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Ukrainian Food Journal is an open-access, peer-reviewed scientific journal published by the National University of Food Technologies (Kyiv, Ukraine). The journal accepts original research articles, short communications, review papers, news, and literature reviews across all areas of food science, including food technology, engineering, nutrition, food chemistry, economics, and management.

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Editorial

Let us consider a phenomenon we have increasingly encountered in our editorial practice, which demands a substantial investment of time: the use of artificial intelligence (AI) in the preparation of scientific texts. At present, approximately every second manuscript we receive exhibits indications of AI-generated content. Notably, this trend is observed not only in submissions from PhD students but also in manuscripts written by experienced and well-established researchers.

We have received manuscripts in which the reference lists contained non-existent publications, while citing the names of well-known researchers and the titles of reputable journals. We have recently received a manuscript presented as original research that was actually based on two publications by a Chinese scientist. The AI-generated content indicated this by adding the note "(Author's data, 2025)" after each table. However, this issue appears to have gone unnoticed by both the PhD student and the supervisor. Also worth mentioning is a manuscript submitted under the names of two well-known American cardiologists and lacking author contact information, which contained a significant amount of poorly presented "experimental material" related to the issue of hamburger portion size in population nutrition.

These cases highlight not only the limitations and risks associated with the uncritical use of artificial intelligence, but also the growing need for stricter editorial screening procedures, enhanced reviewer awareness, and greater author accountability in the preparation of scientific manuscripts.

Sometimes, reviewers rely on artificial intelligence to evaluate submitted manuscripts. While such cases are relatively rare, many authors are increasingly using AI to describe data, formulate conclusions, and even compile reference lists.

Despite its broad capabilities, artificial intelligence often produces inappropriate responses, misinterpret research results, and may even generate fabricated findings that are subsequently incorporated into publications. In our opinion, the use of AI should be limited to auxiliary functions such as translation, grammar and punctuation checking, spelling and stylistic correction, and assistance with reference formatting. AI can also be useful for creating and improving graphical materials and, with caution, for assisting in the analysis of large datasets.

At the same time, we strongly discourage the use of AI for generating the main body of a scientific text, formulating hypotheses, or analyzing data without thorough verification by the author. Artificial intelligence should serve as a tool to support scientific work, but it must not replace the author of a scholarly publication.

We also call for reflection on the future, particularly for young researchers who risk losing essential skills in critical thinking and synthesis, expression of ideas and scientific analysis, and about the role and authority of the scientist.

The Ukrainian Food Journal will adhere to generally accepted practices regarding the use of artificial intelligence in publications, and we expect authors to comply with the principles of scientific ethics and academic integrity.

Sincerely,
Editor-in-Chief *Olena Stabnikova*

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Sustainable food design via fermentation of plant-based matrices

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Abstract

Introduction. Climate change and food security challenges have increased interest in plant-based diets; however, matrix complexity often limits nutrient bioavailability and functionality. Fermentation offers a targeted strategy to overcome these constraints.

Materials and methods. The study analyzed peer-reviewed scientific publications on the fermentation of plant-based food matrices and their nutritional, functional, and technological implications, based on literature searches conducted in PubMed, Scopus, Web of Science, ScienceDirect, and open-access sources, focusing primarily on publications from 2005 to 2026 using relevant keywords.

Results and discussion. Fermentation induces targeted biochemical changes in plant-based food matrices, leading to reported increases in protein digestibility of approximately 10–25%, improvements in mineral bioaccessibility of 15–50%, and reductions in antinutritional factors of up to 60–80%, together with enhanced techno-functional and sensory properties. In addition, fermentation promotes partial proteolysis and the release of free amino acids and bioactive peptides, contributing to improved nutritional value and potential bioactivity. It also facilitates the redistribution of phenolic compounds, increasing the proportion of soluble fractions and associated antioxidant capacity. Moreover, fermentation can modify carbohydrate structures, including partial hydrolysis of complex polysaccharides, which may improve digestibility and reduce gastrointestinal discomfort.

It also contributes to the development of desirable flavor compounds through microbial metabolism, enhancing consumer acceptability of plant-based products. The magnitude of these effects is strongly matrix-dependent and influenced by raw material composition, microbial strains, and processing conditions, while integration with technologies such as extrusion, baking, and drying supports the development of functional foods and plant-based meat analogues; however, variability among studies, lack of standardized protocols, and limited industrial-scale validation remain important challenges.

Conclusions. Fermentation is a versatile, low-energy approach that enables matrix-level optimization of the nutritional, functional, and sensory properties of plant-based foods, while requiring standardized and scalable evaluation for effective implementation.

Introduction

Global drivers for the transformation of food systems

In international and regional policy issues, the global transformation of food systems has emerged as a strategic priority because of challenges such as climate change, the growth of global populations, and food security. The Food and Agriculture Organization of the United Nations (FAO), the World Health Organization (WHO), and the European Union highlight the urgent challenge of developing sustainable food systems. Through policy frameworks such as the European Green Deal and the Farm to Fork Strategy, the EU specifically addresses the need to meet growing food demand without compromising nutritional quality for an expanding population (Cotta, 2025; FAO, 2022). One constant issue highlighted in these frameworks is the ever-widening gap between the requirements for international protein and supply in sustainable animal-based proteins in low- and middle-income regions. For this reason, plant-based diets are progressively established as a cornerstone of future diets in addition to animal products as a means of substituting for animal food in the modern world and as resilient and sustainable systems of food production (Prócel and Padilla, 2025; Verni et al., 2022).

Protein gap and the role of plant-based foods

International reports consistently highlight a growing mismatch between global protein demand and the availability of sustainable protein sources. Animal-based proteins, while nutritionally valuable, are associated with high environmental costs and limited scalability. As a result, plant-based protein systems are increasingly promoted not only as alternatives but as essential components of future resilient food systems, particularly in regions facing nutritional and economic constraints (Capcanari et al., 2025).

Plant sources, in particular cereals and legumes, shape the bulk of this transition, because they are widely produced, have a relatively low environmental impact, and offer complementary nutritional profiles. In combination, cereal-legume systems improve the quality of protein via optimized essential amino acid composition, and dietary fiber, minerals, and bioactive nutrients that are biologically significant to human health.

From a food system perspective, cereals and legumes are attractive raw materials because they:

– are widely produced and globally available;

– have a relatively low environmental footprint (Plaza-Bonilla et al., 2018);

– provide complementary amino acid profiles when combined (Han et al., 2021);

– represent suitable substrates for bioprocessing and functional optimization (Machida et al., 2022).

However, the application of cereals and legumes in food systems is limited by the inherent complexity of their food matrices, where interactions between proteins, starch, minerals, and antinutritional factors reduce nutrient bioavailability, impair techno-functional performance, and negatively affect sensory quality.

Therefore, this review aims to provide a structured and comprehensive synthesis of current knowledge on fermentation-induced biochemical transformations in cereal, legume-, and mixed plant-based food matrices, with a particular emphasis on their nutritional, functional, and technological implications. Special attention is given to matrix-dependent effects, fermentation strategies, integration with complementary processing technologies, and current limitations relevant to sustainable food design.

Materials and methods

This review relies on an analysis of various relevant scientific publications about the fermentation of plant-based food matrices with respect to their nutritional, functional, and technological effects. All the studies were peer-reviewed articles and review papers published by several authors worldwide with a focus on cereals, legumes, mixed cereal–legume systems, and selected plant-derived matrices. The search for literature was conducted using PubMed, Scopus, Web of Science, and ScienceDirect, along with relevant open-access sources, focusing on studies published mainly from 2005 to 2025. Keyword combinations on plant-based foods, fermentation, food matrix, nutritional quality, functional properties, antinutritional factors, sensory attributes, and sustainable food design were employed. Publications were included in the review due to their significance for fermentation-induced biochemical transformations and reported influence on nutritional quality, techno-functional performance, and product-related properties. In view of variability in experimental design and methodology, the results were compared according to commonality of trends, technological relevance, and limitations of the study from a matrix-oriented and design-driven approach.

Results and discussion

Plant-based food matrices as targets for fermentation

Concept of food matrix in plant-based foods. Food matrix is the concept of the three-dimensional organization of nutrients and non-nutritive components within a food (the physical structure and chemical interactions involved) used in food science. Instead of a reductionist mentality that views nutrients as having their own function, a food matrix perspective suggests that the interaction relationship among macromolecules (proteins, carbohydrates, and lipids), micronutrients, and bioactive compounds found present in the matrix determines nutritional, functional, and physiological activities. This organization is very complicated in plant-based foods, where cell walls, storage organelles, as well as a large number of structurally bound compounds exist (Alrosan et al., 2023; Nkhata et al., 2018). And in that sense, there is a significant differentiation between an ingredient and a food matrix. Ingredients (e.g., protein isolates, starches, fiber fractions) are commonly derived via intensive fractionation processes that disrupt native interactions and simplify composition. Such ingredients do provide some degree of technological predictability, of course, but they generally do not have the structural complexity or nutritional synergy that results from whole or minimally processed matrices. Plant-based food matrices (whole flours, meals, fermented batters) retain protein–carbohydrate-mineral-phytochemical interactions which strongly influence digestibility, bioaccessibility, and metabolic responses. Excessive fractionation can indeed enhance functionality, but according to the available literature on cereal- and legume-based foods, it may compromise sustainability and nutritional integrity, reinforcing the importance of matrix-oriented processing strategies (Tsafrakidou et al., 2020; Verni et al., 2022).

The protein-carbohydrate-bioactive interactions are fundamental for plant matrices of nutrition. Storage proteins in legumes and cereals are commonly known to have phytic acid, polyphenols, and non-starch polysaccharides that can form insoluble or poorly digestible complexes. The amount of phytic acid in raw legumes and cereals is between 0.5 and 2.5 g/100 g dry matter; condensed tannins (0.2–1.5 g/100 g depending on species and variety) are also expected. Such interactions decrease mineral bioavailability and protein digestibility, resulting in lower nutritional efficiency despite high protein content (Nkhata et al., 2018; Sakandar et al., 2023).

Table 1

Structural organization and interactions within plant-based food matrices

Matrix component	**Type of interaction**	**Structural role in the matrix**	**References**
Proteins	Protein–protein, protein–polyphenol	Network formation, water binding	Alrosan et al., 2022; Wang et al., 2022; Zhang et al., 2021
Starch	Starch–protein, starch–fiber	Viscosity, gelation, texture	Ren et al., 2024; Yildiz et al., 2013; Zhang et al., 2016
Non-starch polysaccharides	Fiber–water interactions	Hydratation, matrix rigidity	Li et al., 2023; Türker et al., 2023
Minerals	Mineral–phytate complexes	Spatial immobilisation within matrix	Lopes et al., 2021; Pragya et al., 2023; Raes et al., 2014
Polyphenols	Polyphenol–protein complexes	Structural stabilisation, sensory impact	Feng et al., 2023; Perez-Gregorio and Simal-Gandara, 2017

Fermentation is therefore particularly relevant, as it targets these matrix-level interactions rather than isolated components, enabling controlled biochemical restructuring of plant-based food matrices.

Types of fermentable plant-based food matrices

Plant-based food matrices used as substrates for fermentation can be classified according to their botanical origin, macronutrient composition, and structural organization. Legumes, cereals, pseudocereals, mixed cereal–legume systems, and plant-derived by-products differ substantially in protein content, carbohydrate profile, presence of antinutritional factors, and matrix complexity, which in turn determines their response to microbial metabolism during fermentation. This classification framework allows a matrix-oriented understanding of fermentation effects, highlighting how different plant substrates exhibit distinct nutritional, functional, and sensory outcomes following bioprocessing.

Legume-based matrices. Legumes represent one of the most intensively studied plant matrices for fermentation-based food development due to their high protein content (typically 18–35 g/100 g dry matter) and their central role in plant-based dietary patterns. Legumes can be characterized by high levels of antinutritional factors, too, including phytates, trypsin inhibitors (up to 20–40 mg/g in raw seeds), raffinose-family oligosaccharides (2–8% dry matter), and saponins, also in the legume matrix. The compounds negatively affect digestibility, mineral absorption, and sensory acceptance (Sakandar et al., 2023; Verni et al., 2022). Beyond conventional soaking and cooking treatments, De Pasquale et al. (2020) demonstrated that lactic acid fermentation is particularly effective in reducing saponins, which are biologically active glycosides known for their antinutritional effects, especially when applied in combination with starch gelatinization. Compared to gelatinization alone, fermentation resulted in a more pronounced degradation of antinutritional factors and contributed to a significant improvement in in vitro protein digestibility of legume flours (De Pasquale et al., 2020). Fermentation of legume matrices has been shown to reduce phytate content by 30–70%, depending on fermentation time, microbial strains, and pH evolution; it decreases trypsin inhibitor activity and oligosaccharide levels as well. Furthermore, proteolysis that is induced by fermentation enhances the free amino acids and small peptides, allowing for improved digestibility and, in some cases, improved umami perception. These effects make legumes ideal candidates for fermentation-driven matrix optimization (Adebo et al., 2022; Garrido-Galand et al., 2021).

Cereal and pseudocereal matrices. The vast majority of cereals and pseudocereals come in the form of carbohydrates with starch contents ranging from 60 to 75%, and protein contents between 7 to 15%. Although they are less protein-rich than legumes, cereals are major sources of dietary energy as well as micronutrients. Similar to legumes, cereal matrices contain phytic acid (typically 0.3–1.5 g/100 g) and phenolic molecules, limiting mineral bioaccessibility (Nkhata et al., 2018; Oboh et al., 2009).

Fermentation of cereal matrices—namely sourdough fermentation—has been shown to reduce phytic acid concentration by 40–90% when optimised, primarily due to endogenous and microbial phytase activity at acidic pH levels. Fermentation also alters starch–protein interactions, which in turn influence viscosity, glycemic response, and dough rheology. Fermentation in these contexts improves texture and flavor complexity, thereby aiding shelf life in breads and cereal-based porridges, giving added value to the aforementioned transformations (Gobbetti et al., 2020; Mykhonik and Hetman, 2022; Wanink et al., 1994).

Mixed cereal–legume matrices. Mixed cereal–legume matrices integrate the complementary nutritional properties of these two groups and are of growing interest as sustainable options other than animal-based protein systems. These matrices generally obtain improved amino acid balance, with legumes counterbalancing lysine deficiency in cereals and cereals providing sulfur-containing amino acids. In blended matrices, protein contents commonly range from 15 to 25 g/100 g depending on formulation (Chinma et al., 2025; Kaleda et al., 2020).

Fermentation of cereal–legume blends introduces additional complexity, as microbial metabolism must adapt to heterogeneous substrates. Experimental evidence supporting these synergistic effects in cereal–legume matrices was provided by Rizzello et al. (2014), who investigated sourdough fermentation of wheat flour enriched with chickpea, lentil, and bean flours at a total replacement level of 15% (w/w). The authors demonstrated that sourdough-fermented wheat–legume bread exhibited significantly higher concentrations of total free amino acids, enhanced phytase and antioxidant activities, and improved in vitro protein digestibility compared to yeast-fermented wheat bread. Moreover, sourdough fermentation contributed to a marked reduction of the starch hydrolysis index, indicating a lower predicted glycaemic response, while maintaining good sensory acceptability and crumb structure (Rizzello et al., 2014).

However, in most investigations synergistic effects are reported, such as increased degradation of phytates, increased digestibility of protein, and a more balanced sensory profile than with single raw material systems. They are of high potential to be used in sustainable food design as nutritionally adequate systems can be processed with moderate intensity and have functional versatility (Garrido-Galand et al., 2021; Patel et al., 2013).

Plant-based by-products and underutilized matrices. Plant-derived by-products, including bran, hulls, and milling fractions, are becoming more widely acknowledged as fermentable matrices loaded with high fiber and polyphenol contents. Although these matrices are usually characterized by lower amounts of protein, they can have significant levels of bound phenolic compounds and minerals. Fermentation has been reported to generate 20–60% augmented free soluble phenolics, combined with improved antioxidant activity, substantiating their valorization in circular food systems (Oboh et al., 2009; Szymandera-Buszka et al., 2021). These matrices are best incorporated at moderate levels into composite formulations due to technological and sensory limitations, rather than used as stand-alone substrates. However, their incorporation is also compatible with sustainability goals and demonstrates fermentation as an essential facilitator of by-product utilization.

Solid-state fermentation (SSF) represents a particularly suitable approach for the valorization of lignocellulosic agro-industrial residues (Stabnikova et al., 2010). In SSF systems, microorganisms grow directly on moist solid substrates that serve simultaneously as physical support and nutrient source. Filamentous fungi such as *Aspergillus* spp. and *Rhizopus* spp. demonstrate strong lignocellulose-degrading potential and organic acid production capacity, enabling structural disruption of plant cell walls and enhanced release of bound nutrients (Vassilev and De Oliveira Mendes, 2018).

Fermentation as a matrix-oriented biochemical tool

Fermentation represents a matrix-oriented biochemical approach that allows for modifiable rearrangement of plant-based food systems by way of microbial metabolism and enzyme activity. In cereal-based fermentations, lactic acid bacteria (LAB), yeasts, and filamentous fungi constitute the principal microbial groups involved, each contributing distinct metabolic pathways and enzymatic systems. LAB may be classified as homofermentative or heterofermentative depending on their metabolic end-products (Stabnikov et al., 2025), while yeasts contribute primarily to carbon dioxide production and flavor formation (Prakash, 2016). Unlike isolated nutrients, fermentation addresses all the interactions in the food matrix, including protein–carbohydrate–mineral and protein–polyphenol complexes, which play an important role in nutritional and functional performance of cereal- and legume-based foods. A clear illustration of this matrix-oriented processing strategy was provided by De Pasquale et al. (2020), who combined starch gelatinization with lactic acid fermentation to enhance the nutritional quality of legume flours. The authors showed that preliminary gelatinization increased substrate accessibility for microbial proteolysis, while subsequent fermentation intensified the degradation of antinutritional factors, particularly saponins, resulting in higher protein digestibility compared to raw or solely fermented flours (De Pasquale et al., 2020). In plant-based matrices, fermentation promotes a limited range of core biochemical events that carry wide relevance: acidification, activation of endogenous and microbial enzymes (e.g., phytases, proteases, amylases), partial hydrolysis of macromolecules, and redistribution of low-molecular-weight compounds. These changes are mainly pH-controlled, substrate availability, and microorganism–matrix specific and together induce adaptation in digestibility, bioaccessibility, and techno-functional properties reported in the following sections. Crucially, the strength and direction of fermentation effects are dependent on the matrix. Diverse results may also be obtained in legume-, cereal-, or mixed-origin matrices where the same fermentation conditions yield divergent outcomes due to differences in protein composition, starch structure, cell wall architecture, and antinutrient profiles. This matrix dependence explains the variability across studies and reinforces the feasibility of integrating fermentation strategies into a food design framework, where biochemical transformations are targeted toward relevant nutritional and functional outcomes rather than implemented as generic processing steps.

