = 74; records excluded at title/abstract or document-title level, n = 37; reports sought for retrieval, n = 37; reports not retrieved, n = 3; reports assessed for eligibility, n = 34; reports excluded after full assessment, n = 13; documents included in the qualitative synthesis, n = 21. The included corpus comprised EU legislation/standards and institutional documents, n = 14, and peer-reviewed analytical studies or methodological reporting guidelines, n = 7. The structure of the

documentary corpus and its analytical role are summarised in Table 1.

Eligibility was limited to documents that could support one of four analytical functions: legal framing; European public cross-border notification patterns; Romanian public cross-border visibility or publicly documented capabilities; and matrix-specific analytical interpretation. Publications were excluded when they discussed food fraud generically without relevance to authentication evidence, lacked a public source, or could not be linked to one of the selected product groups: honey, meat, fish/seafood and dairy products.

The analysis separated results from interpretation. Results were reported in two distinct blocks: European patterns and Romanian public cross-border visibility. Discussion then interpreted the analytical implications of the documentary evidence, with explicit limits on inference. ACN values were treated as public notification counts and visibility indicators, not as direct measures of national risk, prevalence, enforcement quality or market integrity.

Table 1. Documentary corpus and analytical role

Source group	Exact source examples	Role in analysis	Limitation
EU legislation and standards	Regulation (EC) No 178/2002; Regulation (EU) 2017/625 on official controls; the CEN/CENELEC framework for food-sector standardisation	Provides the legal basis for official controls, traceability, integrity, and the use of analytical evidence in conformity assessment	It does not always provide product-specific authenticity thresholds and does not replace analytical validation on real matrices
ACN/FFN	The European Commission's monthly reports on agri-food fraud suspicions; the ACN network, including RASFF, AAC, and FFN	Indicates fraud typologies, vulnerable sectors, and cross-border trends; useful for justifying the choice of the analytical domain	It covers only cross-border cases communicated through the network and may omit purely national irregularities
JRC/EFSA	The JRC page on food authenticity and quality; JRC materials on reference materials; the report on the suitability of analytical methods for assessing food authenticity	Supports methodological selection, choice of reference materials, and assessment of whether methods are suitable for screening or confirmation	These are mainly guidance and methodology documents, so they must be complemented by validation on local products and contexts
Romanian public documents	ANSVSA legislative pages; Law No. 150/2004 on food safety; orders and public documents on control, traceability, and supervision	Anchors the analysis in the Romanian institutional and regulatory framework, including control responsibilities and national implementation	They are dispersed across institutions and are sometimes more procedural than analytical; updating may be uneven
Peer-reviewed analytical studies	Studies on food authenticity assessment, analytical methods, reference materials, and Romanian consumer contexts	Provides empirical support for method performance and for scientific contextualisation of fraud and authenticity	Results may vary significantly across products, laboratories, and experimental designs; transferability is not always direct

RESULTS AND DISCUSSIONS

European patterns in public fraud-suspicion data

European public fraud-suspicion data show that authenticity-related non-compliances are structurally relevant across the agri-food chain. The ACN Overview 2023 reported categories such as grey-market issues, misdescription, mislabelling, misbranding, adulteration, product tampering, document forgery and counterfeit (European Commission, 2024a). Monthly agri-food fraud-suspicion reports for 2024-2026 continued to show that public cases are distributed across multiple product groups, including fruits and vegetables, supplements, cereals, honey, meat, fish and dairy products (European Commission, 2024b, 2025a, 2025b, 2025c, 2026a) (Table 2).

The European pattern should be understood as visibility in public cross-border suspicion reporting. It does not provide full market prevalence and does not include all domestic cases, private findings, unresolved investigations or non-public enforcement information. Within this limit, the public data are useful for identifying recurrent vulnerabilities and for benchmarking the kinds of evidence needed for authentication.

Table 2. ACN notification counts (2024) as visibility indicators rather than measures of national risk

Member State	ACN notifications in 2024	Source	Indicator definition	Interpretive note
Germany	1,907	European Commission, 2025d, *Alert and Cooperation Network Annual Report 2024* europa+1	Public notification/visibility count in the ACN for 2024 europa+1	High visibility in the ACN does not equal highest national risk; it may also reflect stronger reporting, control intensity, and cross-border trade exposure food.europa+1
Netherlands	1,155	European Commission, 2025d, *Alert and Cooperation Network Annual Report 2024* europa+1	Public notification/visibility count in the ACN for 2024 europa+1	A high count reflects visibility in the network, not an exhaustive measure of fraud prevalence or enforcement performance food.europa+1
Italy	965	European Commission, 2025d, *Alert and Cooperation Network Annual Report 2024* europa+1	Public notification/visibility count in the ACN for 2024 europa+1	Comparable only as a network-based visibility indicator, not as a direct national prevalence rate food.europa+1
Belgium	863	European Commission, 2025d, *Alert and Cooperation Network Annual Report 2024* europa+1	Public notification/visibility count in the ACN for 2024 europa+1	Cross-country comparisons should be read cautiously because notification levels depend on trade routes and control/reporting practices food.europa+1
France	742	European Commission, 2025d, *Alert and Cooperation Network Annual Report 2024* europa+1	Public notification/visibility count in the ACN for 2024 europa+1	A lower count than the top-ranked states does not necessarily mean lower underlying risk food.europa+1
Poland	689	European Commission, 2025d, *Alert and Cooperation Network Annual Report 2024* europa+1	Public notification/visibility count in the ACN for 2024 europa+1	The indicator reflects publicly visible notifications in the ACN, not the full burden of control issues food.europa+1
Spain	671	European Commission, 2025d, *Alert and Cooperation Network Annual Report 2024* europa+1	Public notification/visibility count in the ACN for 2024 europa+1	Useful for visibility ranking, but not for estimating market-wide fraud prevalence food.europa+1
Romania	<261 / not listed in public Top 10	European Commission, 2025d, *Alert and Cooperation Network Annual Report 2024* europa+1	Public notification/visibility count in the ACN for 2024 europa+1	Romania is not listed in the public Top 10, so the available public figure is below the threshold of that ranking; this should not be interpreted as zero or negligible risk europa+2

Romanian public cross-border visibility

Romanian public cross-border visibility appears in selected EU monthly fraud-suspicion reports through product-specific cases. These include lamb meat associated with document forgery, whole lamb carcasses without health marks, seafood cases involving water addition or lower net weight, and crackers associated with a traceability defect involving an unauthorised operator (European Commission, 2025a, 2025b, 2025c, 2026a).

These Romanian examples are not treated as prevalence estimates. They indicate that Romania can appear as country of origin, destination or notifying country in public cross-border reporting (Table 3). The analytical implication is that Romanian authentication work should prioritise evidence chains linking laboratory testing, documentation and digital traceability, especially for product groups where EU reports and scientific studies indicate recurrent vulnerabilities. At the same time, these cases illustrate the operational relevance of integrating compositional analysis with administrative and traceability controls, as cross-border notifications often reflect combined analytical and documentary non-compliances rather than isolated laboratory findings.

Table 3. Romanian cross-border visibility in ACN/FFN reports: selected 2024 cases and interpretive limits

Product/category	Month/year and source report	Romanian role	Reported issue	Interpretive limit
Feed materials: sunflower and rapeseed oils (feed)	March 2024, Monthly report on EU Agri-Food Fraud suspicions studocu	Originating country; notified by the Netherlands studocu	Fluazifop-P residues not compliant with EU MRLs; one case also reported orthophenylphenol studocu	This is a public cross-border visibility case, not a direct estimate of the full Romanian feed-control burden food.europa+1
Feed materials: sunflower and rapeseed oils (feed)	March 2024, Monthly report on EU Agri-Food Fraud suspicions studocu	Originating country; notified by the Netherlands studocu	Repeated non-compliant pesticide-residue findings linked to the same commodity group studocu	The report aggregates non-compliances with fraud suspicions and does not establish final fraud confirmation food.europa+1
Fruits and other food categories under ACN visibility	2024 monthly reports, ACN/FFN public reports food.europa+2	Romania appears as origin or involved Member State in cross-border notifications food.europa+2	Cross-border non-compliances with fraud suspicions shared through ACN components food.europa	The monthly reports exclude purely national cases and therefore underrepresent non-shared domestic incidents food.europa

Analytical interpretation by matrix and fraud type

Honey remains a demanding authentication matrix because adulteration can involve sugar syrups designed to mimic genuine honey. The EU coordinated action From the Hives highlighted the need for advanced methods such as EA/LC-IRMS, LC-HRMS and NMR because conventional compositional criteria may be insufficient in complex adulteration scenarios (European Commission, Joint Research Centre, 2023a). Romanian honey studies based on comprehensive profiling and UV-Vis spectroscopy coupled with chemometrics indicate relevant national analytical research, but these studies should be interpreted as matrix-specific scientific evidence rather than national prevalence estimates (Geană and Ciucure, 2020; Geană et al., 2024). These analytical characteristics and associated fraud risks are positioned within the broader matrix-fraud typology presented in Figure 2.

Meat and fish vulnerabilities are linked to substitution, misdescription, documentary irregularities and compositional discrepancies. DNA barcoding and PCR-based approaches can support species authentication, while HPLC-UV fingerprinting coupled with chemometrics is also relevant for meat-species and quality-attribute authentication. Documentary and traceability evidence remain necessary when the issue concerns origin, health marks, operator identity or net weight (Popa et al., 2017; Santomá-Martí et al., 2025; Food Safety Authority of Ireland, 2024).