While Figure 1 presents a conceptual classification of plant-based matrices as fermentation targets, Table 2 integrates these matrix categories with representative fermentation strategies and reported biochemical transformations described in the literature.

Table 2

Overview of plant-based food matrices, fermentation strategies, and key biochemical targets

Plant-based matrix	Raw material examples	Fermentation type/ microorganisms	Biochemical targets	Key reported outcomes	References
Legumes	Chickpea, lentil, pea, faba bean	Lactic acid fermentation (*Lactiplantibacillus plantarum*, *L. brevis*, *Levilactobacillus fermentum*)	Phytic acid, trypsin inhibitors, raffinose-family oligosaccharides, storage proteins	Decrease in phytate (≈30–70%), reduction of trypsin inhibitor activity, increased protein digestibility, and free amino acids	Karovičová and Kohajdova, 2007; Nkhata et al., 2018; Verni et al., 2022
Cereals	Wheat, rye, oat, barley	Sourdough fermentation (LAB + yeasts)	Phytic acid, starch–protein interactions	Phytate reduction (≈40–90%), improved mineral bioavailability, enhanced dough rheology, and bread quality	Nkhata et al., 2018; Verni et al., 2022
Pseudocereals	Buckwheat, quinoa, amaranth	LAB fermentation	Bound phenolics, protein–polyphenol complexes	Increased free phenolic compounds, enhanced antioxidant activity, improved protein functionality	Oboh et al., 2009; Tsafrakidou et al., 2020
Cereal–legume blends	Wheat–pea, rice–bean, maize–soy	Controlled LAB fermentation	Antinutritional factors, protein–carbohydrate complexes	Improved amino acid balance, reduced antinutrients, more balanced sensory profile	Abdalla et al., 2025; Chinma et al., 2025
Plant-based by-products	Bran, hulls, milling fractions	Solid-state fermentation (LAB, fungi)	Bound phenolics, fiber matrix	Increase in free phenolics (≈20–60%), enhanced antioxidant activity, valorization of by-products	Oboh et al., 2009; Tsafrakidou et al., 2020
Mixed plant matrices	Legume flours, cereal meals	LAB fermentation + extrusion	Protein structure, flavour precursors	Improved texture, reduced beany flavour, enhanced umami perception	Licandro et al., 2020; Tsafrakidou et al., 2020
Legume proteins	Pea, lupin, chickpea proteins	LAB fermentation	Storage proteins, allergenic fractions	Improved solubility and emulsifying properties, reduced allergenicity	Gänzle, 2022
Fermented composite foods	Bread, porridges, extruded snacks	Sourdough / mixed fermentation	Starch–protein network	Improved viscosity control, digestibility, and sensory acceptability	Nout, 2009
Legume flours (pre-treated matrices)	Chickpea, lentil, pea	Starch gelatinization + lactic acid fermentation (LAB)	Saponins, protein–antinutrient complexes, storage proteins	Enhanced degradation of saponins, improved accessibility of storage proteins, increased in vitro protein digestibility, matrix-dependent nutritional optimization	De Pasquale et al., 2020
Cereal–legume composite foods (baked products)	Wheat–chickpea, wheat–lentil, wheat–bean blends	Sourdough fermentation (LAB-dominated)	Phytic acid, starch–protein network, storage proteins	Increased free amino acids, improved protein digestibility, enhanced phytase and antioxidant activity, reduced starch hydrolysis index, improved sensory acceptability	Rizzello et al., 2014
Legume matrices (soybean)	Soybean	LAB fermentation (*Lactobacillus plantarum*, α-galactosidase-producing strains)	Phytates, tannins, trypsin and protease inhibitors, raffinose-family oligosaccharides	Strong reduction of antinutritional factors, enzyme-driven improvement of nutritional quality and gastrointestinal tolerance	Adeyemo and Onilude, 2013
Cereals and pseudocereals	Wheat, maize, buckwheat, amaranth	Lactic acid fermentation (LAB, mixed cultures)	Phytates, enzyme inhibitors, protein–mineral complexes	Consistent reduction of antinutritional factors, improved mineral bioavailability and functional quality across matrices	Liptáková et al., 2017

Beyond botanical classification, the response of plant-based matrices to fermentation is strongly influenced by the metabolic capabilities and enzymatic profiles of the microorganisms involved. Understanding matrix–microorganism specificity is therefore essential for rational fermentation design.

Matrix constraints and fermentation-driven transformations

Strong interactions of proteins, starch, minerals, and phytochemicals, which are often mediated by antinutritional agents such as phytic acid, tannins, protease inhibitors, and α-galactosides, are commonly reported in reviews to restrict nutrient bioavailability, protein digestibility, and technological functionality but also to drive unfavorable sensory characteristics like bitterness, astringency, and legume-derived off-flavours (Nkhata et al., 2018; Sakandar et al., 2023; Verni et al., 2022). The main matrix-level limitations associated with cereal- and legume-based systems and their nutritional and technological implications are summarized in Table 3. These constraints represent key structural and biochemical barriers that justify the application of targeted bioprocessing strategies such as fermentation. These limitations are further illustrated schematically in Figure 1, highlighting the mechanisms of mineral chelation by phytic acid and protein binding by tannins that underpin reduced nutritional performance.

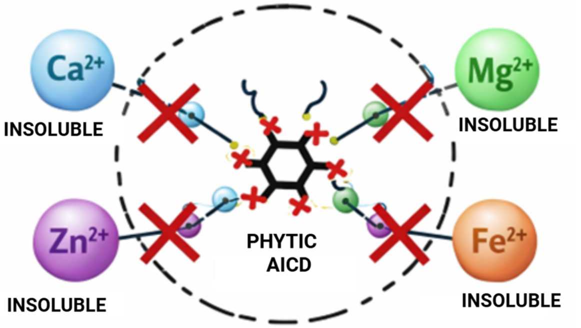
Table 3

Major limitations associated with cereal- and legume-based food matrices

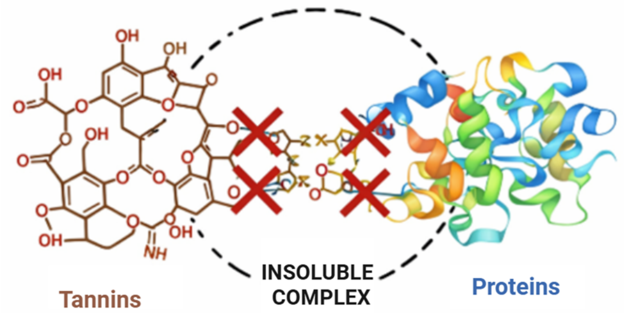
Matrix component	**Limiting factor**	**Consequence**	**References**
Phytic acid	Mineral chelation	Reduced Fe, Zn bioaccessibility	Sarkhel and Roy, 2022; Zhang et al., 2022
Tannins	Protein binding	Lower digestibility	Gilani et al., 2005, 2012; Kumar et al., 2022
Tannins	Iron chelators	Iron absorption inhibition (100 mg can inhibit it by up to 88%)	Okaiyeto et al., 2025
Raffinose-family oligosaccharides	Poor digestion	Gastrointestinal discomfort	Elango et al., 2022; Sanyal and Bishi, 2021
Protein–starch complexes	Structural rigidity	Reduced techno-functionality	Abdel-Aal, 2024; Navneet and Joye, 2025

Addressing these matrix-level constraints requires targeted processing strategies capable of modifying molecular interactions within the food matrix rather than simply altering ingredient composition.

Microbial metabolism, especially that of lactic acid bacteria, induces acidification, activates endogenous and microbial enzymes, and promotes controlled hydrolysis of macromolecules, enabling the degradation of antinutritional compounds, the release of bound minerals and phenolics, and partial proteolysis of storage proteins (Adebo et al., 2022; Alrosan et al., 2023).



Mineral chelation by phytic acid



Protein binding by tannins

Figure 1. Major matrix-level limitations of cereal- and legume-based foods and their implications for nutritional and functional performance
(created by the authors)

Early comprehensive reviews highlighted that legume- and cereal-based fermented foods have been integral components of traditional diets across Asia, Africa, and the Near East for centuries, primarily due to their improved digestibility, enhanced sensory attributes, and increased nutritional value compared to raw materials (Reddy et al., 1983). Building on this traditional knowledge, a growing body of experimental evidence demonstrates that fermentation markedly reduces key antinutritional factors in cereals and legumes, including phytic acid, tannins, trypsin inhibitors, and raffinose-family oligosaccharides, thereby improving mineral bioavailability and gastrointestinal tolerance.

Beyond antinutrient degradation, fermentation induces profound modifications at the protein level, where controlled proteolysis alters macromolecular interactions within the food matrix and directly affects techno-functional properties such as solubility, water-holding capacity, and emulsifying behavior. However, these effects are strongly dependent on the botanical origin of the substrate, the metabolic capacity of the selected microbial strains, and fermentation conditions. Consequently, increasing emphasis is placed on matrix-specific and process-controlled fermentation strategies rather than the application of generalized, one-size-fits-all fermentation approaches (Emkani et al., 2022; Garrido-Galand et al., 2021; Sakandar et al., 2023).

In addition to increasing nutritional quality, fermentation plays a key role in defining the functional and sensory properties of plant-based foods. In cereal-legume systems, fermentation alters the mechanism of interaction between starch and protein and hydration properties, which then influence viscosity, rheology, and texture – variables that are of particular concern to porridges, baked goods, and extruded products. It is well established that sourdough fermentation has proven to be a promising, sustainable means of utilizing the potential of legumes, pseudo-cereals, and milling by-products in baking applications, enabling higher inclusion levels while maintaining acceptable texture, aroma complexity, and shelf life (Chinma et al., 2025; Gobbetti et al., 2020; Rizzello et al., 2014).

Moreover, fermentation also aids in the biotransformation and redistribution of phytochemicals in plant-derived matrices. Studies concerning legumes and underutilized plant species have shown that via fermentation more free soluble phenolic substances are obtained at the expense of bound fractions, leading to an increase in the antioxidant activity. These effects are due to microbial and endogenous enzymatic activity disrupting cell wall structures and phenolic–macromolecule complexes. At the same time, critical assessments underline that increases in antioxidant capacity measured in vitro do not necessarily translate into proven physiological effects, reinforcing the need for cautious interpretation (Oboh et al., 2009; Verni et al., 2019).

In recent years, more and more research has stressed an integrated approach of fermentation together with complementary processing technology as a way for sustainable food design. Hybrid methods, which integrate fermentation with extrusion, baking, or controlled drying techniques, enable any of the biochemical changes seen during fermentation to be converted into tangible changes in texture, flavor development, and product stability. These methods also help to valorize the production of high protein, high fiber plant food items, which are convenient and shelf-stable, while the need for additives or intensive refining is minimized, and are largely consistent with sustainability targets in international food policy frameworks (Patel et al., 2013).

Together, available evidence provides the justification to view fermentation not only as a traditional process, but also as a strategic tool for the sustainable development of plant-based food products. By inducing controlled biochemical transformations within cereal-, legume-, and mixed plant-based food matrices, fermentation can improve nutritional quality, functional performance, sensory acceptance, and product safety. However, the reported effects of fermentation remain highly variable and strongly dependent on matrix composition, microbial strains, and processing conditions, while challenges related to process standardization and industrial scalability persist (Alrosan et al., 2023; Tsafrakidou et al., 2020).

Matrix–microorganism specificity in plant-based fermentation

The biochemical and functional outcomes of plant-based fermentations are determined not merely by the presence of microorganisms, but by the specificity of interactions between microbial enzymatic systems and the structural organization of the plant matrix. This matrix–microorganism specificity is increasingly recognized as a central determinant of fermentation efficiency, metabolic pathways, and nutritional outcomes.

Plant-based matrices differ considerably in macronutrient composition, endogenous enzyme activity, antinutritional factor content, and physical structure. Cereals are typically starch-dominant (60–75%), with moderate protein levels and active endogenous amylases, while legumes contain higher protein concentrations (18–35%) alongside phytates, trypsin inhibitors, raffinose-family oligosaccharides, and phenolic compounds. These compositional differences strongly influence microbial growth dynamics and enzymatic expression profiles (Gupta and Abu-Ghannam, 2012; McFeeters, 2004; Rombouls and Nout, 1995).

Lactic acid bacteria. In cereal fermentations, lactic acid bacteria (LAB), particularly representatives of the genera *Lactobacillus, Leuconostoc, Pediococcus,* and *Lactococcus*, dominate acidification processes. Homofermentative strains primarily convert hexoses into lactic acid, whereas heterofermentative strains produce lactic acid, acetic acid, ethanol, and CO_2, affecting dough rheology and sensory development (Blandino et al., 2003; McFeeters, 2004). The balance between these metabolic pathways influences texture, leavening, and flavor complexity.

In legume matrices, however, LAB performance depends strongly on strain-specific enzymatic systems. Strains of *Lactiplantibacillus plantarum* and *L. casei* exhibit variable proteolytic systems, phytase activity, and α-galactosidase production. These enzymatic differences determine the extent of phytate degradation, protein hydrolysis, and oligosaccharide breakdown (Demirci et al., 1998; Gupta and Abu-Ghannam, 2012; Liu et al., 2018).

Importantly, excessive proteolysis under prolonged fermentation can negatively influence sensory quality due to the accumulation of bitter peptides, highlighting the need for controlled fermentation windows.

Yeasts and symbiotic interactions. In cereal-based sourdough systems, *Saccharomyces cerevisiae* and non-*Saccharomyces* yeasts (e.g., *Candida* spp.) coexist with LAB in stable consortia. Yeasts metabolize maltose and glucose into ethanol and CO_2, contributing to leavening and volatile flavor compounds, while LAB lower pH and modify protein–starch interactions. Symbiotic interactions enhance fermentation stability and product quality (McFeeters, 2004; Mehta et al., 2012). Yeast–LAB interactions are less common in pure legume fermentations but become relevant in cereal–legume blends.

Filamentous fungi. In solid-state fermentations (e.g., tempe-type systems), *Rhizopus oligosporus* restructures dense protein matrices through mycelial penetration, enhancing nutrient accessibility. *Aspergillus oryzae* and *A. sojae* contribute strong proteolytic and amylolytic activities in soybean fermentations (Mehta et al., 2012). These systems differ fundamentally from LAB fermentations in enzyme spectrum and structural impact.

Process environment as modulator. Beyond microbial taxonomy and enzymatic repertoire, fermentation outcomes are strongly modulated by the physicochemical environment of the process. Parameters such as pH evolution, water activity, substrate particle size, oxygen availability, salt concentration, and temperature dynamically influence microbial growth kinetics and metabolic flux distribution (Berenjian, 2019). These variables determine not only the rate of acidification but also the expression and activity of key enzymes involved in phytate degradation, proteolysis, and carbohydrate hydrolysis.

Experimental studies on repeated-batch fermentations with Lactobacillus plantarum and related strains have demonstrated that medium composition and environmental conditions significantly alter lactic acid production rates and metabolic pathways, leading to variability in biochemical transformation efficiency (Singh et al., 2024). Likewise, marked differences have been observed between submerged and solid-state fermentation systems, where oxygen diffusion, moisture gradients, and substrate structure generate distinct enzymatic profiles and structural modifications within plant matrices (Hur et al., 2014; Rombouls and Nout, 1995).

These observations confirm that fermentation performance cannot be generalized across plant-based systems. Rather, it emerges from the coordinated interaction between botanical matrix composition, microbial enzymatic capacity, and tightly controlled process parameters. This matrix-dependent behavior has been experimentally demonstrated in cereal, pseudocereal, and legume substrates co-fermented with *Propionibacterium freudenreichii* and *Levilactobacillus brevis*, where vitamin B12 production varied markedly depending on buffering capacity, pH evolution, and substrate composition (Xie et al., 2021). Recognition of this matrix–microorganism–environment interplay is fundamental for rational fermentation design and for the development of structurally optimized, nutritionally enhanced, and sustainable plant-based food products.

A structured overview of matrix–microorganism interactions across major plant-based fermentation systems is presented in Table 4.

As shown in Table 4, fermentation performance is not uniform across plant matrices. Differences in substrate composition and microbial enzymatic potential result in distinct structural modifications and nutritional responses, reinforcing the importance of matrix-specific fermentation strategies.

Nutritional and functional implications of fermentation-induced biochemical transformations

Biochemical transformation due to fermentation in plant-based food matrices results in greater nutritional quality and increased performance of food, but the effects do vary significantly from raw material to raw material, from microbial cultures to processing conditions. Fermentation results need to be considered at the matrix level, not as isolated nutrient changes, as there is a close interdependence between composition, structure, and processing history (Tsafrakidou et al., 2020).

Protein digestibility and amino acid availability. Digestibility of protein content following fermentation is one of the reported nutritional benefits consistent between cereal-, legume-, and mixed matrices. A series of studies reports in vitro protein digestibility enhancement from 10 to 25 percentage points in fermented legume flours compared with unfermented ones, depending upon the time to ferment and microbial strains used. These enhancements are usually ascribed to partial proteolysis and disentanglement of protein–antinutrient complexes; however, the degree of proteolysis differs significantly between matrices (Tsafrakidou et al., 2020). In addition to digestibility, some authors have found that the availability of free amino acids and low-molecular-weight peptides is promoted after fermentation, with increments in the range of 20–60% relative to the raw materials. Yet reviews have shown that augmented availability of amino acids is not synonymous with sensory acceptance, as excessive proteolysis can cause bitterness or undesirable flavor development in certain legume matrices (Rizzelo et al., 2014).

Mineral bioaccessibility. Fermentation has been extensively studied as a way to enhance mineral bioaccessibility in plant-based foods by reducing phytic acid and other chelating compounds. Several authors have reported reductions in phytic acid content of 30–70% in fermented legume matrices and up to 40–90% in fermented cereal-based systems, particularly under conditions favoring phytase activity.

Table 4

Matrix–microorganism specificity in plant-based fermentation systems

Matrix Type	Dominant microorganisms	Key enzymatic activities	Structural impact	Nutritional outcomes	References
Cereals (wheat, rye)	*Lactobacillus* spp., *Saccharomyces cerevisiae*	Amylases, phytases, proteases	Acidification, starch hydrolysis, gluten network modification	Decrease in phytate content (40–90%), improved rheology	Blandino et al., 2003; McFeeters, 2004
Legumes (chickpea, lentil)	*L. plantarum*, *L. casei*	Proteases, phytases, α-galactosidase	Protein–phytate complex disruption	Increase in digestibility (10–25%), ↓ RFOs	Gupta and Abu-Ghannam, 2012
Cereal–Legume blends	LAB + yeasts	Combined proteolytic and amylolytic activity	Balanced acidification, matrix softening	Improved amino acid balance	Chinma et al., 2025; Patel et al., 2013
Solid-State Fermentation (soy/tempe)	*Rhizopus oligosporus*	Strong proteases, amylases	Mycelial penetration, increased porosity	Improved nutrient accessibility	Mehta et al., 2012
Bran / By-products	LAB, fungi	Esterases, phenolic-degrading enzymes	Cell wall disruption	Increase in free phenolic content (20–60%)	Oboh et al., 2009
Pseudocereals (quinoa, buckwheat)	*L. plantarum*, *Weissella* spp.	Phytases, phenolic esterases	Release of bound phenolics	Improved antioxidant activity	Tsafrakidou et al., 2020
Pea protein isolates	LAB strains (strain-dependent)	Proteolytic systems	Modification of protein solubility	Increase in protein solubility (15–50%), improved emulsification	Aderinola and Duodu, 2022
Fermented soy (koji systems)	*Aspergillus oryzae*	Strong proteases, amylases	Intensive macromolecule hydrolysis	Increase in free amino acids content, flavor compounds	McFeeters, 2004
Repeated-batch LAB systems	*L. plantarum*	Lactic acid production, metabolic flux adaptation	Altered acidification kinetics	Process optimization potential	Demirci et al., 1998
High-moisture cereal fermentation	LAB consortia	Phytase activation	Enhanced mineral release	Increased Fe, Zn bioaccessibility	Nkhata et al., 2018

The direct effect of the fermentation reaction is that the estimated bioaccessibility of mineral sources, including iron, zinc and calcium, has been reported to improve with relative improvements in mineral bioaccessibility usually between 15 and 50%, depending on the matrix and analytical technique (Adebo et al., 2022). However, some studies have noted that reducing phytate does not independently predict mineral uptake, with residual polyphenols and fiber composition in this respect remaining modulating factors for mineral binding (Raes et al., 2014). This highlights the importance of integration of matrix composition assessment, as opposed to dependence on single metrics.

Reduction of antinutritional factors and gastrointestinal tolerance. Other authors mentioned that fermentation leads to enhanced gastrointestinal tolerance of cereal- and legume-based foods due to lower levels of raffinose-family oligosaccharides and protease inhibitors. The reported decreases in α-galactosides are between 20 and 60%, and trypsin inhibitor activity may be reduced by 25–80%, depending on fermentation intensity. These effects are primarily attributed to the activity of microbial α-galactosidases and proteases, which hydrolyze fermentable oligosaccharides and enzyme inhibitors that otherwise contribute to bloating, flatulence, and impaired protein digestion. In this context, fermentation has been described as an ex situ digestion step that improves tolerance of plant-based foods, particularly in cereal- and legume-rich diets, by reducing FODMAPs (fermentable oligosaccharides, disaccharides, monosaccharides, and polyols) and immune-reactive proteins (Gänzle, 2020). These alterations are particularly significant in legume-containing formulations geared towards vulnerable populations or high-consumption scenarios. Consistent with these observations, Adeyemo and Onilude (2013) reported reductions of phytates (from 1.16 to 0.04 mg/g), tannins (from 1.93 to 0.12 mg/g), and trypsin inhibitor activity (from 1.20 to 0.01 mg/g) in soybeans fermented with α-galactosidase-producing *L. plantarum* strains, supporting fermentation as an effective strategy for improving gastrointestinal tolerance in legume-rich formulations (Adeyemo and Onilude, 2013). Similar conclusions were drawn by Gänzle (2020), who emphasized the relevance of fermentation-mediated degradation of raffinose-family oligosaccharides and protease inhibitors for improving digestive tolerance in sensitive individuals consuming plant-based foods.

Functional and technological implications

Hydration and rheological properties. Hydration behavior and rheological properties of plant-based matrices are drastically influenced by fermentation-induced restructuring. These have been attributed to changes in protein unfolding, partial hydrolysis of starch, and fiber structure modification and shown by increases in water absorption capacity of fermented flours in the order of 10–40% by several studies (Emkani et al., 2022). It is known that fermentation achieves a decrease in excessive viscosity of cereal–legume systems at an equivalent solids content, and improves nutrient density without compromising processability (Garrido-Galand et al., 2021). These effects may become an important consideration in all fermented batters, porridges and bakery applications, as viscosity control and water management are important aspects for the quality of the product as well as energy efficiency of the product.

Techno-functional properties of proteins. From a functional perspective, fermentation is said to affect protein solubility, emulsifying capacity, and foaming behaviour (Alrosan et al., 2023). A number of authors showed that protein solubility increased during fermentation by around 15–50%, particularly in legume matrices, with the highest increase in solubility observed, followed by a drop-off under highly acidic conditions. Enhancement of emulsifying activity and stability is most commonly observed within the moderate proteolysis stage, while excessive hydrolysis may impede the formation of networks and the integrity of the structure (De Pasquale et al., 2020). These results indicate that fermentation might benefit protein functionality within a defined processing window and therefore emphasize the optimization of the process.

Sensory-related functional outcomes. While not a primary sensory analysis per se, a combination of studies reported indirect sensory improvements associated with fermentation-induced biochemical alterations. In fermented cereal and pseudocereal products, these biochemical changes have been associated with improved flavor complexity, reduced bitterness, and enhanced consumer acceptance, highlighting the dual nutritional and sensory benefits of fermentation-driven matrix modification (Liptáková et al., 2017).

A reduction of beany or raw legume notes and the emergence of mild acidic and savory characteristics have been observed, especially in cereal–legume blends and fermented dough preparations. Nevertheless, some authors also point to bitter compounds in the fermented products when fermentation is prolonged or poorly controlled, with trade-offs in relation to nutritional enhancement and sensory quality identified.

These biochemical changes undergone by fermentation translate into nutritional and functional improvements at the plant-based food matrix scale, highlighting that fermentation should not be considered merely a preservation method, but rather an aspect of its conceptual design. Both the nutritional and technological effects of fermentation are mediated through the collective transformations of proteins, carbohydrates, minerals, and bioactive constituents of the matrix and are dependent on the composition of the raw materials, microbial consortia, and the environmental conditions of the fermentation process. Improved digestibility of protein across cereal, legume, and mixed-origin matrices is one of the strongest reported nutritional impacts of fermentation.

The digestibility of proteins in fermented legume flours was investigated in experimental studies such as (Nkhata et al., 2018; Verni et al., 2022), and fermented legumes exhibited 10–25 percentage points greater in vitro digestibility than unfermented flours. It is known that the disruption of protein–antinutrient complexes and an increase in enzymatic accessibility occur in the microbial and endogenous proteases-mediated proteolysis. At the same time, several authors present up to 20–60% increase in free amino acids and low-molecular-weight peptides, depending on fermentation duration and strain chosen (Abdalla et al., 2025; Chinma et al., 2025). However, reviews also warn that over-proteolysis is harmful to sensory quality by virtue of the production of bitter peptides in legume-rich matrices, suggesting the importance of fermentation strategy control.

Mineral bioaccessibility is another prominent health benefit of fermentation-induced phytic acid degradation. As indicated in numerous literature studies, phytate-content reductions of 30–70% in legume matrices and 40–90% in cereal-based systems are reported, especially for conditions that maximise phytase activity (Oboh et al., 2009; Verni et al., 2022). Consequently, there has been a reported 15–50% improvement in bioaccessibility of the minerals (iron, zinc, calcium) based on matrix composition and analytical methodology (Nkhata et al., 2018; Tsafrakidou et al., 2020). Importantly, several authors stress that phytate reduction alone does not fully predict mineral uptake. Residual polyphenols, fiber architecture, and protein interactions remain critical modulators of mineral binding. This demonstrates that it is essential to evaluate mineral bioaccessibility within the whole food matrix and not based on isolated compositional indicators alone.

Apart from nutrients alone, fermentation enhances the complete health potential of the nutritional composition of the plant-based matrix with respect to the reduction of the non-nutritional content due to anti-nutritional content to the gastrointestinal problems caused by fermentation. Categorical reductions in raffinose-family oligosaccharides (α-galactosides) have been documented with yields between 20% and 60%, and the action of trypsin inhibitor can be decreased by 25–80%, which is in part due to the fermentation kinetics and substrate (Chakraborty et al., 2022; Karovičová and Kohajdova, 2007; Nkhata et al., 2018). The latter becomes particularly important in legume-containing formulations for high consumption practices or for nutritionally sensitive populations.

Functionally and technologically, fermentation-mediated reorganization of macromolecules also influences hydration properties, rheology, and functionality of proteins. Studies on cereal–legume batters have shown that enzymatic enhancement of starch hydrolysis accelerates microbial metabolism, promotes faster acidification, and significantly modifies viscosity and gas retention behavior (Iyer and Ananthanarayan, 2008). The fermentation process is well-studied in many studies, which showed up to 10-40% increase in water absorption capacity of fermented flours, attributed to protein unfolding and partial starch hydrolysis along with fiber modification (Knez et al., 2023; Verni et al., 2022). These transformations generally lead to decreased viscosity at equal solids content, allowing higher density of nutrients and processability in batter, porridge, and fermented doughs.

Similarly, the solubility and interfacial properties of proteins are also influenced by fermentation. For instance, cereal–legume-based non-dairy probiotic beverages fermented with *Lactobacillus acidophilus* showed increased antioxidant capacity, total phenolic content, and viable cell counts as substrate concentration increased (Chavan et al., 2018). In legume-based systems, protein solubility increased by around 15–50% during moderate fermentation. Before excessive acidification occurred, this was observed as an optimal change (Aderinola and Duodu, 2022; Verni et al., 2022). Comparable improvements in free amino acid availability and protein functionality have been reported in cereal–legume sourdough systems fermented with *Lactobacillus plantarum*, where controlled fermentation enhanced both nutritional and technological properties (Coda et al., 2010).

Enhanced emulsifying performance and foam stability are frequently associated with limited proteolysis, whereas an excessive level of hydrolysis can impair network formation and structural integrity. Together, these results indicate that fermentation can be strategically applied to personalize techno-functional properties in a specified processing window. Finally, fermentation-mediated biochemical modifications also indirectly influence sensory properties such as texture and flavor development. Several authors reported the attenuation of beany or raw legume notes and the emergence of mild acidic and savory properties in cereal–legume blends and fermented dough systems. Still, a long or poorly regulated fermentation might result in the formation of bitter compounds, showing the need for a trade-off between nutritional benefit and sensory reception.

As a whole, these findings suggest that fermentation is indeed a form of matrix-level intervention with the potential to enhance nutritional quality and functional performance at the same time. Once incorporated into a food design system, fermentation facilitates the specific manipulation of biochemical reactions to promote certain nutritional and technological goals, instead of acting as a one-size-fits-all process.

Fermentation as a tool for sustainable food design

Fermentation has been increasingly acknowledged as a crucial enabling technology within sustainable food design, especially in plant-based food systems. Now, far removed from its previous role as a preservation technique, the notion of fermentation is understood in ways that are described as low energy, clean label and functionality-enhancing and is aligned directly to current sustainability targets, in terms of environmental impact reduction, limited list of ingredients and nutritional performance.

Fermentation as a low-energy and clean-label process. Fermentation runs at a less harsh temperature (about 25–40 °C) than conventional thermal or chemical processing, as well as with less mechanical input and lower energy requirements. There are multiple life cycle and process-oriented studies, and fermentation is seen as a resource-efficient technology when included in existing production chains (Verni et al., 2022). Different from enzymatic treatments, which utilize purified enzymes or chemical modifications, fermentation enables in situ enzyme production by microorganisms for use in clean-label formulations without the need for declared additives. Fermentation enables the functional and sensory-enhancing effect through biological transformation rather than ingredient enrichment from a clean-label perspective. A variety of authors emphasize that fermented plant-based products tend to reduce the role of stabilizers, emulsifiers and flavor-masking agents in the formulation of a product, thereby addressing consumer demand for minimally processed foods with recognizable processing histories (Knez et al., 2023; Tsafrakidou et al., 2020).

Relevance for functional foods and plant-based meat analogues. Fermentation also has a particularly strategic role in the development of functional foods and plant-based meat analogues. In protein-rich matrices, controlled fermentation has been observed to allow for improved emulsifying capacity, water-holding ability, and gel formation, all of which are important properties for the structuring of meat analogues. Moderate fermentation tends to improve protein network formation, whereas excessive proteolysis affects texture, thus highlighting the importance of process optimization (Aderinola and Duodu, 2022). Fermentation also plays a role in the development of products with increased protein quality, lower antinutritional content, and better micronutrient bioaccessibility, all within the framework of the functional food paradigm. Meanwhile, flavor complexity obtained through fermentation aids in a decrease of added flavor and salt in plant-based analogues, meeting both sensory and nutritional targets.

The integration of fermentation into plant-based food design results in simultaneous nutritional, functional, and sensory outcomes, as summarized in Table 4.

Integration of fermentation in plant-based product development. Fermentation plays an instrumental role in various growing applications in plant food, such as flours, doughs, beverages, snacks, and protein-rich formulas. Fermentation has been reported to improve protein digestibility (+10–25 percentage points), mineral bioaccessibility (+15–50%) and functional hydration properties (+10–40% water absorption capacity) within cereal–legume matrices, thereby making the matrix more suitable for downstream processes (Chinma et al., 2025; Nkhata et al., 2018). Most importantly, several authors stress the need to promote fermentation upstream in product design, rather than as a corrective step at the final formulation stage. Early fermentation allows the transformation of the food matrix prior to extrusion, baking, or drying, making the processed product easier to process and less complicated to formulate. In legume-rich environments, fermentation attenuates beany flavors and potential antinutritional limitations that impose barriers to inclusion (Karovičová and Kohajdova, 2007; Verni et al., 2019), ensuring the effective application of this approach.

Synergies between fermentation and complementary technologies. Increasing literature indicates the complementary impact of fermentation and various processing technologies like extrusion and baking, as well as drying. Fermented raw materials in extrusion procedures are frequently produced with improved flow behavior, decreased viscosity and enhanced expandability of the obtained ingredients to allow the production of texturized plant proteins and protein-rich snacks. For instance, lactic acid fermentation of pea protein followed by high-moisture extrusion has been shown to increase free glutamate levels, reduce characteristic pea-like off-flavors, and improve structural organization and texture of plant-based sausage analogues in a strain-dependent manner (Valtonen et al., 2023). There is research support that fermentation before extrusion can enhance the solubility of protein by 15–50% and can also decrease shear-induced protein aggregation and thus improve the texture and digestibility of extruded products (Licandro et al., 2020; Tsafrakidou et al., 2020). In baking applications, sourdough fermentation still stands out among the established types of fermentation-based food design. The integration of fermented legume flours into wheat-based doughs has also been demonstrated to significantly enhance nutritional density while still having a satisfactory condition of rheology and sensory profile (Chinma et al., 2025; Verni et al., 2022), including at 15–30% legume. Fermentation in combination with controlled drying practices (e.g., vacuum or low temperature drying) has also been investigated for the design of instant cereal–legume blends and fermented flours that retain functional and nutritional integrity in a package with minimal shelf space (Nkhata et al., 2018).

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Current limitations and research gaps in fermentation-based plant food design

Although the nutritional and functional effects of fermentation have attracted an increasing amount of support from experimental data in relation to plant-based food systems, some drawbacks and unaddressed gaps impair the interpretation, comparability, and application of the published results (Gänzle, 2020; Adebo et al., 2022). Upon further review of the literature, it is found that observed benefits are quite context-dependent and should be regarded in a larger context of matrix composition, processing environment, and product intended use (Gänzle, 2020).

Table 4

Nutritional, functional, and sensory outcomes associated with fermentation-based plant food design (literature synthesis)

Product category	Raw material base	Nutritional outcome (reported trends)	Functional / technological outcome	Sensory outcome	References
Fermented legume flours	Chickpea, lentil, pea	Improved protein digestibility; reduced antinutritional factors	Increased hydration capacity; improved processability	Reduced beany notes	Karovičová and Kohajdova, 2007; Nkhata et al., 2018; Verni et al., 2022
Bakery products (sourdough)	Wheat–legume blends	Enhanced mineral bioaccessibility; improved protein quality	Improved dough rheology and gas retention	Mild acidic and savory notes	Chinma et al., 2025; Verni et al., 2022
Extruded plant-based products	Cereal–legume matrices	Improved protein quality and digestibility	Improved expansion behavior and texture uniformity	Cleaner flavor profile	Licandro et al., 2020; Tsafrakidou et al., 2020
Instant cereal–legume blends	Cereals, legumes	Reduced viscosity at high solids content	Faster rehydration; improved flow properties	Improved mouthfeel	Nkhata et al., 2018; Nout, 2009
Plant-based meat analogues	Legume protein concentrates/ isolates	Enhanced protein functionality	Improved emulsification, gelation, and water retention	Improved juiciness and flavor complexity	Aderinola and Duodu, 2022; Gänzle, 2022
Fermented functional foods	Mixed plant matrices	Increased micronutrient bioaccessibility; reduced antinutrients	Stable matrix structure	Balanced acidity	Chinma et al., 2025; Knez et al., 2023

Reported outcomes reflect general trends described across multiple independent studies and may vary depending on matrix composition, microbial strains, and processing conditions.

The high variability in the experimental design, including the origin of raw material, particle size, pre-treatments, microbial species, fermentation time, temperature, and analytical steps, is among the most serious limitations of the studies in this paper (Adebo et al., 2022). For those reasons, reported enhanced protein digestibility, mineral bioaccessibility, and techno-functional properties are frequently quite broad in range, which complicates cross-study comparisons and meta-analytical interpretation (Adebo et al., 2022; Raes et al., 2014). A few authors have observed that fermentations under the same conditions might produce different results from cereal, legume, or mixed matrices depending on the fundamental protein composition, starch structure, cell wall architecture, and antinutrient profiles of the material (Gänzle, 2020).

This variability is intimately linked to the absence of standardized fermentation protocols optimized for plant-based food matrices (Adebo et al., 2022). Fermentation parameters are often optimized within individual studies, but very few attempts to achieve harmonization of methodologies or create reference conditions that would enable reproducibility and benchmarking across laboratories have been made. This limitation is especially salient in studies assessing protein digestibility and mineral bioaccessibility, where differences in in vitro models, enzyme systems, and extraction protocols can significantly influence reported outcomes (Raes et al., 2014). As a result, the gains made in one experimental setting may not be directly transferable to other contexts.

The other important limitation concerns the lack of generalizing results from laboratory scale to industrial processing conditions (Gobbetti et al., 2020). The majority of the research in fermentation is at bench scale with controlled and simplified systems, while industrial fermentation technology has higher variability of raw material quality, process stability, and energy efficiency. Reasons such as scale-up feasibility, robustness of the process, and integration into existing production lines have not been addressed, especially for extrusion, drying, or high-throughput baking (Gobbetti et al., 2020). This knowledge gap constrains the translation of attractive fermentation strategies into commercial plant-based products.