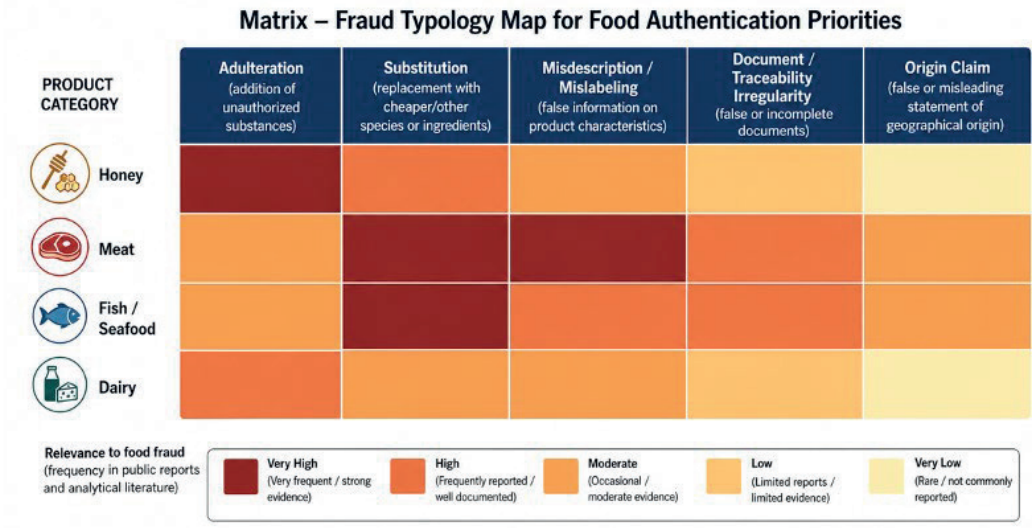

Figure 2. Matrix-fraud typology map for food authentication priorities

Dairy authentication combines compositional integrity, species origin, denomination protection and farm-level vulnerability. Romanian public documents indicate analytical capabilities including LC-MS/MS, GC-MS/MS, atomic absorption spectrometry, HPLC, PCR species determination, QTOF analysis for milk-species origin and testing for vegetable fats in dairy products. DNA-based evidence from dairy authentication studies confirms the relevance of targeted molecular methods for detecting undeclared milk species in processed dairy products. These are capabilities indicated by public documents and supporting scientific literature, not an exhaustive audit of national laboratory performance (Autoritatea Nationala Sanitara Veterinara si pentru Siguranta Alimentelor, 2016, 2023; Roumani et al., 2026).

Governance interpretation

The governance issue is not only analytical. Regulation (EU) 2017/625 requires competent authorities to consider possible intentional violations of agri-food chain rules, while Implementing Regulation (EU) 2019/1715 provides the digital infrastructure for cross-border information exchange. In this context, laboratory findings, documentary controls and digital traceability should be treated as complementary evidence layers rather than isolated tools.

The JRC technical report on Article 9(2) of Regulation (EU) 2017/625 emphasises the need for coherent approaches to fraudulent and deceptive practices, while EFSA materials on traceability-data standardisation point to the importance of interoperable and machine-readable information flows (Winkler et al., 2023; European Food Safety Authority, n.d.). For Romania, this supports a policy direction in which publicly documented laboratory capability is connected more explicitly with operator records, consignment documentation, TRACES/iRASFF data flows and targeted authentication testing.

This also implies a shift towards risk-informed and intelligence-led control strategies, where cross-border notifications, historical operator profiles and commodity-specific vulnerabilities are used to prioritise inspections and analytical testing. Strengthening interoperability between national databases and EU-level systems can enhance the timeliness and consistency of information exchange, while improving the evidentiary value of combined datasets in fraud-suspicion cases. At the same time, clear protocols for data integration and interpretation remain necessary to ensure that analytical results, documentary evidence and digital signals are assessed coherently and proportionately within official control systems.

CONCLUSIONS

The revised analysis supports a set of interrelated conclusions. Romania should be characterised through public cross-border visibility and capabilities indicated by publicly available institutional and scientific documents, rather than through unsupported estimates of prevalence or system-wide performance. European ACN and monthly fraud-suspicion data provide a useful basis for identifying patterns of public visibility, but they do not constitute direct indicators of national food fraud risk and must be interpreted within clearly defined analytical limits.

The matrices examined - honey, meat, fish/seafood and dairy products - remain analytically relevant because they combine recurring visibility at EU level with distinct authentication challenges that require matrix-specific methodological approaches. At the same time, the interpretation of documentary evidence benefits from a clear separation between reported results and analytical inference, particularly in cross-border contexts where notification data reflect reporting practices, trade exposure and control intensity as much as underlying irregularities.

The findings also underline the importance of integrating laboratory-based analytical methods with documentary and traceability evidence, especially in cases where fraud suspicion involves both compositional anomalies and administrative inconsistencies. Public ACN/FFN reporting should therefore be understood as a structured visibility system rather than a comprehensive surveillance dataset, which implies that conclusions at national level must remain cautious and explicitly qualified.

Finally, the analytical framework applied in this study - combining matrix-specific authentication methods with transparent documentary review - offers a reproducible approach that supports both scientific robustness and policy-relevant interpretation. Its value depends on methodological transparency, including explicit search strategies, PRISMA-based reporting, clearly defined indicators and a consistent distinction between European-level patterns and Romanian cross-border visibility.

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MICROWAVE-ASSISTED EXTRACTION OF BIOACTIVE COMPOUNDS FROM *Pelargonium* spp.: POLYPHENOL CONTENT, ANTIOXIDANT CAPACITY, AND ANTIMICROBIAL ACTIVITY

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Abstract

This study evaluated microwave-assisted hydromethanolic extracts from ten Pelargonium species as potential sources of bioactive compounds. Significant differences among extracts were observed ($p < 0.05$). Pelargonium 'Lizzy' and 'Cola Bottles' exhibited the highest total phenolic contents (126.09 and 122.16 mg GAE/g extract, respectively). Antioxidant activity ranged from 144.84 to 260.40 mg QE·g^{-1} extract (DPPH assay) and from 760.50 to 2940.90 µmol Fe^{2+}·g^{-1} extract (FRAP assay). Antimicrobial activity varied according to both the extract and the bacterial strain tested. P. odoratissimum showed the strongest inhibition against Escherichia coli and Salmonella Typhimurium, while Pelargonium 'Nova', 'Lizzy', and 'Cola Bottles' exhibited the most pronounced activity against Staphylococcus aureus, Enterococcus faecalis, and Proteus vulgaris using the agar well diffusion method. MIC and MBC results confirmed antibacterial potential, with lower inhibitory and bactericidal concentrations corresponding to larger inhibition zones, particularly for Pelargonium 'Cola Bottles', 'Nova', 'Lizzy', and P. roseum. These findings highlight the potential of selected Pelargonium species as sources of natural bioactive compounds.

Key words*: Pelargonium, plant extracts, polyphenols, antioxidant activity, antimicrobial activity.*

INTRODUCTION

The growing interest in natural antimicrobial agents has stimulated extensive research on medicinal plants and agro-industrial by-products (Diguta et al., 2014; Purcaru et al., 2017; Grosu et al., 2024, Megdar et al., 2025).

The genus *Pelargonium* L'Hér. ex Aiton, belonging to the family *Geraniaceae*, comprises more than 280 species predominantly native to Southern Africa and widely cultivated worldwide as ornamental, aromatic, and medicinal plants (Meyers et al., 2006; Saraswathi et al., 2011). In recent years, interest for *Pelargonium* has expanded beyond horticultural applications due to the remarkable diversity of secondary metabolites and their associated biological properties. Several studies have reported that *Pelargonium* species contain phenolic acids, flavonoids, tannins, coumarins, and terpenoids that contribute to a wide range of pharmacological activities, including antioxidant, antimicrobial, anti-inflammatory, anticancer, and cytotoxic activities (Hamidpour et al., 2017; Roman et al., 2023; Asker & Al Haidar, 2024; Balabanova et al., 2025; Celi et al., 2025). Furthermore, *Pelargonium* extracts and essential oils have demonstrated insecticidal activities, extending their potential applications beyond pharmaceutical and food-related fields (M'hamdi et al., 2024).

Natural antioxidants derived from plant sources have attracted considerable attention as safer alternatives to synthetic compounds used in food, cosmetic, and pharmaceutical industries. Oxidative stress, resulting from the excessive accumulation of reactive oxygen species, plays a crucial role in the development of numerous chronic diseases.

Plant polyphenols are recognized as major contributors to antioxidant activity through their ability to donate electrons or hydrogen atoms and neutralize free radicals (Roman et al., 2023; Gevrenova et al., 2024). Previous investigations have demonstrated that *Pelargonium* species are rich sources of phenolic compounds, including gallic acid, protocatechuic acid, caffeic acid derivatives,

kaempferol, myricetin, and isorhamnetin (Dimitrova et al., 2015; El Aanachi et al., 2020; Gevrenova et al., 2024). Positive correlations between total phenolic content and antioxidant activity have been reported for various *Pelargonium* extracts (Ali et al., 2020; Ennaifer et al., 2020; Gevrenova et al., 2024).

The extraction method employed represents a critical factor influencing the yield and quality of bioactive compounds recovered from plant material (El Maaiden et al., 2022; Bitwell et al., 2023; Zhou et al., 2023; Pogorzelska-Nowicka et al., 2024). Conventional extraction techniques, such as Soxhlet extraction and maceration, often require prolonged processing times, elevated temperatures, and large solvent volumes, which may lead to degradation of thermolabile compounds (Zhang et al., 2018; Bitwell et al., 2023). In response to these limitations, microwave-assisted extraction (MAE) has emerged as a promising green alternative, offering advantages including reduced extraction time, lower solvent consumption, and enhanced extraction efficiency (El Maaiden et al., 2022; Boztaş, et al., 2024; Pogorzelska-Nowicka et al., 2024). The mechanism underlying MAE involves the direct interaction of microwave energy with polar molecules in the solvent and plant matrix, generating rapid and uniform heating that disrupts cell walls and facilitates the release of intracellular compounds (Zhang et al., 2018; Zhou et al., 2023). Recent studies have demonstrated the effectiveness of MAE for the recovery of polyphenols from various plant matrices, with optimized conditions yielding significantly higher extraction efficiencies compared to conventional methods (El Maaiden et al., 2022; Bitwell et al., 2023; Zhou et al., 2023; Pogorzelska-Nowicka et al., 2024). Furthermore, microwave-assisted processing has been successfully applied to *Pelargonium graveolens*, improving the recovery of volatile and non-volatile phytochemicals while reducing extraction time and energy consumption (Boztaş et al., 2024).

The adoption of green extraction technologies is increasingly encouraged by the food and pharmaceutical industries, as these approaches improve extraction efficiency while reducing environmental impact and solvent consumption, thereby supporting the sustainable valorisation of plant-derived bioactive compounds (El Maaiden et al., 2022; Pogorzelska-Nowicka et al., 2024).

Methanolic extracts of *Pelargonium graveolens* obtained through conventional extraction methods have shown pronounced antibacterial, antifungal, antioxidant, and enzyme-inhibitory activities (Ben Hsouna & Hamdi, 2012; Dimitrova et al., 2015; Ali et al., 2020; Ennaifer et al., 2020).

Additional studies have highlighted the therapeutic potential of *Pelargonium* species in the management of inflammatory and metabolic disorders (Martins et al., 2017; Neagu et al., 2018; Amel et al., 2022).

Despite the increasing number of studies focused on individual *Pelargonium* species, comparative investigations involving multiple species cultivated under similar environmental conditions remain relatively scarce. This aspect is particularly important because phytochemical composition and biological activity may vary considerably among species, cultivars, and genotypes. Such variability is influenced by genetic background, cultivation practices, environmental conditions, and nutrient management strategies (Mazeed et al., 2022; Siddiqui et al., 2024). Therefore, screening diverse *Pelargonium* species may facilitate the identification of promising sources of natural antioxidants and antimicrobial compounds for future breeding programs, food applications, and biotechnological exploitation (Roman et al., 2023; Celi et al., 2025).

The present study aimed to comparatively evaluate the total phenolic content, antioxidant activity, and antimicrobial potential of microwave-assisted methanolic extracts obtained from ten *Pelargonium* species maintained in a commercial breeding and propagation collection.

MATERIALS AND METHODS

Ten *Pelargonium* genotypes/cultivars were obtained from a specialized horticultural collection located in the Netherlands, where plants are maintained for propagation and breeding purposes (Table 1). Healthy aerial parts were collected and processed for extract preparation.

Table 1. *Pelargonium* cultivars used in this study

Code	Species
P1	*Pelargonium* 'Chocolate'
P2	*Pelargonium* 'Watermelon'
P3	*Pelargonium tomentosum*
P4	*Pelargonium odoratissimum*
P5	*Pelargonium* 'Orange Fizz'
P6	*Pelargonium* 'Cola Bottles'
P7	*Pelargonium* 'Nova'
P8	*Pelargonium* 'Lizzy'
P9	*Pelargonium roseum*
P10	*Pelargonium graveolens*

Preparation of plant extracts

The aerial parts of the investigated *Pelargonium* species were freeze-dried (FreeZone 6, Labconco, USA) at −50°C under a vacuum pressure of 0.04 mbar for 22 h. Following lyophilization, the plant material was milled to a particle size of <1 mm and used for subsequent extraction.

The extracts were obtained using 70% (v/v) methanol as extraction solvent at a plant material-to-solvent ratio of 1:30 (w/v). Microwave-assisted extraction (MAE) (Ethos Easy - Advanced Microwave Digestion SystemMilestone™ SpA, Italy) was performed using an extraction power of 500 W at 120°C. The total extraction time was 80 min and consisted of three stages: 10 min of sample preheating to the selected temperature, 60 min of extraction under continuous microwave irradiation at 70°C, and 10 min of cooling.

The extracts were centrifuged for 10 min at 6000 rpm (Universal 320 R, Hettich, France) and the supernatants filtered through 0.45 µm sterile membranes (Sartorius, Germany). The solvent was removed under reduced pressure at 40 °C using a rotary evaporator (Büchi R-210, Switzerland), followed by freeze-drying.

The dried extracts were resuspended in sterile distilled water (100 mg·mL^{-1}), filter-sterilized through 0.22 µm membranes, and stored at +4°C until analysis.

Determination of total polyphenol content

The total polyphenol content (TPC) of the *Pelargonium* extracts was determined using the Folin-Ciocalteu colorimetric method. Briefly, 20 µL of plant extract was mixed with 1580 µL of distilled water, 100 µL of Folin-Ciocalteu reagent, and 300 µL of 20% (w/v) sodium carbonate solution. The reaction mixtures were thoroughly homogenized and incubated in the dark for 2 h at room temperature.

After incubation, the absorbance was measured at 765 nm using a UV-Vis spectrophotometer (Thermo Scientific Helios, England). A calibration curve was prepared using gallic acid standard solutions, and the TPC was expressed as milligrams of gallic acid equivalents per g of extracts (mg GAE·g^{-1} extract). The polyphenol content was calculated according to the following equation:

$$\text{TPC (mg GAE}\cdot\text{g}^{-1}\text{ extract)} = \frac{A_{765}\text{-}0.0082}{0.001}$$

where:

- A_{765} is the absorbance of the sample measured at 765 nm;
- GAE represents gallic acid equivalents.

The calibration curve obtained for gallic acid was $y = 0.001x + 0.0082$ ($R^2 = 0.9991$).

Determination of antioxidant activity by the DPPH Assay

The antioxidant activity of the *Pelargonium* extracts was evaluated using the DPPH (2,2-diphenyl-1-picrylhydrazyl) radical scavenging assay. This method is based on the ability of antioxidant compounds to neutralize the stable DPPH free radical, resulting in a colour change of the solution from deep violet to yellow.

For the analysis, 50 µL of plant extract was mixed with 1950 µL of DPPH solution prepared in ethanol. The reaction mixtures were incubated in the dark at room temperature for 30 min to ensure complete reaction between the antioxidant compounds and the DPPH radical.

After incubation, the absorbance was measured at 515 nm using a UV–Vis spectrophotometer (Thermo Scientific Helios, England). Absolute ethanol was used as the blank. The percentage of radical scavenging activity was calculated based on the decrease in absorbance relative to the control.

Antioxidant activity was expressed as quercetin equivalents (QE) and calculated using the calibration equation:

$$\text{AAE (QE)} = \frac{\%\Delta A_{515}\text{-}3.4954}{0.0811}$$

where:

- AAE (QE) represents the antioxidant activity expressed as quercetin equivalents;

- % ΔA_{515} represents the percentage inhibition determined at 515 nm.
The resulting linear regression equation was: y = 0.0811x + 3.4954.

Determination of antioxidant activity by the FRAP assay

The antioxidant activity of the *Pelargonium* extracts was evaluated using the Ferric Reducing Antioxidant Power (FRAP) assay. This method is based on the reduction of the ferric-tripyridyltriazine complex (Fe^{3+}–TPTZ) to the ferrous form (Fe^{2+}–TPTZ) by antioxidant compounds present in the extracts, resulting in the formation of an intense blue-coloured complex.
The FRAP reagent was freshly prepared by mixing acetate buffer (pH 3.6), 10 mM TPTZ solution prepared in 40 mM HCl, and 20 mM $FeCl_3 \cdot 6H_2O$ solution in a ratio of 10:1:1 (v/v/v).
For the analysis, 100 µL of appropriately diluted plant extract was mixed with 3.0 mL of freshly prepared FRAP reagent. The reaction mixtures were incubated at 37°C for 4 min. After incubation, the absorbance was measured at 593 nm using a UV–Vis spectrophotometer (Thermo Scientific Helios, England). A reagent blank consisting of 3.0 mL FRAP reagent and 100 µL distilled water was used for baseline correction.
The antioxidant activity was expressed as Fe^{2+} equivalents and calculated using a calibration curve prepared with ferrous sulphate ($FeSO_4$). The concentration of Fe^{2+} equivalents was determined according to the calibration equation:

$$\text{FRAP } (Fe^{2+} \text{ equivalents}) = \frac{A_{593} - 0.0038}{1.4953}$$

where:
- FRAP (Fe^{2+} equivalents) represents the antioxidant activity expressed as ferrous ion equivalents;
- A_{593} represents the absorbance measured at 593 nm.
The resulting linear regression equation was: y = 1.4953x + 0.0038.

Determination of antibacterial activity of *Pelargonium* extracts

Antimicrobial activity of the extracts was evaluated against Gram-positive bacteria (*Staphylococcus aureus* ATCC 6538, *Enterococcus faecalis* ATCC 29212, and *Listeria monocytogenes* ATCC 13932) and Gram-negative bacteria (*Escherichia coli* ATCC 8739, *Pseudomonas aeruginosa* ATCC 27853, *Proteus vulgaris* ATCC 13315, *Salmonella enterica* subsp. *enterica* Typhimurium ATCC 14028, and *Serratia marcescens* ATCC 14754). All strains were obtained from the American Type Culture Collection (ATCC). Briefly, bacterial inoculum was prepared from fresh overnight cultures grown on Tryptic Soy Agar (TSA; Scharlab S.L., Barcelona, Spain). The standardized cell suspensions were adjusted to the turbidity of a 0.5 McFarland standard, corresponding to approximately $1–2\times10^8$ CFU·mL^{-1}, prior to antimicrobial activity testing.