An application-oriented limitation that recurs and is consistently observed is insufficient incorporation of biochemical, functional, and sensory considerations into the same research. Though these were investigated for individual factors (e.g., phytate reduction, protein solubility, water absorption capacity), fewer investigations systematically link these biochemical reactions to techno-functional performance and consumer-relevant sensory characteristics (Gänzle, 2020; Gobbetti et al., 2020). As discussed in a number of reviews, increases in nutritional quality don’t always translate to acceptable texture, flavor, or overall product quality. Excessive proteolysis or acidification may enhance digestibility while simultaneously impairing sensory acceptance, implying that efficient strategies for balancing these processes are a critical concern.

Lastly, current investigations generally focus on the occurrence of single-strain fermentations in a controlled environment, which may lack the complexity necessary to comprehend the traditional or industrial fermentation environments. Mixed microbial consortia, back-slopping practices, and dynamic microbial succession – common in real-world fermentations – remain insufficiently characterized in relation to matrix restructuring and product performance (Gobbetti et al., 2020). Overcoming these limitations, fermentation can move beyond purely empirical methods to be a predictive, design-driven technology.

Conclusions

Fermentation is an efficient and sustainable bioprocess for the design of plant-based foods, as it enables the modification of food matrices rather than the manipulation of isolated nutrients. At the matrix level, fermentation induces biochemical changes such as phytate degradation, limited proteolysis, and carbohydrate restructuring, which contribute to increased protein digestibility, improved mineral bioaccessibility, and reduced levels of antinutritional factors in cereal-, legume-, and mixed plant-based systems.

These nutritional improvements are closely linked to enhanced functional and technological properties, including protein solubility, water and oil absorption capacity, emulsification, gelation, texture, and flavor development. However, the extent and nature of these effects are highly matrix-dependent and influenced by the composition of raw materials, microbial strains, and processing conditions, highlighting the need for design-led and controlled fermentation strategies.

Furthermore, the integration of fermentation with complementary processes such as extrusion, baking, and drying expands its applicability in the development of functional foods and plant-based meat analogues. Despite significant progress in this field, challenges remain, including high variability among studies, the lack of standardized fermentation protocols, and limited validation at the industrial scale. Therefore, future research should prioritize standardized and scalable approaches, along with comprehensive assessment of biochemical, functional, and sensory outcomes, to fully exploit fermentation as a strategic tool in sustainable food design.

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Physicochemical, rheological and sensory properties of cottage cheese beverage enriched with fresh and freeze-dried guava puree

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Abstract

Introduction. Dilution of the curd matrix in cottage cheese beverages often leads to reduced stability. The incorporation of guava puree may enhance physicochemical stability and improve rheological behavior of such systems.

Materials and methods. Cottage cheese was produced by acid coagulation of pasteurized milk and diluted to obtain a beverage base. Fresh and freeze-dried guava puree were incorporated at concentrations of 5, 7, and 9%. Physicochemical characteristics, water-holding capacity, rheological properties, total antioxidant activity, and sensory attributes were evaluated using standard analytical methods.

Results and discussion. The incorporation of guava puree significantly affected the physicochemical properties of cottage cheese-based beverages. Increasing the fruit concentration led to a gradual decrease in pH from 4.33 in the control sample to 4.17 and 4.18 in beverages containing 9% fresh and freeze-dried guava puree, respectively, while titratable acidity increased from 85 to 115 °T due to naturally occurring organic acids in guava. The addition of guava puree also increased the dry matter content of the beverages, with the highest value of 19.24% observed in samples containing 9% freeze-dried puree compared with 13.64% in the control. At comparable formulation levels, beverages prepared with freeze-dried guava puree showed higher dry matter content and lower serum separation than those containing fresh puree. The control sample exhibited the highest separated water value (3.0 mL per 100 mL), whereas the lowest value (1.4 mL per 100 mL) was observed in the beverage containing 9% freeze-dried puree, indicating improved physical stability of the diluted curd matrix. Rheological analysis revealed non-Newtonian shear-thinning behavior of all beverages, which was adequately described by the Ostwald–de Waele model. The incorporation of guava puree increased the consistency index, indicating enhanced resistance to flow. Fruit enrichment significantly increased the antioxidant potential, with total antioxidant activity rising from 71.7 µg/g dry matter in the control to 123.9–157.7 µg/g dry matter, depending on the type and concentration of guava puree. Sensory evaluation showed that guava puree improved the appearance, flavor, and texture of the beverages, with enriched samples receiving higher scores than the control. The formulation containing 7% freeze-dried guava puree achieved the highest overall acceptability (4.7 on a five-point scale) and exhibited a balanced fruity flavor and smooth, homogeneous texture.

Conclusions. The incorporation of guava puree improves the physicochemical stability, rheological characteristics, and antioxidant potential of cottage cheese beverages. Freeze-dried puree demonstrates greater effectiveness in achieving structural uniformity and higher sensory acceptability than fresh puree.

Introduction

Fermented dairy products play an important role in human nutrition as sources of high-quality proteins, minerals, and biologically active compounds (Berulava et al., 2024; Fox et al., 2017; Walstra et al., 2006). Among them, acid-curd cheeses occupy a specific position within the dairy sector due to their high protein content, low fat level, and favorable digestibility. Cottage cheese, a typical fresh acid-curd cheese, is widely consumed and frequently recommended for children, elderly individuals, and consumers seeking nutritionally balanced diets. Despite these advantages, traditional cottage cheese products are generally characterized by a mild flavor profile and limited sensory diversity, which may restrict their potential for product diversification and market expansion.

In recent years, increasing consumer demand for convenient and functional foods has stimulated the development of drinkable dairy products (Tamime and Robinson, 2007). Cottage cheese-based beverages, obtained by diluting and homogenizing the curd matrix, represent a promising approach to combine the nutritional value of acid-curd cheese with the sensory appeal and ease of consumption of liquid dairy products (Fox et al., 2017; Walstra et al., 2006). However, dilution of cottage cheese disrupts the acid-induced protein network, which may reduce structural stability and increase the tendency toward phase separation (Lucey, 2004; Sodini et al., 2004). As a result, cottage cheese-based beverages often face challenges related to physical uniformity and water retention, requiring appropriate formulation strategies to improve physicochemical stability and overall product quality.

One of the most effective strategies for enhancing the sensory characteristics of dairy beverages is the incorporation of fruit-derived ingredients (Dickinson, 2013; Martínez et al., 2012; Tamime and Robinson, 2007). Fruits contribute natural sweetness, color, and aroma, while also supplying vitamins, dietary fibre, and bioactive compounds (Gubsky et al., 2025; Martínez et al., 2012; Stabnikova et al., 2023, 2024; Tamime and Robinson, 2007). The enrichment of fermented dairy products with fruit components has been extensively studied, particularly in yoghurt and probiotic milk systems, where positive effects on consumer acceptance and nutritional value have been consistently reported (Chauhan et al., 2013; Patil et al., 2009; Walkunde et al., 2009). However, acid-curd systems such as cottage cheese differ fundamentally from yoghurt in terms of protein structure, buffering capacity, and acid-protein interactions. Consequently, technological responses observed in yoghurt-based beverages cannot be directly transferred to cottage cheese beverages.

Guava (*Psidium guajava* L.) is a tropical fruit known for its high nutritional value and distinctive sensory characteristics. It is particularly rich in vitamin C, phenolic compounds, carotenoids, and dietary fibre, which contribute to its antioxidant properties and potential health benefits (Jiménez-Escrig et al., 2001; Martínez et al., 2012; Thaipong et al., 2006). Previous studies have demonstrated the feasibility of incorporating guava pulp or puree into various dairy products, mainly yoghurt and fermented milk, resulting in improved sensory attributes and enhanced nutritional profiles (Chauhan et al., 2013; Hui et al., 2022; Patil et al., 2009; Walkunde et al., 2009; Xu et al., 2026). Despite these promising findings, the application of guava in acid-curd dairy beverages remains poorly investigated, especially in relation to its impact on the physicochemical stability and structural properties of diluted cottage cheese systems.

In addition to fruit selection, the technological form of fruit ingredients represents a critical factor influencing product quality. Fresh fruit purees are commonly used in dairy formulations but contain high moisture levels and may exhibit variability in composition and stability. In contrast, freeze-dried fruit ingredients offer concentrated solids, reduced water activity, and improved storage stability, while retaining a high proportion of bioactive compounds (Karam et al., 2016; Kumar and Sagar, 2014). The use of freeze-dried fruits has demonstrated technological advantages in several dairy applications; however, comparative studies evaluating the effects of fresh and freeze-dried fruit ingredients in cottage cheese-based beverages are still limited.

From a technological standpoint, the interaction between fruit components and the acid-curd protein matrix is of particular importance. Guava contains organic acids and soluble dietary fibre that may influence acidity balance, dry matter content, and water-binding properties of cottage cheese beverages (Jiménez-Escrig et al., 2001; Martínez et al., 2012; Sendra et al., 2008; Turgeon and Beaulieu, 2001). The form in which guava is incorporated may significantly influence physicochemical stability, texture, and sensory perception. Therefore, a systematic comparison of fresh and freeze-dried guava puree in a cottage cheese beverage matrix is necessary to provide insights into formulation optimization and industrial feasibility.

Therefore, the objective of this study was to develop a cottage cheese-based beverage enriched with guava puree and to evaluate the effects of guava form (fresh and freeze-dried) and concentration on physicochemical, rheological, and sensory properties, as well as water-holding capacity. The study provides matrix-specific technological insight into the application of tropical fruit ingredients in acid-curd dairy beverages.

Materials and methods

Materials

Pasteurized cow's milk with a fat content of 3.2% (Vinamilk, Vietnam Dairy Products Joint Stock Company, Ho Chi Minh City, Vietnam) was used as the primary raw material for cottage cheese production. The milk was obtained from a local dairy processor and stored at 4±1 °C prior to use. A commercial mesophilic lactic acid starter culture containing *Lactococcus lactis* subsp. *lactis* and *Lactococcus lactis* subsp. *cremoris* was used for milk fermentation. Calcium chloride ($CaCl_2$, food-grade) was applied to improve curd formation.

Guava fruits (*Psidium guajava* L.) at commercial maturity were used as the fruit ingredient. Two forms of guava puree were prepared and evaluated: fresh guava puree and freeze-dried guava puree. Granulated sucrose (food-grade) was used as a sweetening agent. All reagents used for physicochemical analyses were of analytical grade.

Preparation of guava puree

Fresh guava fruits were washed thoroughly under running potable water, manually peeled, and deseeded. The edible portion was cut into small pieces and homogenized using a laboratory blender (HR3652, Philips, Netherlands) until a uniform puree was obtained. The fresh puree was used immediately for beverage formulation to minimize enzymatic browning and nutrient degradation.

For the preparation of freeze-dried guava puree, fresh guava puree was frozen at -40 °C for 24 h and subsequently subjected to freeze-drying using a laboratory freeze dryer under vacuum conditions until a constant weight was achieved. The freeze-dried material was ground into a fine powder and stored in airtight containers at room temperature, protected from light and moisture. Prior to use, the freeze-dried guava powder was rehydrated with distilled water to obtain a dry matter content comparable to fresh guava puree (≈ 16.46±0.01% dry matter).

Production of cottage cheese

Cottage cheese was produced using conventional acid-curd technology. Pasteurized milk was heated to 30±1 °C and inoculated with the mesophilic starter culture at the dosage recommended by the manufacturer. Calcium chloride was added at a concentration of 0.02% (w/w). The milk was gently mixed and allowed to ferment at 30 °C until the pH reached approximately 4.6.

After coagulation, the curd was cut into uniform cubes (approximately 1.5-2.0 cm) to facilitate whey separation. The curd-whey mixture was gently stirred and allowed to rest to enhance syneresis. Whey was drained, and the curd was washed once with cold potable water to reduce acidity and obtain the characteristic mild flavor of cottage cheese. The washed curd was drained thoroughly and stored at 4 °C for no longer than 12 h before beverage preparation.

Preparation of cottage cheese-based beverage

The cottage cheese-based beverage was obtained by diluting the cottage cheese system to achieve a drinkable consistency, followed by homogenization to ensure uniform dispersion. Dilution was applied solely to modify the physical structure of the cottage cheese system and did not involve additional fermentation or compositional modification. Dilution of the cottage cheese matrix resulted in a smoother and more drinkable texture (Fox et al., 2017; Walstra et al., 2006).

Guava puree (fresh or freeze-dried, rehydrated) and sucrose were added to the beverage base according to the experimental design. The mixture was homogenized using a laboratory-scale homogenizer (T25 digital ULTRA-TURRAX, IKA-Werke GmbH & Co. KG, Germany) at 10,000 rpm for 2 min to ensure uniform dispersion of fruit particles and stabilization of the beverage matrix. No stabilizers or artificial colorants were used in order to evaluate the intrinsic effects of guava puree on product quality.

Experimental design

A formulation-based experimental design was used to evaluate the incorporation of guava puree into a cottage cheese beverage system. Guava puree concentration (5%, 7%, and 9%, w/w) and guava form (fresh or freeze-dried) were considered as the main formulation variables.

Sucrose was added at a constant level of 5% (w/w) in all fruit-containing formulations in order to provide a balanced sweetness profile and comparable sensory conditions among samples. Sugar was therefore treated as a fixed formulation component rather than an experimental factor.

Each formulation was prepared using either fresh or freeze-dried guava puree, resulting in six experimental samples. An additional control sample without guava or sucrose was also prepared. The formulation matrix of the cottage cheese beverages is presented in Table 1.

All formulations were produced in triplicate on different production days to ensure experimental reproducibility. After preparation, the beverages were stored at 4±1 °C and analyzed within 24 h.

Table 1
Formulation matrix of cottage cheese-based beverages

Sample	Guava puree (%, w/w)	Sugar (%, w/w)	Guava form
F0	0	0	- (Control)
F5	5	5	Fresh
F7	7	5	Fresh
F9	9	5	Fresh
FD5	5	5	Freeze-dried
FD7	7	5	Freeze-dried
FD9	9	5	Freeze-dried

Note: F0, control sample without guava and sugar addition;
F, fresh guava puree; FD, freeze-dried guava puree.

Physicochemical analysis

All physicochemical analyses were carried out according to standard methods (AOAC, 2016). The pH of the samples was measured at 20±1 °C using a calibrated digital pH meter (SevenCompact S220, Mettler Toledo, Switzerland). Titratable acidity was determined by titration with 0.1 N NaOH and expressed in degrees Thörner (°T), where 1°T corresponds to 0.009 g lactic acid per 100 mL of sample. Dry matter content was measured by oven-drying samples at 105 °C using a laboratory drying oven (UN55, Memmert GmbH, Germany) until constant weight was achieved. Serum separation (mL/100 mL), used as an inverse indicator of water-holding capacity (WHC), was determined by centrifugation of 10 g samples at 3000 × g for 10 min at 20 °C using a laboratory centrifuge (5804R, Eppendorf AG, Germany), and expressed as milliliters of separated water per 100 mL of sample.

All physicochemical analyses were performed in triplicate, and results were expressed as mean values with standard deviations.

Rheological analysis

Rheological properties of cottage cheese beverages were determined using a rotational viscometer (Rheotest-2, VEB MLW Medingen, Germany). Measurements were carried out at different rotor speeds corresponding to various shear rates. Instrument readings were converted into shear stress (τ, Pa) and apparent viscosity (η, Pa·s) using the instrument calibration constants.

Flow behavior was described using the Ostwald-de Waele (power law) model:

$$\tau = K \cdot \gamma^n,$$

where τ is shear stress (Pa), γ is shear rate (s^{-1}), K is the consistency index ($Pa \cdot s^n$), and n is the flow behavior index. Effective viscosity at a shear rate of 1 s^{-1} was used for comparative evaluation of sample consistency (Rao, 2014; Steffe, 1996). Measurements were conducted at 20±2 °C in triplicate.

Total antioxidant activity

Total antioxidant activity (TAA) of the beverage samples was determined by coulometric titration with electrogenerated bromine using an “Expert-006” coulometric analyzer. The method is based on the reaction between antioxidant compounds present in the sample and bromine generated electrochemically in the titration cell.

The total antioxidant capacity was calculated according to Faraday's law based on the amount of electricity consumed during bromine generation. Results were expressed as micrograms of antioxidant equivalents per gram of dry matter (μg/g dry matter).

All analyses were performed in triplicate and the results were reported as mean±standard deviation.

Sensory evaluation

Sensory evaluation was conducted by a trained panel consisting of 10-12 members experienced in dairy product assessment. The evaluation was carried out in a sensory analysis laboratory under controlled conditions in accordance with international sensory analysis guidelines (ISO 8586, 2012).

Samples were coded with random three-digit numbers and presented in a randomized order at 10±2 °C (ISO 8589, 2008). Panelists evaluated appearance, aroma, flavor, texture, and overall acceptability using a 5-point hedonic scale, where 1 indicated "dislike extremely" and 5 indicated "like extremely" (Meilgaard et al., 2016; Stone et al., 2012). Drinking water was provided for palate cleansing between samples.

Statistical analysis

Experimental data were analyzed using two-way analysis of variance (ANOVA) to evaluate the effects of guava form (fresh vs. freeze-dried) and guava concentration (5-9%). Differences between means were considered statistically significant at $p < 0.05$. Statistical analysis was performed using SPSS software (IBM Corp., USA).

Results and discussion

Physicochemical characteristics of cottage cheese beverages

The physicochemical properties of cottage cheese-based beverages enriched with guava puree are presented in Table 2. The results indicate that both the form and concentration of guava puree significantly influenced pH, titratable acidity, dry matter content, and water-holding capacity of the beverages ($p < 0.05$). These parameters are important indicators of technological quality and structural stability of acid-curd dairy beverages.

Table 2

Physicochemical properties of cottage cheese beverages enriched with guava puree

Sample	pH	Titratable acidity (°T)	Dry matter (%)	Serum separation (mL/100 mL)
F0	4.33±0.02^{a}	85±1^{a}	13.64±0.30^{a}	3.0±0.1^{a}
F5	4.27±0.02^{b}	96±2^{c}	17.76±0.35^{c}	2.0±0.1^{c}
F7	4.22±0.02^{c}	103±2^{d}	17.36±0.30^{bc}	1.9±0.1^{c}
F9	4.17±0.02^{d}	115±2^{f}	15.77±0.28^{b}	1.7±0.1^{d}
FD5	4.28±0.02^{b}	92±1^{b}	19.24±0.32^{d}	1.7±0.1^{d}
FD7	4.23±0.02^{c}	99±2^{cd}	18.86±0.31^{d}	1.6±0.1^{d}
FD9	4.18±0.02^{d}	111±2^{e}	17.21±0.29^{bc}	1.4±0.1^{e}

Note: Values are expressed as mean±standard deviation ($n = 3$). Different superscript letters within the same column indicate significant differences ($p < 0.05$) according to Tukey's test.
°T: degrees Thörner (1°T = 0.009 g lactic acid per 100 mL).