Agar well diffusion assay

Antimicrobial activity was evaluated using the agar well diffusion method with minor modifications based on Balouiri et al. (2016).
For the assay, 1 mL of each standardized cell suspension was inoculated into sterile 90-mm Petri dishes, followed by the addition of 25 mL of Tryptic Soy Agar at 42-45°C. Plates were gently homogenized and allowed to solidify.
Wells (6 mm diameter) were aseptically prepared, and 100 µL of each extract was added. Plates were incubated for 24 h under optimal growth conditions. Antimicrobial activity was determined by measuring inhibition zone diameters (mm).

Determination of minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC)

The minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of the extracts were determined using the broth microdilution method in sterile 96-well microplates, according to the protocol described by Grosu et al. (2024), with minor modifications. Briefly, bacterial inoculum was prepared from fresh overnight cultures grown on Tryptic Soy Agar. The standardized cell suspensions were diluted in Mueller-Hinton broth (MHB, Accumix®, Microxpress, Verna, Goa, India) to obtain a final inoculum concentration of approximately 5×10^5 CFU·mL^{-1} in each well after addition to the microplate. The lyophilized extracts were

reconstituted in sterile distilled water and subsequently subjected to two-fold serial dilutions in sterile MHB using 96-well microtiter plates. The tested concentration range was 12.5-0.024 mg·mL^{-1}. Each well contained the appropriate concentration of extract and bacterial inoculum, resulting in a uniform final volume of 100 µL in each well. Positive controls consisted of inoculated medium without extract, while negative controls contained uninoculated medium. Following inoculation, the microplates were incubated at 37°C for 18-24 h under aerobic conditions. Subsequently, 20 µL of 0.01% (w/v) resazurin solution were added to each well, and the plates were further incubated for an additional 2 h at 37°C to allow colour development. Resazurin was used as a bacterial viability indicator, whereby metabolically active cells reduced the blue dye to pink resorufin. At the highest extract concentrations, a yellowish coloration was occasionally observed, likely due to interactions between phytochemical constituents and the resazurin reagent (Neufeld et al., 2018; Akter et al., 2019); therefore, MIC values were interpreted in conjunction with visible growth inhibition and subsequently confirmed by MBC determination. The MIC was defined as the lowest concentration showing no detectable bacterial growth, as indicated by the absence of resazurin reduction and confirmed by visual inspection of the wells. To determine the MBC, aliquots from wells corresponding to the MIC and higher concentrations were aseptically streaked onto TSA plates and incubated at 37°C for 24 h. The MBC was defined as the lowest concentration of the extract that completely prevented bacterial regrowth, as evidenced by the absence of visible colonies on the agar surface.

2.6. Statistical analysis

Statistical analyses were performed using one-way analysis of variance (ANOVA). Significant differences among means were determined using Tukey's honestly significant difference (HSD) post hoc test at a significance level of $p < 0.05$. Results are expressed as mean ± standard deviation of three independent determinations. Different letters indicate statistically significant differences between samples.

RESULTS AND DISCUSSIONS

Total polyphenol content of *Pelargonium* extracts

To assess the phytochemical characteristics of the investigated samples, dry matter content and TPC were determined prior to the evaluation of their biological activities (Table 2).

Dry matter content varied between 8.55% and 15.50% among the investigated *Pelargonium* extracts; however, the observed variation was not directly associated with TPC, suggesting that phenolic accumulation is primarily genotype-dependent rather than related to biomass dry matter levels (Table 2).

Similar observations have been reported for *Pelargonium* species and other aromatic plants, where secondary metabolite production depends on genetic and physiological factors in addition to biomass composition (Gevrenova et al., 2024; Balabanova et al., 2025).

The TPC varied significantly among the investigated *Pelargonium* extracts ($p < 0.05$), indicating substantial differences in the accumulation of phenolic compounds among the studied genotypes. The highest TPCs were determined in the extracts obtained from *Pelargonium* 'Lizzy' (P8) and *Pelargonium* 'Cola Bottles' (P6), reaching 126.09 and 122.16 mg GAE/g extract, respectively (Table 2).

Moderate phenolic concentrations were observed in extracts of *Pelargonium* 'Orange Fizz' (P5), *Pelargonium* 'Nova' (P7) and *Pelargonium roseum* (P9), which formed the same statistical group. Extracts of *Pelargonium tomentosum* (P3) and *Pelargonium* 'Chocolate' (P1) exhibited intermediate values, whereas the extract of *Pelargonium odoratissimum* (P4) showed a significantly lower phenolic content (Table 2).

The lowest TPCs were found in extracts of *Pelargonium graveolens* (P10) and *Pelargonium* 'Watermelon' (P2), which did not differ significantly from each other. The approximately 2.5-fold variation between the highest and lowest values highlights the strong influence of genotype on the biosynthesis and accumulation of phenolic compounds within the *Pelargonium* genus.

Tabel 2. Total polyphenol content of *Pelargonium* extracts

Sample	Dry matter (%)	TPC (mg GAE·g⁻¹ extract)
P1	14.15	79.02 ± 2.65[c]
P2	13.30	49.99 ± 2.30[e]
P3	12.94	80.47 ± 0.11[c]
P4	8.55	66.63 ± 3.05[d]
P5	11.13	105.26 ± 2.60[b]
P6	12.92	122.18 ± 6.02[a]
P7	9.24	103.67 ± 9.88[b]
P8	15.50	126.09 ± 11.79[a]
P9	14.60	102.75 ± 5.20[b]
P10	14.04	50.71 ± 3.20[e]

Considering the well-documented role of phenolic compounds as antioxidants and antimicrobial agents, the elevated phenolic contents observed in *Pelargonium* 'Lizzy' and *Pelargonium* 'Cola Bottles' extracts may contribute to their enhanced biological activity. The results indicate that the selected *Pelargonium* extracts constitute promising sources of natural bioactive compounds for food and pharmaceutical applications. Similar observations were reported for *Pelargonium graveolens* extracts, in which phenolic compounds represented major contributors to the biological activity of the plant material (Ali et al., 2020; Ennaifer et al., 2020; Gevrenova et al., 2024).

The significant differences observed in TPC among the investigated *Pelargonium* extracts are consistent with previous reports demonstrating considerable phytochemical variability within the genus. Dimitrova et al. (2015), El Aanachi et al. (2020) and Gevrenova et al. (2024) reported that *Pelargonium* species are rich sources of phenolic acids and flavonoids, although their concentrations strongly depend on genotype, extraction conditions and environmental factors.

Antioxidant activity determined by DPPH assay

The antioxidant capacity of the investigated *Pelargonium* extracts was evaluated using DPPH radical scavenging and ferric reducing antioxidant power (FRAP) assays to assess their ability to neutralize free radicals and act as reducing agents (Table 3).

The DPPH assay revealed notable radical-scavenging activity in all *Pelargonium* extracts. The highest antioxidant activity was observed for P1 (260.40 mg QE·g⁻¹ extract), followed by P5 (242.19 mg QE·g⁻¹ extract) and P4 (196.50 mg QE·g⁻¹ extract). Lower values were recorded for P9, P7, P10, and P8. Interestingly, the DPPH results did not fully follow the same trend as the TPC, suggesting that radical-scavenging activity may depend not only on the total amount of phenolics, but also on the specific structure and reactivity of individual antioxidant compounds present in each extract.

Tabel 3. Antioxidant activity of *Pelargonium* extracts

Sample	Antioxidant activity (mg QE·g⁻¹ extract)	Antioxidant activity (μmol Fe^{2+}·g⁻¹ extract)
P1	260.40 ± 0.34[a]	1005.30 ± 2.40[h]
P2	168.45 ± 0.11[e]	1150.20 ± 5.10[g]
P3	160.77 ± 0.11[f]	1729.26 ± 11.35[e]
P4	196.50 ± 0.26[c]	1268.10 ± 1.80[f]
P5	242.19 ± 0.16[b]	1916.10 ± 1.50[d]
P6	169.19 ± 0.11[d]	2595.90 ± 40.80[b]
P7	154.62 ± 0.20[h]	2256.00 ± 154.80[c]
P8	159.61 ± 0.21[g]	2940.90 ± 3.30[a]
P9	144.84 ± 0.57[i]	1232.10 ± 5.10[f]
P10	154.92 ± 0.10[h]	760.50 ± 5.70[i]

The FRAP assay showed substantial differences in ferric reducing antioxidant power among the investigated extracts. The highest FRAP values were obtained for P8 (2940.90 μmol Fe^{2+}·g⁻¹ extract), P6 (2595.90 μmol Fe^{2+}·g⁻¹ extract), and P7 (2256.00 μmol Fe^{2+}·g⁻¹ extract), indicating a strong reducing capacity. The lowest value was recorded for P10 (760.50 μmol Fe^{2+}·g⁻¹ extract), followed by P1 and P2 extracts.

A positive correlation was observed between TPC and antioxidant activity measured by the FRAP assay. Extracts with high TPC values, particularly *Pelargonium* 'Lizzy' (P8) and *Pelargonium* 'Cola Bottles' (P6), exhibited correspondingly elevated FRAP values, indicating that phenolic compounds are major contributors to the reducing capacity of these extracts.

These findings indicate that phenolic compounds contribute substantially to the reducing capacity of *Pelargonium* extracts. Similar relationships have been previously documented in *Pelargonium* species and other phenolic-rich plant matrices (Ali et al., 2020; El Aanachi et al., 2020; Gevrenova et al., 2024; Grosu et al., 2024).

In contrast, the correlation between TPC and DPPH radical-scavenging activity was less pronounced. Although *Pelargonium* 'Chocolate' (P1) extract exhibited the highest DPPH activity (260.40 mg QE·g^{-1} extract), its TPC was lower than that of *Pelargonium* 'Cola Bottles' (P6) and *Pelargonium* 'Lizzy' (P8) extracts. This observation suggests that antioxidant activity is influenced not only by the total concentration of phenolic compounds but also by their qualitative composition, molecular structure, and specific reaction mechanisms involved in each assay. Comparable antioxidant activities have been reported for *Pelargonium graveolens* extracts rich in polyphenols and flavonoids (Hamidpour et al., 2017; Ennaifer et al., 2020; Gevrenova et al., 2024).

Our results demonstrate that *Pelargonium* extracts represent valuable sources of natural antioxidants and highlight significant phytochemical variability among the investigated genotypes.

The strong reducing capacity observed for extracts of *Pelargonium* 'Cola Bottles' (P6), *Pelargonium* 'Nova' (P7), and *Pelargonium* 'Lizzy' (P8) indicates their potential as promising candidates for future studies focused on the isolation and characterization of bioactive compounds with antioxidant properties.

Evaluation of antibacterial activity of *Pelargonium* extracts

The antibacterial potential of the investigated *Pelargonium* extracts was assessed using agar well diffusion (Table 4), minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) assays (Tables 5 and 6).

Table 4. Antimicrobial activity of the investigated extracts against pathogenic bacteria

Pathogens	P1	P2	P3	P4	P5	P6	P7	P8	P9	P10
S. aureus ATCC 6538	8.50 ± 0.41^{c}	10.50 ± 0.41^{b}	8.33 ± 0.24^{c}	8.33 ± 0.24^{c}	10.17 ± 0.24^{b}	12.50 ± 0.00^{a}	12.17 ± 0.29^{a}	12.50 ± 0.87^{a}	12.00 ± 0.50^{a}	8.17 ± 0.76^{c}
Ent. faecalis ATCC 29212	8.33 ± 0.24^{d}	8.33 ± 0.24^{d}	9.50 ± 0.41^{c}	9.00 ± 0.41^{d}	10.33 ± 0.24^{c}	11.50 ± 0.50^{b}	14.17 ± 0.29^{a}	14.17 ± 0.29^{a}	12.17 ± 0.29^{b}	7.33 ± 0.29^{e}
L. monocytogenes ATCC 13932	8.17 ± 0.24^{a}	7.33 ± 0.24^{b}	8.33 ± 0.24^{a}	6.33 ± 0.24^{b}	6.33 ± 0.24^{b}	9.00 ± 0.50^{a}	8.17 ± 0.29^{a}	8.00 ± 0.50^{a}	8.83 ± 1.04^{a}	6.50 ± 0.50^{b}
E. coli ATCC 8739	2.67 ± 0.24^{f}	4.17 ± 0.29^{e}	4.33 ± 0.24^{e}	19.17 ± 0.62^{a}	18.33 ± 0.24^{a}	5.67 ± 0.76^{d}	8.33 ± 0.29^{c}	8.33 ± 0.29^{c}	12.50 ± 0.00^{b}	4.33 ± 0.29^{e}
P. aeruginosa ATCC 27853	7.33 ± 0.24^{e}	14.33 ± 0.24^{c}	17.17 ± 0.24^{b}	17.33 ± 0.24^{b}	14.17 ± 0.24^{c}	18.33 ± 0.29^{a}	17.00 ± 0.50^{b}	9.83 ± 0.29^{d}	10.17 ± 0.29^{d}	10.50 ± 0.50^{d}
P. vulgaris ATCC 13315	8.33 ± 0.24^{d}	8.33 ± 0.24^{d}	7.17 ± 0.47^{d}	12.33 ± 0.24^{b}	12.33 ± 0.24^{b}	10.17 ± 0.29^{c}	15.30 ± 0.52^{a}	15.17 ± 0.76^{a}	10.67 ± 0.76^{c}	8.33 ± 0.29^{d}
S. Typhimurium ATCC 14028	8.00 ± 0.00^{f}	6.50 ± 0.00^{g}	6.50 ± 0.00^{g}	22.17 ± 0.24^{a}	17.50 ± 0.00^{c}	17.33 ± 0.29^{c}	20.17 ± 0.29^{b}	15.17 ± 0.29^{d}	11.67 ± 0.76^{e}	8.33 ± 0.29^{f}
S. marcescens ATCC 14754	2.30 ± 0.22^{d}	2.50 ± 0.00^{d}	2.33 ± 0.24^{d}	11.33 ± 0.24^{a}	7.33 ± 0.24^{b}	5.67 ± 0.76^{c}	7.33 ± 0.76^{b}	7.00 ± 0.50^{b}	2.50 ± 0.00^{d}	2.50 ± 0.00^{d}

Data are presented as mean ± SD. Values followed by different superscript letters differ significantly ($p < 0.05$). Antimicrobial activity is expressed as inhibition zone diameter (mm).

Extracts that produced larger inhibition zones also exhibited lower MIC and MBC values, confirming their stronger antibacterial efficacy. This relationship was particularly evident for extracts of *Pelargonium* 'Cola Bottles' (P6), *Pelargonium* 'Nova' (P7), *Pelargonium* 'Lizzy' (P8), *Pelargonium roseum* (P9) and *Pelargonium odoratissimum* (P4), which demonstrated both high inhibition zone diameters and low inhibitory or bactericidal concentrations.

Pelargonium odoratissimum (P4) extract showed the strongest activity against *E. coli* ATCC 8739 and *S. typhimurium* ATCC 14028 in the agar diffusion assay, producing inhibition zones of 19.17 mm and 22.17 mm, respectively. However, MIC and MBC values for these microorganisms remained relatively high (>12.5 mg·mL^{-1}), suggesting that although the extract contains compounds capable of diffusing efficiently through agar and inhibiting bacterial growth, higher concentrations are required to achieve complete growth inhibition and bacterial killing in liquid media. This discrepancy may reflect differences in the diffusion behavior and bioavailability of active compounds.

Table 5. MIC values (mg·mL⁻¹) of *Pelargonium* cultivar extracts against the tested bacterial strains

Pathogens	P1	P2	P3	P4	P5	P6	P7	P8	P9	P10
S. aureus ATCC 6538	3.13	3.13	1.56	3.13	1.56	0.78	0.78	0.78	0.78	3.13
Ent. faecalis ATCC 29212	6.25	6.25	3.13	6.25	3.13	1.56	1.56	1.56	1.56	3.13
L. monocytogenes ATCC 13932	6.25	6.25	3.13	6.25	3.13	3.13	1.56	3.13	3.13	6.25
E. coli ATCC 8739	>12.5	>12.5	>12.5	>12.5	>12.5	>12.5	6.25	6.25	>12.5	6.25
P. aeruginosa ATCC 27853	12.5	6.25	6.25	0.78	12.5	12.5	6.25	6.25	12.5	6.25
P. vulgaris ATCC 13315	1.56	1.56	0.78	1.56	0.78	0.78	0.78	0.78	0.78	6.25
S. Typhimurium ATCC 14028	>12.5	>12.5	3.13	>12.5	>12.5	>12.5	6.25	>12.5	>12.5	>12.5
S. marcescens ATCC 14754	>12.5	>12.5	>12.5	>12.5	>12.5	>12.5	>12.5	>12.5	>12.5	>12.5

Data are presented as mean ± SD.

Table 6. MBC values (mg·mL⁻¹) of *Pelargonium* cultivar extracts against the tested bacterial strains

Pathogens	P1	P2	P3	P4	P5	P6	P7	P8	P9	P10
S. aureus ATCC 6538	3.13	3.13	1.56	3.13	1.56	0.78	0.78	0.78	0.78	3.13
P. aeruginosa ATCC 27853	12.5	12.5	6.25	0.78	12.5	12.5	6.25	6.25	12.5	12.5
E. coli ATCC 8739	>12.5	>12.5	>12.5	>12.5	>12.5	>12.5	>12.5	>12.5	>12.5	>12.5
E. faecalis ATCC 29212	6.25	6.25	3.13	6.25	3.13	1.56	1.56	3.13	1.56	6.25
P. vulgaris ATCC 13315	1.56	1.56	1.56	1.56	0.78	0.78	0.78	0.78	0.78	6.25
L. monocytogenes ATCC 13932	6.25	6.25	3.13	6.25	3.13	3.13	3.13	3.13	3.13	6.25
S. Typhimurium ATCC 14028	>12.5	>12.5	3.13	>12.5	>12.5	>12.5	>12.5	>12.5	>12.5	>12.5
S. marcescens ATCC 14754	>12.5	>12.5	>12.5	>12.5	>12.5	>12.5	>12.5	>12.5	>12.5	>12.5

Data are presented as mean ± SD.

A strong correlation between agar well diffusion and microdilution methods was observed for *Proteus vulgaris* ATCC 13315. *Pelargonium* 'Nova' (P7) and *Pelargonium* 'Lizzy' (P8) extracts generated inhibition zones exceeding 15 mm and exhibited low MIC and MBC values (0.78 mg·mL⁻¹), indicating pronounced antibacterial and bactericidal activity. Similar findings were obtained for *Staphylococcus aureus* ATCC 6538 and *Enterococcus faecalis* ATCC 29212, where extracts with larger inhibition zones also displayed lower MIC and MBC values.