A gradual decrease in pH was observed with increasing guava concentration regardless of the form of puree used. The control sample exhibited the highest pH value (4.33), whereas the lowest values were recorded in samples containing 9% guava puree (4.17 for fresh puree and 4.18 for freeze-dried puree). This decrease can be attributed to the presence of organic acids naturally occurring in guava, such as citric and malic acids, which contribute to the overall acidity of the system (Sharma et al., 2009). Similar trends have been reported in fruit-enriched fermented dairy products, where increasing fruit addition leads to measurable reductions in pH due to acid diffusion from the fruit matrix into the dairy phase.

Titratable acidity values increased consistently with increasing guava concentration, confirming the pH results. The control sample showed the lowest acidity (85 °T), while the highest value was observed in the beverage containing 9% fresh guava puree (115 °T). Samples formulated with freeze-dried guava puree exhibited slightly lower acidity at comparable concentrations (92–111 °T) than those containing fresh puree. From a technological perspective, excessive acidity may negatively influence flavor balance and protein aggregation behavior in acid-curd systems, potentially affecting beverage texture. In this respect, formulations containing 7% guava puree may represent a compromise between sufficient fruit flavor and acceptable acidity.

Dry matter content was strongly influenced by both guava concentration and the form of guava puree. In general, samples containing freeze-dried guava puree exhibited higher dry matter values than those prepared with fresh puree at comparable concentrations. The highest dry matter value (19.24%) was observed in the beverage containing 5% freeze-dried guava puree, whereas the control sample showed the lowest value (13.64%). This effect is associated with the low moisture content and high solids concentration of freeze-dried ingredients. Increased dry matter content contributes to improved body, mouthfeel, and structural stability of dairy beverages.

Serum separation (mL/100 mL), used as an indicator of water-holding capacity, was significantly affected by the incorporation of guava puree (Table 2). The control sample showed the highest serum separation (3.0 mL per 100 mL), indicating relatively low structural stability of the diluted curd matrix. The addition of guava puree significantly reduced water separation in all formulations. The lowest value (1.4 mL per 100 mL) was observed in the sample containing 9% freeze-dried guava puree, demonstrating that guava solids may contribute to improved water retention.

The reduction in water separation may be associated with interactions between milk proteins and fruit-derived polysaccharides, which enhance the stability of the beverage matrix. Overall, the results demonstrate that incorporation of guava puree, particularly in freeze-dried form, improves the physicochemical stability of cottage cheese-based beverages by increasing solids content and enhancing water retention within the acid-curd matrix.

Antioxidant activity

The total antioxidant activity of the samples is presented in Figure 1. The results demonstrated that the incorporation of guava puree significantly increased the antioxidant capacity of cottage cheese beverages compared with the control sample ($p < 0.05$).

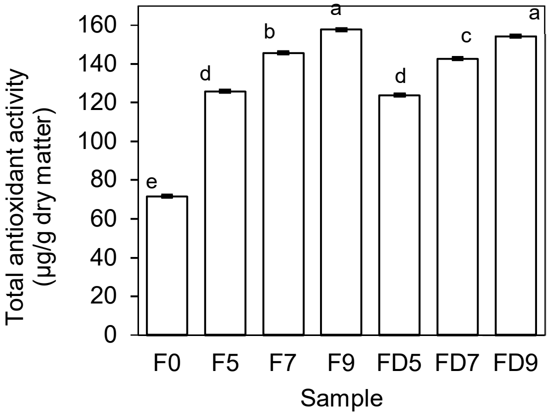

Figure 1. Total antioxidant activity, µg/g dry matter, of cottage cheese beverages enriched with fresh and freeze-dried guava puree

The control sample (F0) showed the lowest antioxidant activity (71.7 µg/g dry matter), while all guava-enriched formulations exhibited substantially higher values. In beverages containing fresh guava puree, antioxidant activity increased progressively with increasing fruit concentration, reaching 125.9, 145.7, and 157.7 µg/g dry matter for samples containing 5%, 7%, and 9% puree, respectively. A similar trend was observed for beverages prepared with freeze-dried guava puree, with values ranging from 123.9 to 154.3 µg/g dry matter.

At comparable formulation levels, samples containing fresh and freeze-dried guava puree showed relatively similar antioxidant activity, indicating that both forms of guava provide substantial amounts of bioactive compounds. The increase in antioxidant capacity can be attributed primarily to the presence of phenolic compounds, vitamin C, and other natural antioxidants naturally occurring in guava fruit.

Overall, the results confirm that the incorporation of guava puree enhances the functional properties of cottage cheese-based beverages by increasing their antioxidant potential.

Rheological analysis

The flow curves of the cottage cheese beverage samples are presented in Figure 2. In all formulations, shear stress increased with increasing shear rate, indicating non-Newtonian flow behavior typical for protein-based dairy dispersions. The presence of guava puree noticeably influenced the magnitude of shear stress across the entire shear rate range. Formulations containing the same concentration of guava puree, regardless of whether fresh or freeze-dried fruit was used, exhibited very similar rheological behavior, resulting in overlapping curves; therefore, only representative curves are shown in the figure for clarity.

Samples containing higher concentrations of guava puree exhibited higher shear stress values compared with the control sample, suggesting a progressive strengthening of the beverage structure. This effect was particularly evident in samples containing 7% and 9% guava puree, which showed steeper flow curves than formulations with lower fruit content. The increase in shear stress can be attributed to the higher concentration of suspended solids and the interaction between milk proteins and fruit-derived polysaccharides.

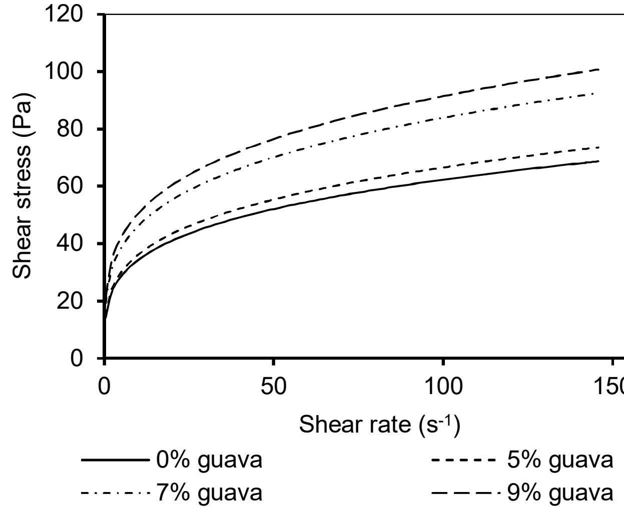

Figure 2. Flow curves (shear stress vs. shear rate) of cottage cheese beverages containing different concentrations of guava puree. Curves represent the control sample and beverages formulated with fresh guava puree (5–9%)

In addition to flow curves, the relationship between apparent viscosity and shear rate was analyzed to further characterize the rheological behavior of the beverages (Figure 3).

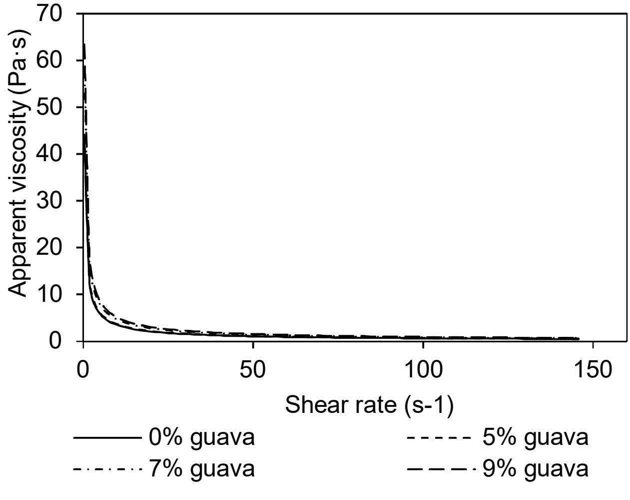

Figure 3. Apparent viscosity curves of cottage cheese beverages with different concentrations of guava puree

As shown in Figure 3, the apparent viscosity of all samples decreased markedly with increasing shear rate, indicating typical shear-thinning behavior. Such behavior is characteristic of structured food systems in which the internal network gradually breaks down and aligns in the direction of flow under mechanical stress.

The rheological data were successfully described using the Ostwald-de Waele (power law) model, and the calculated parameters are presented in Table 3. The flow behavior index (n) values ranged from 0.258 to 0.269, which are all below unity, confirming the pseudoplastic nature of the beverages. These results are consistent with the viscosity curves observed experimentally.

Table 3

Rheological parameters of cottage cheese beverages obtained from the Ostwald-de Waele model

Sample	Consistency index (K) (Pa·s^n)	Flow behavior index (n)	R^2
F0	18.96 ± 0.46^d	0.258 ± 0.004^a	0.945
F5	20.59 ± 0.49^c	0.264 ± 0.003^b	0.965
F7	25.51 ± 0.52^b	0.259 ± 0.004^a	0.948
F9	26.03 ± 0.53^a	0.269 ± 0.003^c	0.956
FD5	20.65 ± 0.48^c	0.263 ± 0.003^b	0.958
FD7	25.55 ± 0.51^b	0.258 ± 0.004^a	0.938
FD9	26.18 ± 0.54^a	0.266 ± 0.003^b	0.946

Note: Values are expressed as mean±standard deviation (n = 3). Different superscript letters within the same column indicate significant differences ($p < 0.05$) according to Tukey's test.

The consistency index (K) increased with increasing guava concentration, ranging from 18.96 Pa·s^n in the control sample to approximately 26 Pa·s^n in samples containing 9% guava puree, indicating a gradual increase in the structural consistency of the beverages. This trend reflects the higher total solids content introduced by guava puree and the formation of a more structured protein-polysaccharide matrix.

At comparable formulation levels, beverages containing freeze-dried guava puree exhibited slightly higher consistency indices than those prepared with fresh puree. This behavior can be explained by the higher solids concentration and enhanced water-binding capacity of freeze-dried ingredients after rehydration.

Overall, the results demonstrate that the incorporation of guava puree modifies the rheological properties of cottage cheese beverages by increasing structural consistency while maintaining desirable shear-thinning behavior, which is advantageous for beverage processing and consumer perception during drinking.

Sensory evaluation

The results of sensory evaluation are summarized in Table 4. The incorporation of guava puree significantly improved all evaluated sensory attributes, including appearance, flavor, texture, and overall acceptability ($p < 0.05$). Control samples without fruit addition were characterized by a relatively neutral flavor profile and lower sensory appeal, whereas guava-enriched beverages received consistently higher scores from the sensory panel.

Appearance scores increased markedly with guava addition due to the attractive color imparted by the fruit. Samples containing freeze-dried guava puree achieved the highest appearance scores, reflecting their uniform color distribution and smooth visual texture. Beverages formulated with fresh guava puree also showed improved appearance compared to the control, although slightly lower scores were observed, possibly due to minor color heterogeneity.

Table 4
Sensory scores of cottage cheese beverages (5-point scale)

Sample	Appearance	Flavor	Texture	Overall acceptability
F0	4.3 ± 0.1^{c}	4.2 ± 0.1^{c}	4.1 ± 0.1^{c}	4.2 ± 0.1^{c}
F5	4.4 ± 0.1^{c}	4.3 ± 0.1^{c}	4.3 ± 0.1^{bc}	4.3 ± 0.1^{bc}
F7	4.6 ± 0.1^{b}	4.5 ± 0.1^{b}	4.4 ± 0.1^{b}	4.5 ± 0.1^{b}
F9	4.5 ± 0.1^{bc}	4.2 ± 0.1^{c}	4.3 ± 0.1^{bc}	4.3 ± 0.1^{bc}
FD5	4.6 ± 0.1^{b}	4.5 ± 0.1^{b}	4.5 ± 0.1^{b}	4.5 ± 0.1^{b}
FD7	4.8 ± 0.1^{a}	4.7 ± 0.1^{a}	4.6 ± 0.1^{a}	4.7 ± 0.1^{a}
FD9	4.6 ± 0.1^{b}	4.4 ± 0.1^{bc}	4.4 ± 0.1^{b}	4.4 ± 0.1^{b}

Note: Values are expressed as mean±standard deviation ($n = 12$).
Different superscript letters within the same column indicate significant differences ($p < 0.05$) according to two-way ANOVA followed by Tukey's post hoc test.

Flavor perception and overall acceptability were strongly influenced by formulation balance. At a low concentration of 5%, guava flavor was perceived as mild and only slightly modified the characteristic dairy taste. Conversely, samples containing 9% guava puree exhibited an overly intense fruit flavor and increased acidity, which partially masked the dairy characteristics. The formulation containing 7% guava puree achieved the highest flavor scores, providing a balanced combination of fruity aroma and mild dairy character. This observation is consistent with previous studies reporting that moderate levels of fruit supplementation result in optimal sensory acceptance, whereas excessive addition may negatively affect flavor balance (Chauhan et al., 2013; Patil et al., 2009; Walkunde et al., 2009).

In addition to flavor and overall acceptability, guava addition influenced the perceived texture and visual appearance of the beverages. Samples containing freeze-dried guava puree appeared more homogeneous and smoother compared to those formulated with fresh puree, which occasionally exhibited slight particulate perception due to residual fibrous components. This enhanced texture perception is closely associated with improved physicochemical stability of the acid-curd matrix, indicating that sensory acceptance is strongly influenced by matrix integrity rather than fruit flavor intensity alone. The improved homogeneity of freeze-dried guava samples may be associated with enhanced dispersion of fruit solids and better interaction with the milk protein matrix, contributing positively to consumer perception of product quality (Martínez et al., 2012).

The observed sensory responses can be attributed to the combined effects of guava concentration and the corresponding sugar adjustment implemented to achieve balanced flavor perception under realistic beverage formulation conditions. Accordingly, sensory outcomes represent formulation-driven effects rather than isolated ingredient responses. Among all tested samples, the beverage containing 7% freeze-dried guava puree with adjusted sugar content exhibited the highest overall acceptability, offering an optimal balance between sensory quality and textural stability.

Comparative evaluation of fresh and freeze-dried guava puree

A comparative evaluation of fresh and freeze-dried guava puree revealed clear differences in their technological performance within the cottage cheese beverage system.

Although both forms of guava improved product quality, freeze-dried guava puree demonstrated several advantages in terms of physicochemical and sensory properties.

Beverages formulated with freeze-dried guava puree exhibited higher dry matter content and improved structural uniformity compared with those prepared with fresh puree. This effect can be attributed to the higher solids concentration and improved dispersion of fruit components after rehydration of freeze-dried material.

From a sensory perspective, formulations containing freeze-dried guava puree were generally preferred by the panelists, showing smoother texture and more balanced flavor characteristics. The more uniform distribution of fruit solids likely promoted improved interaction with the dairy protein matrix.

Optimization of formulation and industrial relevance

Based on the combined evaluation of physicochemical characteristics, rheological behavior, antioxidant activity, and sensory properties, the formulation containing 7% guava puree and 5% sugar provided the most balanced product characteristics, particularly when freeze-dried guava puree was used.

These results indicate that cottage cheese-based beverages can serve as an effective carrier for fruit ingredients, allowing the development of dairy products with improved physicochemical stability and sensory quality.

The evaluation period of 24 h was selected to examine the early-stage stability of the beverage system, during which phase separation and structural rearrangements are most likely to occur in diluted acid-curd matrices. Further studies will be required to assess long-term storage stability.

Conclusions

This study demonstrated the feasibility of formulating a cottage cheese-based beverage enriched with guava puree, with particular emphasis on the technological behavior of a diluted acid-curd system. The incorporation of guava puree affected key physicochemical parameters, including pH, titratable acidity, dry matter content, and water-holding capacity, as well as sensory attributes. Rheological analysis confirmed non-Newtonian shear-thinning behavior of all samples, adequately described by the Ostwald–de Waele model.

At comparable formulation levels, beverages prepared with freeze-dried guava puree exhibited higher dry matter content and reduced water separation compared to those containing fresh puree, indicating improved physical stability of the diluted curd matrix, which is inherently prone to phase separation. In addition, guava enrichment increased the total antioxidant activity due to the presence of natural antioxidant compounds.

Sensory evaluation showed that moderate levels of fruit incorporation improved flavor balance and texture, whereas excessive addition led to increased acidity and an overly intense fruit flavor. Among the tested formulations, the beverage containing 7% freeze-dried guava puree demonstrated the most favorable combination of physicochemical properties and sensory acceptability.

Overall, these findings provide a technological basis for developing fruit-enriched cottage cheese beverages with enhanced physical stability, rheological performance, and antioxidant potential.

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Fermentation-induced changes in the colour of dairy products

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Abstract

Introduction. Colour is a key aspect of dairy product quality, strongly influencing consumers' first impressions of freshness and safety. In heat-treated dairy products, the colour profile is largely determined by the Maillard reaction and caramelization; however, the effects of subsequent fermentation on these pigments remain poorly understood.

Materials and methods. Commercial milk (0.5% and 4.0% fat) and cream (10.0% fat) were heated at 95 ± 2 °C for 4 h. The samples were then fermented using *Streptococcus thermophilus* and *Lactococcus* cultures to produce ryazhenka and fermented sour cream. Instrumental colour analysis was performed in the CIELab system using an LS 173 colorimeter, while pH and titratable acidity were monitored throughout the acidification and fermentation stages.

Results and discussion. The study revealed a non-linear relationship between the acidity of dairy products and colour coordinates. Initial acidification of fermented milk (from pH 6.6 to 5.8) caused a sharp decrease in the a* coordinate to ~1.6 units, indicating the sensitivity of certain chromophore groups of melanoidins to a weakly acidic environment. A further decrease in pH to 4.7–4.6 led to a recovery of the a* coordinate, which reached a maximum of ~3.8 units. Similar trends were observed for the b* coordinate, the peak of which was recorded at pH 4.6–4.7 (~110–120 °T), significantly enhancing the yellow hue. A comparative analysis of the technological stages showed that although fermentation typically «lightens» standard milk bases due to protein aggregation, in fermented products, this effect is partially offset by the ongoing formation of Maillard reaction products. Baked milk demonstrated higher colour saturation (C*) compared to cream due to a higher lactose-to-protein ratio. The whiteness index (WI) decreased consistently at all processing stages, reflecting a steady shift away from the colour of the raw material. It was established that the specific fermentation temperature (37–42 °C for fermented baked milk versus 38–40 °C for sour cream) is an additional factor that intensifies melanoidin formation. The results demonstrate that the final colour of fermented baked milk products is the result of a competitive balance between acid clarification and thermal stabilisation of pigments.

Conclusions. Fermentation significantly alters the colour of fermented milk products; however, continued Maillard reactions during warm incubation can compensate for potential acid-induced discolouration. These findings help maintain stable and appealing sensory characteristics in both ryazhenka and fermented sour cream.

Introduction

Colour is the most important indicator of the quality of dairy products, shaping the consumer's first impression of freshness, naturalness, and safety (Clydesdale, 1991; Milovanovic et al., 2020). Visual perception precedes sensory analysis, so any deviations from the bluish tint of skimmed milk to the excessive yellowness of butter critically influence product choice (Pădureţ, 2021). Colour reflects complex physicochemical processes: protein denaturation, which alters light scattering; the Maillard reaction and the caramelisation of sugars (Newton et al., 2012; Xiang et al., 2021), which form the colour of toasted products; fat oxidation (Xiang et al., 2021); and the degradation of anthocyanins in plant-based fillers (Popov-Raljić et al., 2008).

The colour of milk is determined primarily by genetic factors. Jersey and Guernsey cattle produce milk with a distinct yellow tinge due to their high β-carotene content compared to that of Holstein cattle, as well as goats and sheep, and it appears visually whiter (Chudy et al., 2020; Dufossé et al., 2009). In small ruminants, β-carotene is completely converted into colourless retinol (vitamin A) (Chudy et al., 2020), and small fat globules enhance light scattering, creating a matt effect (Felice et al., 2021).

The animals' diet is also a key factor: grazing increases the carotenoid content in milk, whereas preserved feed and heat treatment lead to their destruction (Lucas et al., 2006; Nozière et al., 2006). The animals' health status, particularly mastitis or infections, can cause a reddish tinge due to the presence of blood or specific microorganisms in the milk (Muhammad et al., 2015).

The chemical composition of the raw material directly influences the product's appearance. The characteristic white colour of milk is due to the scattering of light by fat globules (Fox and McSweeney, 2007) and casein micelles (Misawa et al., 2016; Mistry et al., 1992). An increase in casein content enhances whiteness, whilst an increase in water content makes the product visually more transparent (Walstra et al., 2005). The content of natural pigments, carotenoids (Noziere et al., 2006) and riboflavin (Chudy et al., 2020), gives milk its yellow color.

Processing significantly alters the colour characteristics of milk. Heat treatment triggers the formation of melanoidins, giving the milk creamy or amber tones (Lohinova et al., 2025; Solah et al., 2007). Homogenisation makes the product lighter in colour by breaking down the fat (McSweeney et al., 2015; Milovanovic et al., 2020). Fermentation, as a complex biochemical process, leads to a decrease in pH and casein aggregation, which alters the structure of the protein matrix and is accompanied by a slight reduction in lightness (L*) and a slight increase in yellowness (b*) (Danków et al., 2013).

To adjust the colour of milk and dairy products, natural colourings (annatto, curcumin) and fruit-based fillers are used, which alter the colour tones due to their own pigments (Calvo et al., 2001; da Cunha Júnior et al., 2025; Najgebauer-Lejko, 2014). The colour of protein- and fat-containing products, particularly cheeses, may also change due to the accumulation of protein breakdown products and the oxidation of fats (Lucas et al., 2006). Since fermentation can both form and decolourise pigments, a pressing task has arisen to investigate the influence of this process on the colour of fermented dairy products for the purpose of specifically regulating their appearance.

There is currently a significant gap in the scientific understanding of how colour changes occur in baked dairy products after the baking process. In particular, the transformation of milk colour during cooling following prolonged heat treatment, as well as during subsequent fermentation, remains poorly studied. Determining the kinetics of changes in colour coordinates and individual colour indices at these stages will help establish a clear link between the transformation of milk components and colour intensity, which is essential for stabilising the quality of the final product.

The aim of this study is to investigate the effect of fermentation on the colour characteristics of fermented milk products and to provide a basis for recommendations to maintain stable sensory properties and standardise consumer-relevant characteristics in industrial production.

Materials and methods

Materials

The study used commercial milk samples with fat contents of 0.5% and 2.6%, and cream with a fat content of 10.0%.

Preparation of milk samples

A milk sample with 4.0% fat was prepared by mixing commercial milk (2.6% fat) with cream (10.0% fat) using a Philips blender (model HR3030/00, China). To obtain fermented milk and cream samples, the mixtures were heat-treated for 4 h with stirring at 95 ± 2 °C. The samples were then cooled to specific temperatures: 0.5% fat samples to 20 °C for simulated oxidation, 4.0% fat samples to 40 °C for fermented baked milk, and 10.0% fat samples to 38 °C for baked cream.

Fermentation was carried out using “Vivo” starter cultures (Ukraine). Fermented baked milk was produced with *Streptococcus thermophilus* and *Lactococcus lactis*, while sour cream was produced with *Lactococcus lactis*, *Lactococcus cremoris*, *Lactococcus diacetylactis*, and *Streptococcus thermophilus*. Samples were fermented in a TS-1/80 SPU (Ukraine) thermostat under the following conditions: 40 ± 2 °C until titratable acidity reached at least 80 °T for fermented baked milk, and 38 ± 2 °C until titratable acidity reached at least 60 °T for sour cream.

For simulated acidification, a 10% lactic acid solution was added in increments of approximately 0.2 pH units.

Methods

The active acidity (pH) of the samples was measured using the potentiometric method with an ADWA 1300 pH meter (Romania).

The titratable acidity of the samples was determined using the titrimetric method (Matela et al., 2019).

Colour was measured in the CIELab system using an LS 173 colorimeter (China). For each measurement, 10 ml of the samples was placed in a transparent quartz cuvette to allow light to pass through. Readings were taken for lightness L* (from 0 (black) to 100 (white)), a* (from green to red) and b* (from blue to yellow). Colour measurements were taken every 30 minutes with three repetitions. The results were taken as the arithmetic mean of the data obtained (Alvarado et al., 2025).

Processing of research results

Colour saturation (C*) was calculated using Equation 1 (Pathare et al., 2013):

$$C^* = \sqrt{a^{*2} + b^{*2}} \qquad (1)$$

where C*is the colour saturation;

a^* and b^* are the colour coordinates on the red-green and yellow-blue axes.

The Whiteness Index (WI), according to ASTM E313, is a quantitative measure used to assess the visual «whiteness» of a sample:

$$WI = 100 - \sqrt{(100 - L)^2 + (a^{*2}) + (b^{*2})}, \qquad (2)$$

where WI is the whiteness index, L* is the brightness (0 = black, 100 = white), and a* and b* are the colour coordinates on the red–green and yellow–blue axes, respectively.

Statistical analysis

To ensure the accuracy of the measurements, each experiment was conducted three times; the results were analysed at a confidence level of $P \geq 0.95$. The calculations were performed using PSPP software – the GNU PSPP statistical analysis program, version 1.2.0-g0fb4db.

Results and discussion

Effect of acidity changes on the colour of dairy products

The effect of medium acidity on the colour of dairy products was investigated by directly acidifying baked milk with a characteristic colour (Lohinova et al., 2025). Figure 1 illustrates the dependence of the a* and b* colour coordinates on pH and titratable acidity.

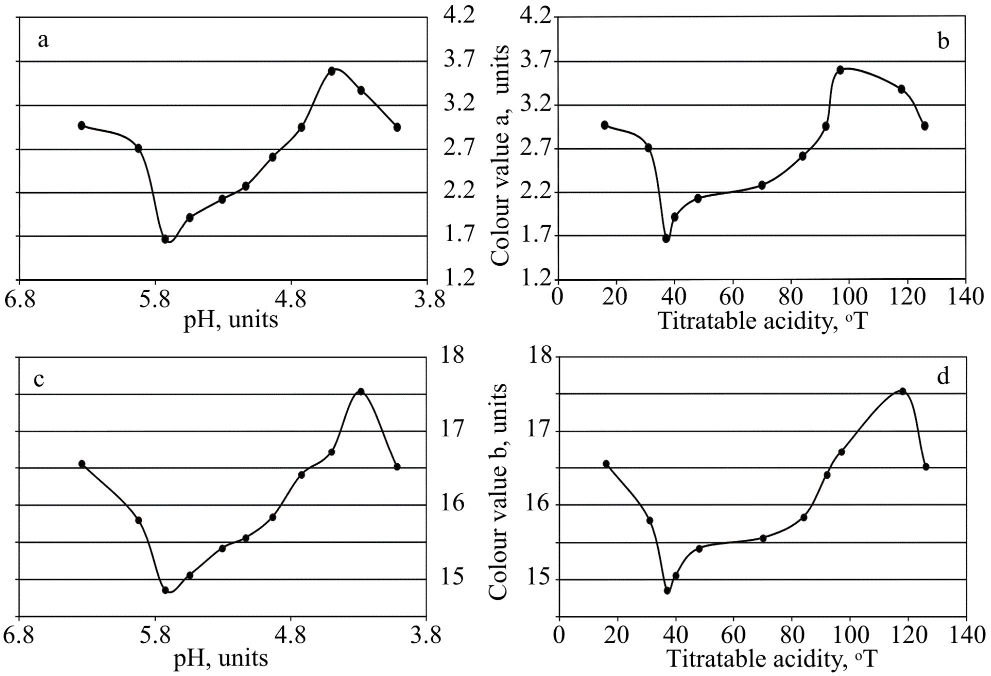


Figure 1. Dependence of the colour coordinates of curdled milk on pH and titratable acidity: (a, b) – a* coordinate; (c, d) – b* coordinate

The results obtained indicate a close correlation between the colour characteristics and the acid-base balance of the product. When the pH of baked milk is reduced from 6.6 to 5.8, a sharp decrease in the a* value to ~1.6 units is observed, which can be explained by a change in the chemical structures of the chromophore groups of melanoidins (Rodríguez et al., 2021), which are less stable in a weakly acidic environment. A further decrease in pH to 4.7–4.6 leads to a gradual increase in the a* coordinate, peaking at ~3,8 units, reflecting a shift in colour towards a reddish hue as the Maillard reaction progresses (Lohinova et al., 2023). When the pH is reduced to ~4.0, the colour intensity decreases slightly but remains higher than the initial values. Acidified fermented milk visually exhibits colloidal instability due to casein coagulation.

The addition of lactic acid to baked milk causes non-linear changes in both the red-green (a*) and yellow-blue (b*) colour coordinates. In the initial stages of increasing acidity (from 16 to 40 °T), a simultaneous decrease in both coordinates is observed. In particular, the red hue (a*) decreases sharply from a moderate level (~2.7–2.9 units) to ~1.6 units, and the yellow hue (b*) also shows an initial decrease. Following this initial decline, both coordinates exhibit a marked increase and reach maximum values at high acidity levels, which may be associated with the intensification of Maillard reactions and the accumulation of melanoidin pigments. The a* coordinate reaches its maximum value (~3.7 units) in the range of ~95–100 °T, corresponding to the most intense cream hue. At the same time, b* gradually increases, reaching maximum values at pH 4.6–4.7 (or ~110–120 °T), indicating a marked intensification of the yellow hue. At the highest acidity levels (≥ 110–120 °T), the intensity of both coordinates decreases slightly, indicating stabilisation or inhibition of processes in a strongly acidic environment.

Figure 2 shows the relationship between colour saturation and whiteness index as a function of pH and titratable acidity.

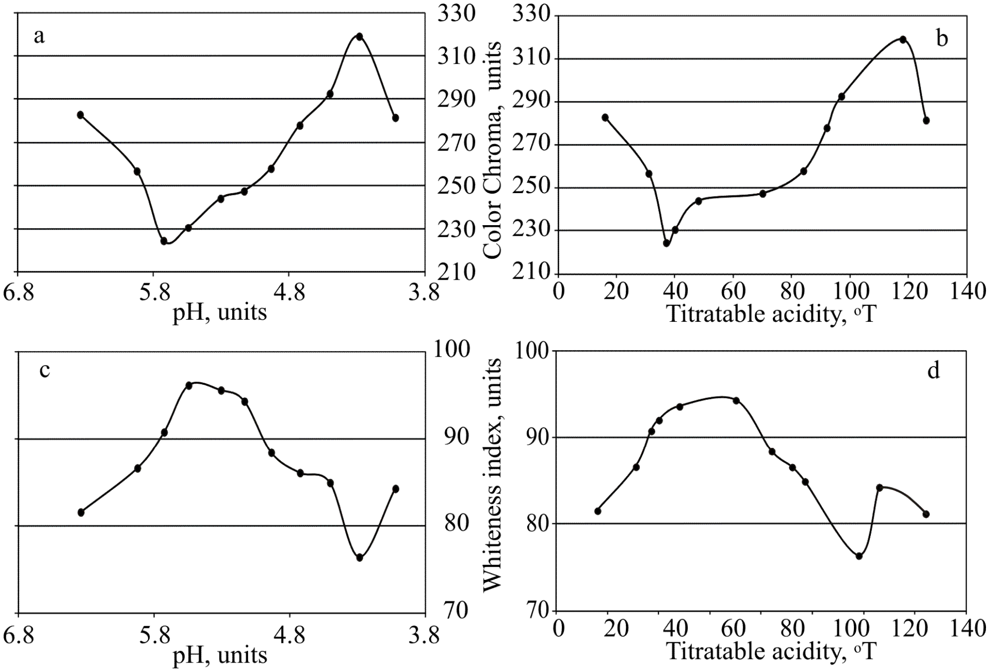


Figure 2. Relationship between colour saturation and whiteness index of curdled milk as a function of pH and titratable acidity:
(a, b) – colour saturation (C*); (c, d) – whiteness index (WI)

Analysis of the data shown in Fig. 2 revealed that as the pH decreased from 6.6 to 5.8, there was a sharp decrease in colour intensity. Subsequently, as the pH decreases in the range 5.8–4.2, the colour saturation gradually increases and reaches a maximum near pH ≈ 4.2, after which it decreases again. This indicates a non-linear nature of the colour change, which is due to colloidal transformations of proteins in the region of their isoelectric point (Francis et al., 2019; Ramezani et al., 2021).

A similar effect was observed when investigating the relationship between colour saturation and the titratable acidity of baked milk. Maximum values are recorded at high acidity (≈ 110 °T), whereas in the middle range (40–60 °T) colour saturation is at its lowest. With a further increase in acidity (10–20 °T), the value rises again. The results indicate that milk colour changes in response to acidity in a non-linear manner and can serve as an indirect indicator of the progression of biochemical processes during fermentation and storage.

Changes in pH affect the intensity of reddish, greenish, and yellowish hues, which directly influences consumer perception of yoghurts, cheeses, dairy desserts, and baked goods (Walstra et al., 2005).

Knowledge of the pH ranges at which colour remains most stable enables the optimisation of production processes and helps to prevent the formation of undesirable colour tones during fermentation, heat treatment or storage (Singh, 2004). A stable colour within a range acceptable to consumers creates an association with naturalness and freshness, an important marketing factor that influences product choice (Ong et al., 2012).

The results obtained not only provide a deeper understanding of the mechanisms underlying colour formation in dairy products, but also allow this knowledge to be applied to improve production processes, thereby enhancing product stability and market appeal.

Effect of fermentation on the colour of fermented dairy products

To assess the effect of the fermentation process on the colour development of fermented products, their colour was measured at various stages of production, as shown in Figures 3 and 4.

The increase in the a* and b* values towards red and yellow hues during baking is due to the Maillard reaction in both milk and cream. The initial colour coordinates of milk and cream differ. Thus, cream has higher values for the a* and b* coordinates, which can be explained by its higher fat content and the presence of β-carotene (Fig. 3b).

The semi-finished products were baked until a similar colour was achieved, as evidenced by the similarity in the b-coordinate values. At the same time, the a* coordinate showed a slight difference between the milk and cream samples – up to 0.4 units. The subsequent cooling stage was accompanied by a slight increase in both coordinates (Fig. 3a, b) as the Maillard reaction continued.

Although the fermentation of baked milk and cream does not follow a linear pattern, as shown in the previous section of this study, a slight increase in the a* and b* colour coordinates can be observed in the final product. Fig. 3 shows that for milk, these values will be higher due to slightly higher recommended fermentation temperatures: 37–42 °C for milk; 31–39 °C for cream, which is the main factor in the formation of colour compounds via melanoidin.

The fermentation process alters the colour of fermented products through protein aggregation, increased acidity, and reduced mineral content. For instance, in milk-cereal substrates, significant increases in lightness (L*) and colour saturation (C*) were observed following fermentation (Pires et al., 2018; Wijesekara et al., 2022). The values of the colour components a* and b* usually decrease — the product becomes «lighter». Furthermore, acidic fermentation products can affect the pigments in additives, such as fruit extracts (anthocyanins), leading to their degradation, an increase in ΔE*, and changes in b* in yoghurts, respectively (Aleman et al., 2023; Chandan et al., 2013).

To ensure a comprehensive assessment, colour saturation and whiteness index characteristics were also analysed (Fig. 5).