Listeria monocytogenes ATCC 13932 showed relatively small inhibition zones and comparatively higher MIC and MBC values, indicating reduced susceptibility to the investigated extracts. Likewise, *Serratia marcescens* ATCC 14754 exhibited the highest resistance, characterized by small inhibition zones and the maximum MBC value (>12.50 mg·mL⁻¹) for all extracts tested.

The combined evaluation of inhibition zone diameters, MIC and MBC values indicates that the extracts of *Pelargonium* 'Cola Bottles' (P6), *Pelargonium* 'Nova' (P7), *Pelargonium* 'Lizzy' (P8), and *Pelargonium roseum* (P9) possess the most promising antibacterial properties. Their low MIC and MBC values, together with their strong inhibition zones, suggest the presence of bioactive compounds capable of exerting both bacteriostatic and bactericidal effects. These findings are consistent with the higher phenolic content previously reported for these extracts, suggesting a role in their antimicrobial activity.

The higher susceptibility of Gram-positive bacteria observed in the present study is consistent with previous reports concerning *P. graveolens*. Dimitrova et al. (2015) reported antibacterial activity of *P. graveolens* leaf extracts primarily against *Listeria monocytogenes*, while little or no activity was observed against *E. coli* and *Salmonella* spp. Similarly, Ennaifer et al. (2020) demonstrated that aqueous preparations of *P. graveolens* (infusion and decoction) exhibited considerable antibacterial activity against *S. aureus*, supporting the high sensitivity of Gram-positive bacteria observed in the present study.

The lower susceptibility of Gram-negative bacteria may be attributed to the structural characteristics of their cell envelope. The

presence of an outer membrane containing lipopolysaccharides constitutes an additional permeability barrier that restricts the diffusion of phenolic compounds and other antimicrobial phytochemicals into the bacterial cell. This mechanism has frequently been proposed to explain the reduced effectiveness of plant-derived extracts against Gram-negative microorganisms. Furthermore, previous investigations on *P. graveolens* extracts have shown that their antimicrobial activity is largely associated with phenolic compounds, flavonoids, and volatile constituents such as citronellol, geraniol, and linalool, which may act individually or synergistically to disrupt bacterial membranes and interfere with essential cellular processes (Ben Hsouna & Hamdi, 2012).

CONCLUSIONS

The present study demonstrated that *Pelargonium* extracts represent valuable sources of bioactive compounds with significant antioxidant and antimicrobial properties. Considerable variability was observed among the investigated extracts, highlighting the influence of genotype on phenolic accumulation and biological activity. *Pelargonium* 'Lizzy' and *Pelargonium* 'Cola Bottles' extracts exhibited the highest total phenolic contents and antioxidant capacities, indicating a strong contribution of phenolic compounds to the antioxidant potential of the extracts.

The antimicrobial assays revealed activity against both Gram-positive and Gram-negative foodborne pathogens, with susceptibility varying among bacterial species. The strongest overall antimicrobial performance was recorded for *Pelargonium* 'Cola Bottles' (P6), *Pelargonium* 'Nova' (P7) and *Pelargonium* 'Lizzy' (P8), which consistently produced large inhibition zones and low MIC and MBC values. These findings indicate that selected *Pelargonium* species represent promising natural sources of antioxidant and antimicrobial compounds. Further research is required to identify the bioactive constituents responsible for these activities and to assess their potential applications in food preservation, nutraceutical formulations, and other value-added products.

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RESEARCH ON MARKETING STRATEGY INNOVATION OF CHINESE ENTERPRISES IN THE CONTEXT OF DIGITAL AND GREEN TRANSFORMATION

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Abstract

With the rapid development of digital technology and the deepening of the concept of sustainable development, the dual transformation of digitalization and greening has become crucial for Chinese enterprises to gain a competitive advantage. This paper uses literature review and theoretical discussion to analyze the problems existing in the marketing process of Chinese enterprises and proposes innovative paths for marketing strategies under the background of dual transformation. Chinese enterprises face problems such as an imperfect green certification system, an incomplete digital marketing system, and insufficient innovation in new media marketing scenarios. The study finds that these problems can be effectively solved by constructing a unified and coordinated green certification standard system, building a digital and intelligent marketing system, and actively exploring innovative digital marketing models. The research results provide practical guidance for the digital and green marketing transformation of Chinese enterprises and contribute new research perspectives to the theoretical research of digital and green marketing, possessing certain theoretical and practical significance.

Key words: *digital transformation, green transformation, marketing strategy innovation, ESG, sustainable marketing.*

INTRODUCTION

Currently, the digital technology revolution and green sustainable development are key to the economic development of countries worldwide. On July 28, 2025, the European High-Level Forum on Standardization released standardization recommendations for the EU's annual work plan for 2026, proposing 100 action initiatives focusing on digital transformation, green transformation, and cross-sectoral coordination. These initiatives aim to provide strategic guidance for EU standard setting and support long-term growth and sustainable development goals. This strategy not only improves business efficiency and economic value but also emphasizes environmental protection and social responsibility to ensure sustainable economic development. As the world's second-largest economy, China actively responds to this trend. The government, in policy documents such as the "Overall Layout Plan for the Construction of Digital China" and the "Action Plan for Carbon Peaking before 2030", explicitly states that enterprises should promote a dual transformation of digitalization and greening.

However, under the dual transformation context, Chinese enterprises still face many challenges in their marketing processes. Many enterprises treat digitalization and greening as separate entities, lacking a synergistic and integrated strategic perspective, and lacking a systematic exploration of marketing strategy innovation mechanisms. Therefore, this study explores how Chinese enterprises can innovate their marketing strategies under the dual transformation context to achieve a win-win situation for economic and environmental benefits, aiming to provide enterprises with a set of intelligent marketing methods that are highly operable and widely adaptable. The research findings can provide practical references for Chinese

enterprises to formulate and implement marketing strategies under the dual transformation context, helping them effectively enhance their market competitiveness and sustainable marketing capabilities.

LITERATURE REVIEW

Evolution of Digital Marketing Research

In recent years, with the development of technologies such as artificial intelligence, big data, and blockchain, data-driven marketing, intelligent recommendation, and immersive experience have become new research directions. The main advantages of digital marketing are: precise reach, interactive experience, and measurable effects. Cui (2024) pointed out that with the rapid development of emerging technologies such as big data and artificial intelligence, the marketing field has undergone significant changes and improvements. As a data-driven marketing method, digital marketing pays more attention to data-driven and effect optimization. By continuously testing and optimizing strategies, it seeks the most profitable marketing strategy combination, and is not limited by channels, resulting in more significant marketing effects. Qiao et al. (2024) believe that digital marketing, as a mainstream marketing method today, has brought disruptive changes to the innovation activities of small and medium-sized enterprises.

Development of Green Marketing Theory

In recent years, under the background of the "dual carbon" goal, scholars' research on green marketing has shown new characteristics. On the one hand, research is paying more attention to the effectiveness evaluation of green marketing, and how to quantify the environmental and economic value of green marketing has become a research hotspot. Huang (2025) believes that in the context of global carbon neutrality, introducing green product design, promoting technological innovation, optimizing supply chain management, and stimulating consumers' green demand can achieve a balance between economic and ecological benefits. On the other hand, research on corporate green marketing innovation strategies is constantly deepening. Zhang (2025) proposes that enterprises should build a new green marketing system centered on products, prices, channels, and promotions, optimize cost control mechanisms, and strengthen industry-university-research collaborative innovation. By establishing green supply chain management, digital marketing transformation, and improving talent training mechanisms, enterprises can effectively enhance their market competitiveness and promote the sustainable development of new material manufacturing enterprises under the "dual carbon" goal.

Research on the Integration of Corporate Marketing Strategies under the Background of Dual Transformation

Although scholars have achieved certain research results in digital marketing and green marketing, few have combined the two in their research. Existing studies mainly focus on integrating digitalization or greening with corporate marketing strategies, which has significant shortcomings. These shortcomings are mainly reflected in: first, few scholars have simultaneously applied digitalization and greening to the marketing process; second, there is insufficient research considering the specific development situation of Chinese enterprises, and no research has been conducted on the impact of China's institutional environment and market characteristics on marketing strategies under the dual transformation. Therefore, this study delves into the innovation of marketing strategies of Chinese enterprises under the background of digitalization and greening, aiming to fill the gaps in existing research.

CURRENT PROBLEMS IN THE MARKETING PROCESS OF CHINESE ENTERPRISE

Incomplete Green Certification System

In the context of the dual transformation of digitalization and greening, the green certification system, as one of the important standards for measuring the environmental protection level of enterprises, plays a key role in green marketing. However, the current green

certification system in China is incomplete, hindering enterprises from carrying out green marketing. Inconsistent certification standards and low international recognition. Currently, China's existing green certifications include green product certification, environmental labelling certification, and energy-saving product certification. There are still significant differences between different certification types, and the evaluation dimensions are not uniform, resulting in enterprises having to bear very high costs if they want to obtain multiple certifications. In terms of international recognition, there are many differences between Chinese green certifications and internationally accepted standards.

Inadequate Digital Marketing System

With the rapid development of big data technology, product information, customer information, and market information can be shared and integrated, making the establishment of a digital integrated marketing system crucial for enterprises. Currently, most Chinese enterprises suffer from untimely communication between their marketing departments and other departments, resulting in severe information asymmetry. Only the marketing department actively participates in marketing activities, while other departments lack marketing responsibilities. Due to the lack of a digital and intelligent marketing system, staff in production, technology, human resources, and finance departments are not involved in marketing activities. Even in a few enterprises that have implemented digital marketing systems, relevant personnel still rely on traditional marketing methods, demonstrating poor ability to leverage digital technology to expand marketing channels. Consequently, a comprehensive, multi-channel digital marketing system has not been formed, resulting in poor marketing effectiveness.