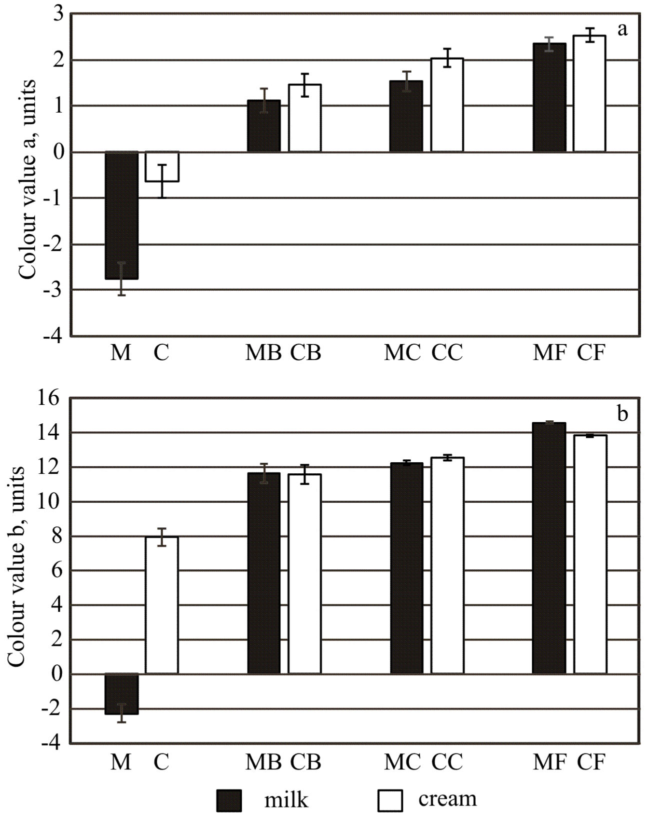
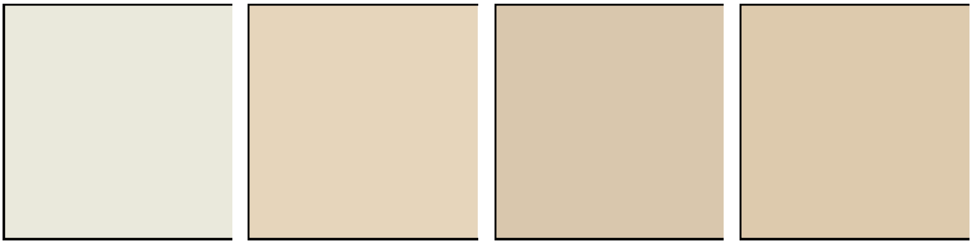
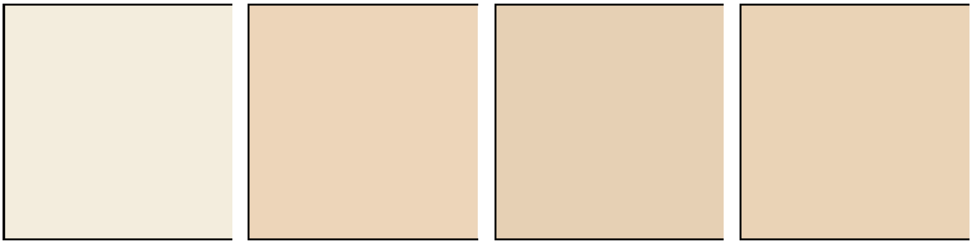


Figure 3. Changes in colour coordinates during technological stages of baking and fermentation of milk (M) and cream (C):
(a) – a* coordinate;
(b) – b* coordinate.
(M – milk;
MB – baked milk;
MC – chilled baked milk;
MF – fermented baked milk;
C – cream;
CB – baked cream;
CC – chilled baked cream; CF – fermented baked cream)



Milk | Baked milk | Chilled baked milk | Fermented baked milk



Cream | Baked cream | Chilled baked cream | Fermented baked cream

Figure 4. Appearance of product samples at various stages of the manufacturing process

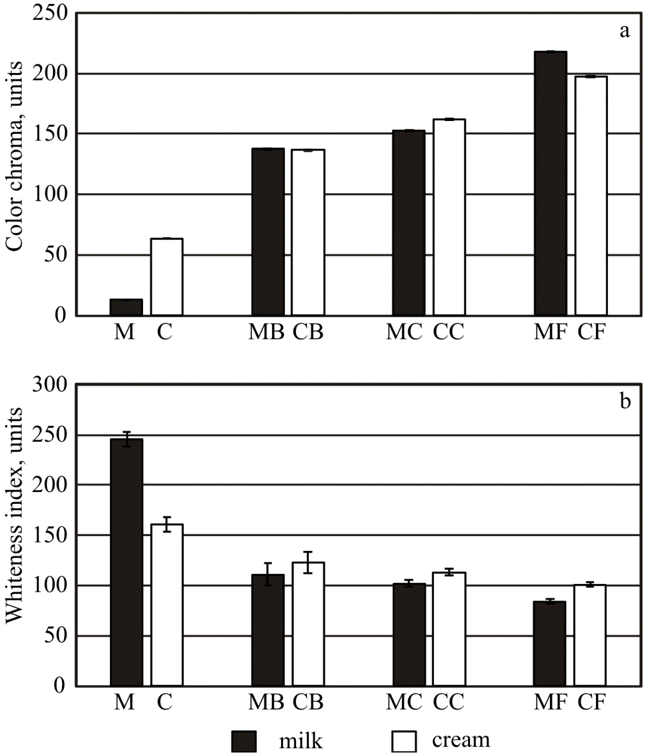


Figure 5. Changes in colour saturation (C*) and whiteness index (WI) during the technological stages of milk (M) and cream (C) baking and fermentation:
(a) – colour saturation;
(b) – whiteness index.
(M – milk;
MB – baked milk;
MC – chilled baked milk;
MF – fermented baked milk;
C – cream;
CB – baked cream;
CC – chilled baked cream;
CF – fermented baked cream)

Colour saturation (Fig. 5a) increases more rapidly during the heat treatment of milk than in cream, due to the Maillard reaction proceeding more actively in milk than in cream. This effect can also be explained by the higher lactose and protein content in milk, which are the main reactants in the reaction (Mandiuk et al., 2024). However, the general trend of a slight increase in colour saturation and, consequently, in colour intensity from the baked semi-finished product to the cooled baked semi-finished product and further to the fermented product remains.

For the whiteness index, determined in accordance with ASTM 313 (Fig. 5b), which inversely reflects the distance from white, a clear decline in characteristics is observed for both milk and cream at all stages. The difference lies in the higher whiteness index value for milk compared to cream (~80 units), as shown in Fig. 5 by lower values of the a* and b* coordinates. In subsequent dairy systems, the trend changes, and higher whiteness indices are characteristic of baked cream, chilled baked cream, and fermented baked cream.

The incomplete nature of the Maillard reaction may persist even after baking and is influenced by cooling rate, temperature, and fermentation duration. To maintain stable consumer-relevant characteristics of fermented dairy products during storage, these factors should be considered when monitoring the various stages of production.

Conclusions

Model oxidation of baked milk showed discolouration only during the initial stages, caused by the breakdown of the chromophore groups of melanoidins at high titratable acidity and low pH. The stability of the Maillard reaction allows the acid-induced discolouration in fermented baked milk to be neutralised. Trends in changes in colour coordinates (a* and b*) and colour saturation during the fermentation of baked milk and cream were similar to those observed in the model samples, with slightly higher values, as the elevated fermentation temperature enhances the intensity of the Maillard reaction.

Descriptions of consumer properties should reflect the range of changes that may occur during production. Consumer preferences regarding the colour of fermented baked dairy products must be extrapolated to the final product, particularly at the spinning stage. Further research should focus on developing industry recommendations for producing fermented baked dairy products from curd with consistent sensory properties.

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Milk protein concentrate and *Aronia melanocarpa*: Effects on coagulation of goat milk proteins

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Abstract

Introduction. The aim of the study was to determine the combined effect of Promilk and *Aronia melanocarpa* powder on the quality and qualimetric characteristics of milk protein concentrates obtained by enzymatic coagulation of goat milk proteins.

Materials and Methods. Enzymatic coagulation was carried out using a rennet enzyme and calcium chloride, with the addition of the milk protein concentrate Promilk and *Aronia melanocarpa* powder. The water-holding capacity of the resulting concentrates was determined using a gravimetric method.

Results and Discussion. The effect of the milk protein concentrate Promilk on the enzymatic coagulation of goat milk was evaluated. The addition of 0.5% Promilk resulted in an average increase in the yield of milk protein concentrate by 22.5±0.2% compared to the control under identical coagulation conditions. This supplementation also enhanced the utilization of milk solids to 71.0±0.2% and increased water-holding capacity to 68.3±0.3%, while reducing the moisture content to 49.0±0.1%. These results indicate a more efficient incorporation of protein fractions into the formation of the protein–fat matrix.

The addition of *Aronia melanocarpa* powder during the production of milk–plant curd modified the organoleptic properties, particularly color, taste, and consistency. Incorporation of up to 0.7% ensured a homogeneous structure, a milky taste with a characteristic aronia aroma, and a pink-red coloration. Increasing the level from 0.5% to 0.7% enhanced color and aroma intensity; however, at 0.9%, a mealy consistency was observed. Within the studied range, the plant additive slightly affected the dry matter content of whey and promoted a decrease in pH during coagulation, which is important for the protein concentration process in goat milk.

Instrumental color analysis demonstrated increased product pigmentation with higher levels of *A. melanocarpa* powder and lower protein content in the medium. Increasing the concentration from 0.7% to 0.9% enhanced reflectance in the red region ($\lambda \approx 650$–750 nm), while changes in the blue–green region ($\lambda \approx 480$–560 nm) were negligible, confirming the predominant expression of aronia pigments at longer wavelengths. These results indicate that *Aronia melanocarpa* can be used to modulate the optical properties and visual quality of milk–plant concentrates. Negative ΔL values, along with increased Δa and decreased Δb, further confirm the shift toward the red–violet region of the color space.

Conclusions. The combined application of Promilk and *Aronia melanocarpa* powder improves the qualitative and qualimetric characteristics of milk protein concentrates obtained by enzymatic coagulation of goat milk.

Introduction

The development of improved methods for extracting milk proteins from goat milk and their application in products such as curd products, semi-finished foods, and sauces is of considerable relevance. The process of enzymatic coagulation of goat milk proteins is less effective than that of cow milk (Nayik et al., 2022). This is due to compositional features such as insufficient content of kappa-casein, lower pH, and high dispersion of fat globules. Technological difficulties arise from the high concentration of β-casein and the low level of α-caseins, which hinder maximum protein coagulation with the formation of a dense curd (Pokhyl et al., 2023; Ryzhkova et al., 2019). The addition of ingredients of different origin (dry milk protein concentrate, plant powders) during the fermentation of goat milk is likely to increase the final yield of the concentrate with appropriate quality characteristics.

It is advisable to improve the technology of milk protein concentrates from goat milk and to increase their biological and energy value through the combined addition of Promilk and *Aronia melanocarpa* powder. Promilk is a soluble milk protein concentrate produced from skimmed milk by ultrafiltration and drying (Khalesi et al., 2021). According to organoleptic characteristics, it is a powder ranging from white to slightly creamy in color with a neutral taste and odor. Unlike casein, this protein contains a high proportion of isolated κ-casein fractions (approximately 90% of the total protein content), which are responsible for curd formation, structure, and product yield. Promilk is widely used in the production of fermented milk products, curd products, and beverages based on cow milk (Kadhim et al., 2023). The main advantages of this ingredient include stabilization of milk proteins, improved water-holding capacity, low required dosage, ease of application, high solubility, and safety. According to the manufacturer, Promilk can be used, depending on the product, as an emulsifier, stabilizer, skim milk powder replacer, improver of structural and organoleptic properties, and a moisture-binding agent (Khalesi et al., 2021). However, the incorporation of berry powders obtained by drying and grinding into dairy formulations remains limited and requires further investigation (Berulava et al., 2024).

Black chokeberry (*Aronia melanocarpa*) has already been used as a functional ingredient in different food products due to its high content of polyphenols and anthocyanins, which determine its biological activity, intense coloration, and potential health benefits (Artamonova et al., 2025; Khomych et al., 2024; Koshak and Pokrashinskaya, 2020; Pasichniy et al., 2022; Stabnikov et al., 2025; Wieloch, 2025; Xu et al., 2025). A number of studies (Cușmenco and Bulgaru, 2020; Pădureț et al., 2024; Plessas et al., 2024; Uzunsoy, 2022) are devoted to investigating the effect of various forms of black chokeberry (berries, juice, puree, fermented extracts) on the physicochemical, antioxidant, and sensory properties of yogurts and yogurt-like products. The authors note that the addition of black chokeberry leads to noticeable product coloration (purple/purplish-red shades), an increase in the total content of phenolic compounds and antioxidant activity, as well as changes in rheological indicators due to an increase in the proportion of dry matter (Pădureț et al., 2024).

Uzunsoy (2022) highlighted the potential for enhancing the functionality of dairy products and the technological compatibility of *Aronia melanocarpa* with protein matrices. Despite its benefits, challenges remain in stabilizing anthocyanins in dairy systems due to their sensitivity to pH, oxidation, and interactions with proteins. Wieloch (2025) emphasizes the chemical composition of *Aronia melanocarpa*, particularly its polyphenolic fractions and anthocyanins, and their role in product color formation and antioxidant activity.

Black chokeberry powder is a homogeneous, fine-dispersed substance that is readily soluble in water and alcohol and is quickly absorbed by the human body. Commercially produced, it is used in the food industry as a colorant for beverages (including alcoholic ones), creams, and dough. The powder is obtained through an innovative low-temperature dehydration process combined with simultaneous grinding of the raw material. According to the manufacturer, the powder contains 56% dietary fiber, of which 3% is a soluble fraction. Insoluble fiber consists mainly of cellulose and hemicelluloses, with cellulose accounting for about 27–28% of the powder mass and hemicelluloses (xylans, arabinogalactans) approximately 17–18% (Jurendić et al., 2021; Schmid et al., 2020). Soluble fiber is represented mainly by pectic polysaccharides (1.7–2.0%), arabans and galactans (0.5–0.8%), and a small amount of β-glucans and other soluble polysaccharides (0.1–0.2%) (Schmid et al., 2021). Thus, the total polysaccharide content in black chokeberry powder is about 53% of dry matter, with a predominance of insoluble polysaccharides (Jurendić and Ščetar, 2021; Schmid et al., 2020). The powder also contains a wide natural complex of vitamins (P, C, E, K, B1, B2, B6, and beta-carotene), macro- and microelements, and sugars (glucose, sucrose, and fructose) (Saracila et al., 2024). According to Samilyk et al. (2025), the content of organic acids in *Aronia melanocarpa* powder is as follows, %: malic – 0.268; citric – 0.280; tartaric – 0.304; acetic – 0.240; oxalic – 0.180; lactic – 0.36. The total polyphenol content in *Aronia melanocarpa* powder is 41.1 mg/g of dry matter, anthocyanins – 4.42 mg/g, and flavonoids – 6.03 mg/g (Yikilkan et al., 2025). A significant part of polyphenolic compounds is represented by polymeric catechins and tannins, which belong to tannins and influence the functional and structural properties of products (Adamczyk et al., 2025). The content of pectic substances in fresh fruits is 0.4–0.8% of mass (Mezhenskyj et al., 2024). The permissible daily intake of *Aronia melanocarpa* is 50–100 g, since it contains a high concentration of polyphenols, anthocyanins, flavonoids, and tannins (Go et al., 2024), which is a limiting factor for application.

The quality characteristics of *Aronia melanocarpa* powder are presented in Table 1 (adapted from Koshak and Pokrashinskaya, 2020).

Table 1

Quality characteristics of *Aronia melanocarpa* powder

Quality parameters	Characteristics
Organoleptic indicators	
Colour	Brown or burgundy
Odour	Typical of this raw material
Taste	Sour-sweet, slightly astringent
Physicochemical indicators	
Moisture content, % / Ash content, %	4.96 / 1.49
Acidity, degrees recalculated as malic acid	0.12
Microelement content, µg/g	
S / K	2407.00 / 2855.81
Cl / Ca	295.01 / 577.21
Mn / Fe	10.14 / 8.3
Ci / Rb / Zn	2.42 / 2.86 / 2.86

Taking the above into account, *Aronia melanocarpa* powder is advisable to use in the production of dairy protein concentrates to influence the protein–fat matrices of goat milk, impart natural colouring, and improve the qualimetric characteristics of the process. Relevant data are absent in the scientific literature. Therefore, the study of the effect of *Aronia melanocarpa* powder on the quality of dairy protein concentrates is a relevant task.

The aim of this study is to evaluate the combined effect of Promilk and *Aronia melanocarpa* powder on the quality and qualimetric characteristics of dairy protein concentrates obtained through enzymatic coagulation of goat milk proteins.

Materials and methods

Materials

Goat milk with the following chemical composition (%): fat 4.14; protein 3.6 (including 3.0 casein); carbohydrates 4.4; minerals 0.85; and ash 0.82, was used as the raw material in this study.

A cheese enzyme, TM VIVO, consisting of plant-derived chymosin and sodium chloride, was used as a coagulant for milk proteins.

Promilk (Ingredia, France; ISO 9001:2008 certified) with 71% micellar casein, 16% lactose, 1% fat, 4.5% carbohydrates, 8% ash, 5% moisture, 95% dry matter, and pH 6.4–7.05 was used in the study. Promilk is rich in calcium (2460.40 mg%) and provides a balanced amino acid profile in accordance with FAO/WHO standards.

Images of chokeberry fruits and chokeberry powder are shown in Figure 1.

a b

Figure 1. Black chokeberry fruits (a) and chokeberry powder (b)

Preparation of model samples of dairy-plant concentrates

Model samples were prepared under laboratory conditions as follows: Promilk (0.4–0.6%) and black chokeberry powder (0.3–0.9%) were dissolved in goat milk. The mixture was pasteurized at 70–72 °C for 15–30 s, cooled to 32–36 °C, and supplemented with 40% aqueous calcium chloride and 0.04% rennet enzyme. Fermentation was carried out for 50–60 min, after which the curd was cut, heated for compaction, whey was removed, and the concentrate was allowed to self-press for 10–15 min before cooling to 18–22 °C.

The control sample was prepared under identical conditions but without the addition of Promilk or black chokeberry powder powder.

Preparation of Promilk and black chokeberry powder involved dissolution in milk at a 1:8 ratio at 50 ± 2 °C for 20 ± 1 min with mechanical stirring until complete dissolution of the milk solids and swelling of the plant component, followed by mixing with the total volume of goat milk.

Methods

The study employed standardized and validated analytical methods for evaluating technological and quality characteristics, ensuring reliable results and fulfillment of the research objectives.

Yield and properties of dairy and dairy-plant concentrates and whey. The degree of utilization of dry matter of goat milk (A_c) was determined by the formula:

$$A_c = 100\frac{C_d(C_m - C_{whey})}{C_m(C_d - C_{whey})},$$

where C_d is the mass fraction of dry matter in dairy and dairy-plant concentrates, %; C_m is the mass fraction of dry matter in goat milk, %; C_{whey} is the mass fraction of dry matter in whey, %.

The yield of concentrates (Y) was determined by the formula:

$$Y = \frac{M_{mpc} \cdot 100}{M_{mc}} \;\%,$$

where M_{mpc} is the mass of dairy-plant concentrate, g; M_{mc} is he mass of dairy concentrate, g.

The water-holding capacity (WHC) of dairy and dairy-plant concentrates was determined by the mass of water released from the sample during light pressing and absorbed by filter paper (Chubenko et al., 2025).

Moisture content was measured by an accelerated method using KVARTS-21M-33 by drying the sample to constant mass.

Dry matter content in dairy–plant concentrates was determined by the formula:

$$S = 100 - W,$$

where S is the dry matter content, %, and W is the moisture content, %.

Active acidity (pH) of dairy concentrates and whey was determined potentiometrically using a Sartorius PB-20 pH meter.

Dry matter content in whey was determined by the refractometric method (Chubenko et al., 2025).

A**ppearance of concentrates *and whey*** was evaluated visually, and the consistency was assessed organoleptically at a temperature of 18–20 °C.

Sensory indicators of concentrates and whey were evaluated in accordance with ISO 22935-3:2009 and individual sensory attributes (Elsamani, 2016; Grek et al., 2025).

Colour characteristics of dairy and dairy–plant concentrates and whey. The colour of experimental samples was determined using a YL4500 Series Non-Contact Color Meter (China). Measurements were carried out in the CIE Lab system using a standard illuminant D65 and a 2° standard observer. The measurement geometry was 45°/0°, background – white reference, temperature – 20±1 °C. Samples of protein concentrate and whey (with/without Aronia melanocarpa powder) were placed in optical cuvettes of standardized thickness. For whey, the "Transmission" mode was used, ensuring the same optical thickness for all measurements. Based on the measured parameters L, a, b, ΔL, Δa, Δb were calculated relative to the control sample.