Insufficient Innovation in New Media Marketing Scenarios

Currently, many Chinese companies publish the same content on different platforms during their actual marketing processes, resulting in severe homogenization of new media marketing information. Furthermore, companies have poor capabilities in applying technologies such as blockchain, AR/VR, and big data, lacking innovation in marketing content formats and user interaction. Existing marketing content mostly focuses on entertainment, creating overly simplistic consumption scenarios for customers. In terms of brand promotion, they fail to create emotional resonance with customers and lack interaction, leading to low customer trust in the brand.

Frequent Data Privacy Leaks

Frequent data leaks and security issues are common problems faced by enterprises in the marketing process. Data leak are frequent when enterprises use new media marketing technologies to process customer’s information during marketing activities. To achieve precise marketing, enterprises need to leverage digital technology to create customer profiles and target specific customer groups. However, data collection involves a large amount of sensitive user information. If data security is not guaranteed during collection and processing, this sensitive information can be obtained by hackers, easily leading to privacy leaks.

Poor Green Brand Value Marketing Capabilities

In new media marketing, maintaining a green brand image and marketing brand value are crucial. As people's incomes gradually increase, consumers' demands on brands are also rising. While some companies have created their own green brands, their brand value marketing capabilities are very poor. Incomplete consumer analysis, inaccurate product brand positioning, unclear value propositions, and a lack of authenticity in social media content all contribute to errors in product promotion. Furthermore, some social media marketing involves over-promotion and misleading advertising, leading to a decline in consumer trust in green brands and damaging the brand image.

MARKETING INNOVATION STRATEGIES FOR CHINESE ENTERPRISES UNDER THE DUAL TRANSFORMATION OF DIGITALIZATION AND GREENING

Building a Unified and Coordinated Green Certification Standard System

Relevant departments should establish a national-level green certification standard system to systematically optimize existing, fragmented green certification standards. Specific measures include: First, formulating unified green certification standards, using carbon footprint, environmental labelling, and energy-saving performance as unified certification standards. Second, establishing a unified dynamic updating mechanism for green certification standards. Relevant departments should evaluate and update certification standards every two years to ensure they adapt to market development. Third, establishing green certification standards at different levels for different industries.

Building a Digital Marketing System Currently

Chinese enterprises should efficiently utilize big data, integrate existing marketing resources, and build a digital marketing system guided by marketing objectives, led by the marketing department, and supported by other departments. This involves breaking down information barriers, promoting the sharing of customer, market, and product information, and encouraging resources such as production, technology, human resources, and finance to contribute to the company's marketing efforts. Furthermore, enterprises should improve their intelligent product recommendation systems, deeply mine and analyze customer historical purchase data and browsing records, gain insights into consumer behaviour, analyze and predict market trends, and provide data support for marketing decisions.

Actively Innovate Social Media Marketing Scenarios

Enterprises should deeply analyze current social media marketing trends and fully utilize technologies such as short videos, blockchain, AR/VR, and big data. Combined with user behaviour tags and scenario characteristics, they should deepen innovation in content formats, technology applications, user interaction, and data analysis. This will transform users' cognitive surplus, emotional fluctuations, and even biometric data into quantifiable and tradable marketing production factors, thereby attracting traffic and improving user engagement and conversion rates. Strengthen real-time marketing by implementing interactive promotions through live streaming, limited-time discounts, and interactive games to create a sense of urgency and participation, shorten the decision-making process, and drive rapid conversion from "interest" to "purchase."

Strengthen Green Brand Value Marketing

Enterprises should accurately position their differentiated advantages based on product characteristics, clarify brand positioning and unique value proposition through consumer insights and competitive environment analysis, refine the connotation of green brand culture, strengthen systematic brand management, formulate brand visual identity and behavioural code manuals, and enhance long-term brand value through social media operations. A multi-channel communication matrix should be built, consumer research strengthened, and a strategic layout implemented on mainstream platforms such as WeChat, Weibo, and Douyin (TikTok). Based on platform characteristics and different audience groups, differentiated content should be carefully designed, and execution strategies dynamically optimized. Weibo should respond quickly to public opinion, WeChat should provide in-depth interpretations of brand connotation, and Douyin should showcase product advantages through short videos, extending consumption scenarios and directly reaching users to achieve product market expansion and consumer recognition.

Emphasize Privacy Protection and Strengthen Marketing Data

Security to address the challenges of user information privacy leaks and data insecurity faced by enterprises in the process of new media

marketing, enterprises should strengthen user privacy protection and enhance data security. Enterprises should establish a sound new media marketing data security management system, classify and process new media marketing data, set access permissions for personnel, anonymize sensitive user data, and encrypt data storage and transmission to ensure the security of the entire process of new media operation data collection, processing, analysis, transportation, storage, and sharing.

CONCLUSIONS

In the context of dual transformation - digitalization and greening - marketing strategy innovation has become crucial for Chinese enterprises to increase market share and achieve sustainable development. This study finds that digitalization and greening are not independent but rather mutually reinforcing and deeply integrated. Digital transformation can promote green innovation, thereby enhancing enterprises' green competitive advantage in marketing activities. Under the dual transformation of digitalization and greening, enterprises can adopt five main methods for marketing strategy innovation: building a unified and coordinated green certification standard system, constructing a digital marketing system, actively innovating social media marketing scenarios, strengthening green brand value marketing, and strengthening marketing data security. The theoretical contribution of this study lies in enriching sustainable marketing theory and providing practical reference for enterprises to formulate and implement dual transformation marketing strategies. Future research can focus on cross-cultural comparisons and the impact of emerging technologies to further expand the research scope of marketing strategy innovation in the context of dual transformation.

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THE USE OF HOMEOPATHY IN THE AGROECOSYSTEM AND THE ONE HEALTH CONCEPT

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Abstract

The concept of One Health has emerged as a unifying approach to sustain, balance and optimize the health of people, animals and ecosystems. It recognizes that the health of humans, animals, plants, soils and the environment are closely linked and interdependent. This paper explores the use of Homeopathy in agriculture, as a scientific and holistic method which demonstrates and applies this unifying approach. Uniquely, the method uses the same natural remedies for humans, animals, plants and soil basing its indications on the interconnectedness of the ecological elements and on the dynamics of an agroecosystem. At the same time, the homeopathic preparations, are not focused on eliminating or killing organisms but rather work on the reestablishment of the homeostasis of the living organisms at very small doses, meaning they produce no residual harming effects to the environment and are efficient and economical, offering one of the best possibilities for the three pillars of the One Health Approach (people, animals, environment), therefore supporting the food systems and sustainable agricultural practices.

Key words*: Systemic health, homeopathic preparations, biostimulation, agriculture and environment, vitality.*

INTRODUCTION

Within the philosophical and methodological enquiry for the global sustainable food systems, the concept of One Health, emerges as a unifying approach that aims to sustain, balance and optimize the health of people, animals and ecosystems. It recognizes that the health of humans, animals, plants, soils and the environment are closely linked and interdependent (Capra & Luisi, 2014; Adisasmito et al., 2022). The roots of this concept can be traced from ancient times, when Hippocrates, in his work "Air, Waters and Places" recognized the connection between public health and a clean environment.

Within the health-based strategies for agroecosystems, the use of low-residual impact technologies and natural biostimulants is included. One of these methods is Homeopathy and its application to agriculture and environment (Boff et al., 2021). Homeopathy is a science and method of vitalizing the organisms by applying dynamized high dilutions used accordingly to the *simillimum* principle.

In the last 20 years, the research and practice using Homeopathy in agriculture and environment has been implemented and expanded in countries such as Brazil, Mexico, India, Germany, Italy and others (Faedo et al., 2024).

Scientific data and field experience have evidenced that the same homeopathic preparations used for humans are effective also for animals and plants. This is due to homeopathy`s unique methodology which allow an ecological reinterpretation of symptoms observed in complex living beings.

The dynamized high dilutions (DHD) act beyond the threshold of the Avogadro number, where mater becomes electromagnetic information patterns, operating in terms of biostimulation and morphogenesis, signalizing resonance patterns within the complexity of the agroecosystem (Bertalanffy, 1973; Sheldrake, 2013).

Research has evidenced they also contain elemental nanoparticles (Chikramane et al., 2010; Rajendran, 2017); with action via epigenetic mechanisms (Khuda-Bukhsh, 1997; Bellavite et al., 2015).

These theoretical bases of fields of information and organization of complexity in Nature have explained how living organisms depend for their life processes on the constant exchange of energy, matter and information, with the external environment, at all levels.

An interesting approach is explored by Manzalini & Galeazzi (2019), connecting the health of the system through quantum electrodynamics. The authors discuss on how every component has its own field-wave with a specific oscillatory pattern, resonating with other ecological oscillatory patterns. This illustrates how the pattern of the homeopathic remedies resonates across the system/organism, and why Homeopathy as a therapeutic system functioning for humans, animals, soil, plants, and for the whole environment.
So, Homeopathy, the Hippocratic-Hahnemannian Therapeutic System (Hahnemann, 1810), acts as a genuine holistic method which converges with the One Health approach.

OBJECTIVES

The objective of this work is to open the dialogue on how Homeopathy can be used as an agroecological method applied successfully in agriculture and the environment, illustrating this facet by exploring a few practical examples from the scientific literature. As an integrated discipline within the concept of One Health, Homeopathy applied in agriculture can help fostering innovative paths towards sustainability and productivity, with low residual impact for the agroecosystems.

METHODOLOGY

A narrative literature review exploring studies using homeopathic preparations across biological kingdoms is presented.

- **Ecolinguistics, health parameters and ecological correlations**

A typical example of a homeopathic preparation which can be useful both for humans, animals and plants is *Arnica*. It is a remedy which treats successfully the effects of an injury in human patients, but it is used with the same indication in animals and even in plants, for injuries after pruning, repotting, relocation. Another example is *Calendula* as a treatment for injuries especially when lacerations are present, a problem which can be treated in humans, animals or plants, the remedy being the same. *China officinalis* is a good treatment for effects after loss of fluids in humans (e.g. after haemorrhages), but a similar indication is met in plants after juice loss (e.g. after an infestation with sucking pests). *Dulcamara* is ideal for treating damage caused by wet weather and the consequences after a change of weather from warm and dry to cold and damp, this modality being similar both in humans and plants.
These initial examples demonstrate how using ecolinguistics allows one to expand use of classical homeopathic remedies and their symptoms to application on different living organisms.

- **Human and animal studies**

Studies (Frass et al., 2005; Zanasi et al., 2014; Macri, 2019) show that homeopathy is an important help in reducing the antibiotic use in human patients, but also in animals (Weiermayer et al., 2020).
Viksveen (2003), in an overview, explored homeopathy as a potential help to avoid unnecessary use of antibiotics, which offers a possible solution in future.
For animal health, a recent meta-analysis (Zeise & Fritz, 2019) concerning bovine mastitis shows that homeopathic treatments differ from placebo and many case reports point also to the efficiency of homeopathy in treating infections.
Main findings are presented in Table 1.

Table 1. Role of homeopathy in human and animal health (after Tripathi et al., 2024)

Authors	Methods	Results/Conclusions
Frass et al. (2005)	70 patients admitted to the MICU at the "University of Vienna" were evaluated for eligibility, and all eligible individuals were enrolled in the study. Each patient underwent randomization and received treatment; three participants had to be excluded due to incomplete data. All enrolled patients survived.	The use of homeopathic treatment appears to positively impact the extended survival of individuals experiencing severe sepsis. However, conclusive recommendations require additional research. A notable impediment to further research and implementation is the scarcity of trained homeopaths available.
Macri (2019)	A total of 90 children were enrolled in the study and randomly assigned to either the homeopathic treatment group or the control group, with each	Existing literature provides evidence that the utilization of CAM, including homeopathy, has the potential to decrease reliance on antibiotics.

Authors	Methods	Results/Conclusions
	group consisting of 45 patients. Antibiotics were administered to 33.3% of children in the treatment group, whereas 62.2% of children in the control group received antibiotics (*P*=0.006).	This bears favourable implications for strategies aimed at addressing the challenge of antibiotic resistance.
Zeise & Fritz (2019)	Homeopathic studies up to February 2018 were gathered through searches in the Carstens Foundation's online library, published meta - analyses, references from mastitis - related doctoral theses, and online databases like "NLM pubmed.de, orgprints.de, and researchgate.com".	In brief, homeopathy was effective in preventing and treating mastitis, especially in clinical cases. Challenges stem from subclinical cases lacking observable signs, complicating remedy selection.
Viksveen (2003)	An overview of the existing scenario regarding antibiotic resistance, along with an exploration of homeopathy as a potential substitute for antibiotics.	Global antibiotic resistance poses a public health challenge. Increased funding for research on homeopathic remedies, supported by positive study outcomes, is crucial for addressing recurring infectious diseases.
Weiermayer et al. (2018)	On June 17, 2016, a four year-old gelding with an acute injury to the right front leg was brought to an Austrian clinic specializing in equine medicine.	This case report shows the need for further investigations into the efficacy of *Silicea terra* and other homeopathic remedies in instances of multidrug - resistant bacterial infections in horses or animals overall.
Zanasi et al. (2014)	The research, spanning January to December 2012 at a specialized cough management clinic in Bologna, Italy, included 80 consecutive patients with uncomplicated upper respiratory tract infections.	Findings contribute to the increasing evidence supporting the safety and efficacy of homeopathy in managing acute productive cough.

- **Environmental studies**

Research by Aparicio et al. (2020) on *Phosphorus* 30 C and Pinto et al. (2021) on *Artemia salina* with *Mercurius* 30 C and their influence when used in lake and/or sea water in various experiments, underlines the environmental impact of homeopathic preparations, opening a new perspective on the role of homeopathy (Bonamin, 2014; 2020), besides the influence which homeopathic treatments can have by achieving a better health of all the elements of agroecosystems (Table 2).

Table 2. Role of homeopathy for the environment (after Tripathi et al., 2024)

Authors	Methods	Results/Conclusion
Aparicio et al. (2020)	Samples of water were collected from different times and locations within the lake system both before and after seeding. Comparative controls involved obtaining water from a nearby lake that had not undergone any treatment, with a volume of 1385 m^3.	The research suggests that *Phos* 30 C influence can spread across extensive water volumes, inducing system alterations in a connected lake. Monitoring these changes with solvatochromic dye MV allows the assessment of homeopathic medicines in large-scale applications.
Pinto et al. (2021)	*Artemia salina* cysts were exposed to mercury chloride at a concentration of 5.0 µg/mL during the hatching process, with varying potencies of mercury chloride (6CH, 30CH, 200CH) added to seawater. The study conducted four sets of nine experiments, investigating cyst hatching percentage, levels of soluble total mercury, precipitated mercury content, and potential physicochemical indicators of biological activity of mercury chloride.	Mc 30 CH is theorized to enhance *Artemia salina*'s natural resilience, potentially influencing hatching rate and mercury bioavailability, suggesting protective effects.
MV: Methylene violet		

RESULTS AND DISCUSSIONS

According to the World Health Organization (W.H.O.), among areas where One Health approach is particularly important, there are many of the domains where homeopathy applied in agriculture and environment can play a significant role, like: food safety, zoonotic disease control, environmental health and antimicrobial resistance (AMR).

- **Applying one health concepts**

Researchers from Brazil (Boff, 2009; Casali et al., 2009) are among the first who made the assertion that homeopathy can be applied to all the elements of an agroecosystem, after a careful observation, so that a successful homeopathic treatment for plants will influence all other elements, including the farmers and the whole environment (Maute, 2011).

The greatest challenge in using homeopathy in agriculture and environment remains the application of the principle of similarity (Carneiro, 2011), as the symptoms from classical *Materia Medica* are described for humans and from here, different approaches are needed. Ecolinguistics helps now to reinterpret the language for broad application on complex living systems (Table 3).

Table 3 - *Materia Medica* for some farm crops (after De Rezende, 2009)

Homeopathic Remedy	Symptoms
Arnica Montana	In cases of stress such as when transplanting, thinning, pruning, water deficiency and sudden damage by insects/ frosts/ harvests
Belladonna	Too many ants (spray remedy on leaves, plants and pathways)
Carbo vegetabilis	When there is general weakness, e.g. after insect attack, defoliation, water deficiency, close spacing, flower loss, death of buds, plants in compacted soils
Staphisagria	Attack of aphids, nematodes or mites, plants in excess shade, grafted plants and artificial insemination in animals, flea infestations
Nux vomica	Plants and soils polluted by pesticides
Sulphur	Excess transpiration, plants with high fertility needs, itches and scabies in animals (the remedy induces detoxification in plants, soils and animals)
Calcarea carbonica	Plants unresponsive to good fertility, chlorosis, seedlings sensitive to cold, delay in new root growth, slow plant development and leaf yellowing.

Many specialists use their knowledge for humans and make analogies with animals and plants, using repertorisation strategies to indicate the appropriated treatments.

In some cases, there is a literature of *Materia Medica* already developed for plant pathology (Kaviraj, 2006; Bonato, 2007; Tichavsky, 2007; Maute, 2011), but this work is only at its beginning.

To be able to apply dynamized high dilutions in agriculture and have results on a large scale, it is necessary to integrate this with social sciences, as the human factor is crucial and not separated from the environment it is inserted in.

The method needs specialized knowledge, therefore, training farmers and citizens who are interested in using a complex systemic therapeutic method to produce food free from pesticides residues, is needed.

In this way the use of homeopathy in agriculture became an effective social technology (Andrade & Casali, 2011). In Brazil, where homeopathy has been legislated for public use in the national health system, as well as in organic food production systems (2007; 2011), farmers are trained by the Agricultural Research and Rural Extension Service Agency (EPAGRI) of Santa Catarina State/Brazil and other state agencies, adopting homeopathy in their toolkits with outstanding results (Córdoba Correoso et al., 2022).

Homeopathy became a specialization discipline not only for physicians, but also for agronomists, veterinarians, psychologists, nurses, dentists, gardeners and farmers. For instance, since 2012, courses of 6 months (150 hours) were organised by the Laboratory of Homeopathy and Plant Health based in the EPAGRI of Lages, where members of different communities of practices received training on the method and homeopathic kits for general use in farms, plants, soil, animals and the environment.

CONCLUSIONS

From this literature analysis, it is clear that using Homeopathy to treat the environment, plants, animals and humans, focusses on the reestablishment of homeostasis in living

organisms. Its small doses, with no residual effects to the environment, are at the same time a method to increase the sustainability of the agroecosystem, being agronomically efficient and economically feasible.

The literature also identified that more agronomical trials, particularly at bigger scales, mainly on field conditions are needed to comprehend better the possibilities and limitations of using DHDs in agriculture. Nonetheless, the robust research already done in the topic represents basal standpoints to advance the important role which Homeopathy could play on the integration of multidisciplinary disciplines and communities of practices helping to attain the goals of the One Health and a sustainable future for all.

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