The total colour difference ΔE was calculated by the formula:

$$\Delta E = \sqrt{(\Delta L)^2 + (\Delta a)^2 + (\Delta b)^2}$$

Colour perception thresholds ΔE: $\Delta E < 1$ – practically imperceptible difference; $1 \leq \Delta E < 2$ – noticeable for a trained eye; $2 \leq \Delta E < 3.52$ – noticeable for most observers; $\Delta E \geq 3.5$ – clearly noticeable and accepted as significant (Becker et al., 2024; Khalil et al., 2024).

Statistical analysis

All experiments were conducted in triplicate or more, and the results were expressed as mean±standard deviation. Data were analysed using one-way ANOVA and Tukey's HSD tests by the SPSS statistical package. Significant difference was defined at $p \leq 0.05$.

Results and discussion

Influence of the milk protein concentrate Promilk on the enzymatic coagulation of goat milk

The effect of dry Promilk powder on the enzymatic coagulation of goat milk was evaluated. The relationship between the amount of added Promilk and both the yield and the degree of dry matter utilization in milk is shown in Fig. 2.

According to the results (Fig. 2), the addition of 0.5% Promilk to goat milk increases the yield of dairy protein concentrate by an average of 22.5±0.2 % compared to the control under the same enzymatic coagulation conditions. The results indicate that an increase in protein content in milk contributes to more efficient curd formation and better whey retention, which leads to an increase in yield. These results are consistent with data from other researchers – an increase in milk proteins, particularly casein fractions and whey proteins, correlates with an increase in cheese mass yield (Bielska and Cais-Sokolińska, 2023; Bonfatti et al., 2019; Kalit et al., 2021). A similar effect was observed during milk standardization using milk protein concentrate or milk powder, which allows increasing both the actual and moisture-adjusted cheese yield (Guinee et al., 2006).

Presumably, the addition of aggregated milk proteins to goat milk leads to a significant increase in protein content in the concentrate matrix, which is accompanied by intensification of the coagulation process, compaction of the curd during subsequent processing, and, as a result, improvement of the qualimetric indicators of the curd.

Studies analyzing the distribution of casein and fat in cheeses have shown that standardization of milk by protein increases both actual and moisture-adjusted yield (in particular, using Gouda/Cheddar-type cheeses as an example) (Kayihura, 2023). This is consistent with the increase in dairy protein concentrate yield when adding Promilk in an amount of 0.4–0.6 % to goat milk with a fat mass fraction of 4.14 %.

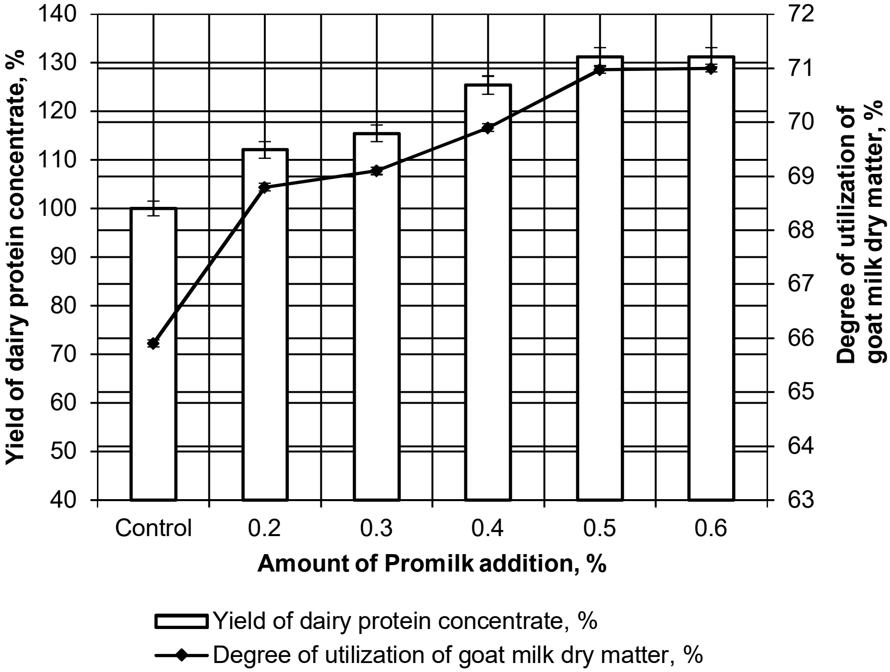


Figure 2. Influence of added Promilk on yield and dry matter utilization of goat milk

With an increase in the amount of Promilk from 0.2 to 0.6%, a gradual increase in the degree of utilization of dry matter of goat milk was observed: from 68.8±0.1% when adding 0.2% Promilk to a maximum value of 71.0±0.2% at a dosage of 0.6%, while the control was 68.2±0.1%. This indicates more complete involvement of protein fractions, including kappa-casein, in the structure of the protein–fat curd and more efficient binding of dry matter. The use of Promilk in an amount of 0.6 % was found to be irrational, since no significant increase in the above-mentioned indicators was observed. The obtained results indicate that a further increase in the amount of Promilk does not provide an adequate effect.

The dependence of the mass fraction of moisture and water-holding capacity of dairy protein concentrates on the amount of added Promilk is shown in Figure 3.

An increase in the amount of Promilk from 0.2% to 0.6% led to a gradual decrease in the mass fraction of moisture of the dairy protein concentrate from 73.3±0.4% (in the control sample) to 48.4±0.3% at the maximum concentration of the technological ingredient. At the same time, the water-holding capacity of the dairy protein concentrate showed an opposite trend, increasing from 64.2±0.1% (in the control sample) to 69.8±0.2% when adding 0.6% Promilk. The obtained results indicate that the addition of the technological ingredient contributes to more effective water binding in the curd structure, improving the ability of the dairy protein concentrate to retain moisture, while a significant increase in concentration above 0.5% does not provide a substantial additional effect. The results are explained by improved protein-water interactions, possible formation of additional protein–fat matrices or structures (frameworks) capable of retaining water.

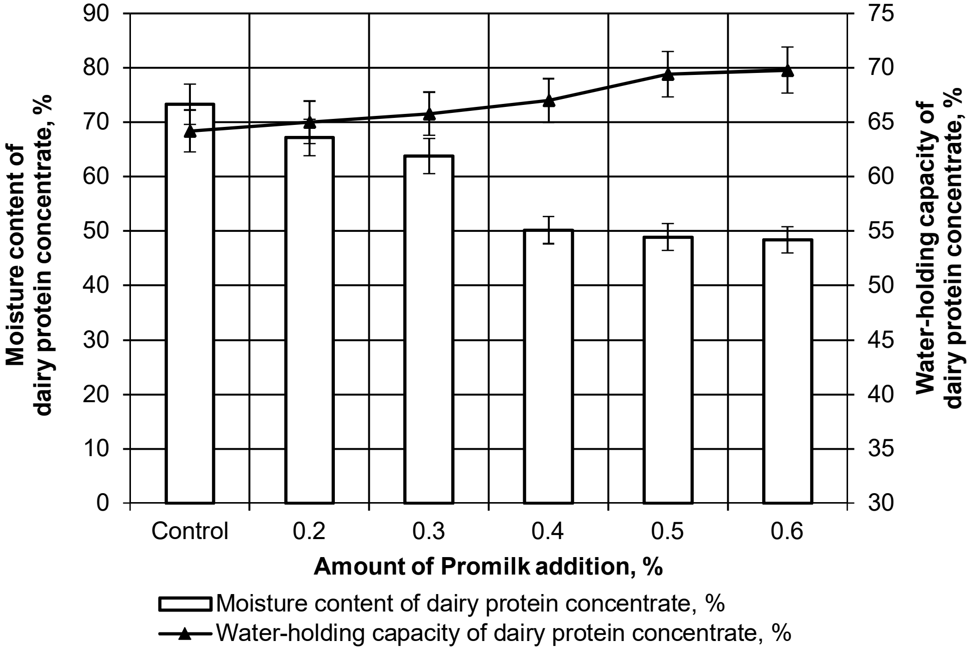


Figure 3. Influence of added Promilk on the moisture content and water-holding capacity of dairy protein concentrates

The obtained information is consistent with studies by scientists (Khatkar et al., 2023; Żulewska et al., 2025). Milk protein concentrates (MPC/WPC) can significantly improve water-holding capacity (WHC), gel formation, and textural properties of dairy products. In particular, the addition of protein (e.g., 1% WPC) to low-fat sour cream provides increased moisture binding and structural stability of the product (Mykhalevych et al., 2022). In fermented products such as yogurts, whey protein concentrates also reduced syneresis and increased viscosity and density of the product (Żulewska et al., 2025).

The efficiency of coagulation is characterized by the qualitative characteristics of whey obtained during the production of dairy protein concentrate with the addition of 0.5% Promilk and without it. The active acidity of the control whey sample was 6.1±0.1 pH units, and that of the experimental sample was 6.0±0.1 pH units, respectively. A decrease in the dry matter content in the experimental whey sample from 7.0±0.1 % to 6.7±0.1% correlates with previously obtained data on the increase in dairy protein concentrate yield, indicating more efficient involvement of goat milk protein fractions in the concentrate. In general, the addition of the technological ingredient does not cause significant changes in all other physicochemical characteristics of whey.

Effect of *Aronia melanocarpa* powder on the fermentation process of goat milk

The study of the effect of *Aronia melanocarpa* powder added to goat milk with Promilk on the qualitative and quantitative indicators of concentrates is the next task. Since data on the application of the above-mentioned powder in the technology of protein products from goat milk are absent.

The qualitative characteristics of dairy protein concentrates with Promilk and *Aronia melanocarpa* powder are presented in Table 2.

Table 2

Qualitative characteristics of dairy protein concentrates with Promilk and *Aronia melanocarpa* powder

Parameters	Control	Amount of added *Aronia melanocarpa* powder, %			
		0.3	0.5	0.7	0.9
Yield, %	131.2±0.2^{a}	131.8±0.2^{a}	132.7±0.1^{ab}	135.3±0.2^{b}	135.3±0.1^{b}
Moisture, %	48.9±0.1^{a}	46.6±0.1^{b}	45.3±0.1^{c}	44.7±0.1^{cd}	43.9±0.1^{d}
Dry matter, %	51.1±0.1^{a}	53.4±0.1^{b}	54.7±0.1^{bc}	55.3±0.1^{c}	56.1±0.1^{d}
Active acidity, pH units	6.3±0.1^{a}	6.2±0.1^{ab}	6.1±0.1^{b}	6.0±0.1^{b}	6.0±0.1^{b}
Degree of utilization of milk dry matter, %	70.98±0.2^{a}	71.6±0.1^{ab}	71.9±0.1^{ab}	72.2±0.,2^{b}	72.5±0.2^{b}
Water-holding capacity, %	69.4±0.1^{a}	70.3±0.1^{a}	71.1±0.1^{ab}	74.1±0.1^{b}	74.8±0.1^{b}

Note: Values represent the mean±standard deviation. Mean values in the same row with different superscripts differ significantly at $p \leq 0.05$.

The addition of *Aronia melanocarpa* powder at 0.3–0.9% affected the properties of dairy–plant concentrates compared to the control. Product yield increased proportionally with the additive, from 131.2±0.2% in the control to 135.3±0.1% at 0.7% and 0.9% additions, indicating more efficient retention of moisture and dry matter in the curd. These findings are consistent with Ryzhkova and Heyda (2023), who reported that organic acids improve the technological properties of goat milk curds. In particular, organic acids (malic, citric, tartaric, acetic, oxalic, lactic) present in significant amounts in *Aronia melanocarpa* powder increase curd density, reducing protein and fat losses into the whey.

With increasing amounts of *Aronia melanocarpa* powder, moisture content decreased from 48.9±0.1% in the control to 43.9±0.1% at 0.9% addition, while dry matter correspondingly increased from 51.1±0.1% to 56.1±0.1%. This suggests a structure-forming effect of the dietary fiber and polyphenolic components in *Aronia melanocarpa*, which likely create a framework that “captures” and retains solids and moisture. These results are consistent with Popescu et al. (2022), who reported that apple pomace reduced whey separation and improved curd texture.

A slight but consistent decrease in pH was observed, from 6.3±0.1 in the control to 6.0±0.1, likely due to the influence of acidic complexes in *Aronia melanocarpa.* The addition of the powder also increased the utilization of goat milk dry matter, from 70.98±0.2% in the control to 72.5±0.2% at the highest dosage, indicating more efficient coagulation and incorporation of protein and fat into the curd structure.

The most pronounced effect was observed on water-holding capacity (WHC). Addition of 0.7–0.9% *Aronia melanocarpa* powder increased WHC from 69.4±0.1% (control) to 74.1–74.8±0.1%, reflecting the hydrophilic properties of polysaccharides and phenolic compounds and their ability to stabilize the protein matrix. These results are consistent with Pădureț et al. (2024), who reported increased WHC and dry matter in yogurt with chokeberry pomace, and Blejan et al. (2024), who observed improved gel stability and texture with blueberry pomace. The effect is attributed to a combined mechanism: dietary fiber and polyphenols form a protein–polyphenol matrix, while organic acids lower pH, enhancing coagulation and curd density.Thus, the optimal addition of chokeberry powder in an amount of 0.7 % ensures improved characteristics of the dairy–plant concentrate, increasing yield, water-holding capacity, and product stability.

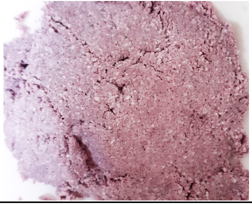
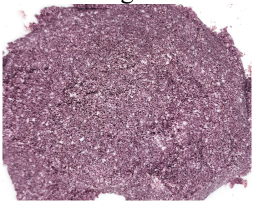
The composition of whey was analyzed, revealing a decrease in dry matter content from 6.6±0.1% in the control to 6.0±0.1% with increasing *Aronia melanocarpa* concentration. Whey pH also decreased consistently, from 6.0±0.1 in the control to 5.6±0.1 at 0.9% chokeberry addition. This effect reflects the influence of organic acids and polyphenolic compounds in *Aronia melanocarpa*, which partially enhance protein coagulation and modify the structural characteristics of the curd (Carboni et al., 2025).

Sensory characteristics of dairy–plant concentrates

The optimal amount of *Aronia melanocarpa* powder was selected to ensure standard sensory characteristics of colored dairy-plant concentrates, which serve as a basis for producing various curd products, sauces, and semi-finished products. Table 3 presents the results of the sensory evaluation of dairy–plant concentrates prepared from goat milk with 0.5% Promilk and *Aronia melanocarpa* powder.

Table 3

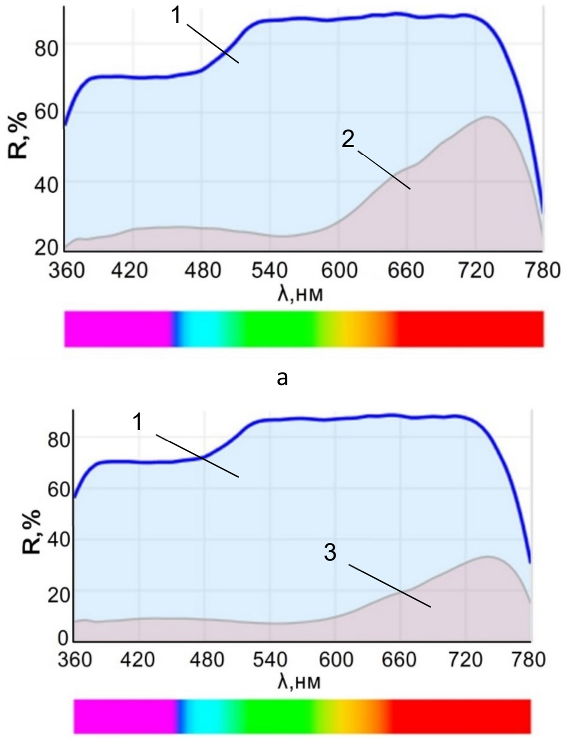
Sensory characteristics of dairy-plant concentrates

Amount of added *Aronia melanocarpa* powder, %	**Consistency and appearance**	**Taste and odor**	**Colour**
0.3	Homogeneous, insufficiently viscous	Milky, weakly expressed taste and aroma of chokeberry powder	Noticeable light pink, uniform throughout the mass
0.5			
0.7	Homogeneous, soft, moderately creamy	Milky with a slight aroma of chokeberry powder	Red-pink, uniform throughout the mass 
0.9	Homogeneous, slightly crumbly, floury	Milky with a pronounced taste and aroma of chokeberry powder	Pronounced, intense dark red with a strong violet hue, uniform throughout the mass 

Harmonious taste and consistency of dairy-plant concentrates were achieved with 0.7% *Aronia melanocarpa* powder. These samples exhibited a pronounced milky flavor with the characteristic aroma of the plant additive, a homogeneous moderately creamy texture, and a uniform purple color. Increasing the addition to 0.9% negatively affected organoleptic properties, producing a floury aftertaste and undesirable texture. Conversely, additions below 0.5% showed little impact on either product structure or sensory quality. Low additions of *Aronia melanocarpa* reduce the positive effects of its biologically active compounds. These findings are consistent with recent studies using chokeberry as a functional ingredient in dairy products (Pădureț et al., 2024). Chokeberry is rich in polyphenolic compounds, which contribute to its intense color and functional properties (Cușmenco and Bulgaru, 2020; Wieloch, 2025).

Influence of *Aronia melanocarpa* powder on the color characteristics of dairy–plant concentrates and whey

The color characteristics of whey and dairy–plant concentrates prepared from goat milk with 0.5% Promilk and varying amounts of *Aronia melanocarpa* powder were evaluated, providing an objective measure of appearance. The reflectance spectra of dairy and dairy-plant concentrate samples as a function of wavelength are shown in Fig. 4.



b

Figure 4. Reflectance spectra of samples depending on wavelength:
1 – dairy concentrate (control); 2 – dairy-plant concentrate with the addition of 0.7% *Aronia melanocarpa* powder (a) during fermentation; 3 – dairy-plant concentrate with the addition of 0.9% (b) *Aronia melanocarpa* during fermentation

The reflectance spectra (R, %) show that the control dairy concentrate (curve 1) has stable high reflectance in the visible spectrum and a slight decrease in the short-wavelength (360–450 nm) region, which is consistent with data from other studies of dairy products (Agiomavriti et al., 2024). The control sample demonstrates an almost uniform reflectance curve across the entire visible spectrum and is characterized by a high, stable reflectance coefficient in the range of 70–85 % over the entire wavelength range of 360–780 nm.

Dairy–plant concentrates with the addition of *Aronia melanocarpa* (curve 2 and curve 3) demonstrate a gradual increase in reflectance at long wavelengths (λ ≈ 600–750 nm), which indicates the influence of the plant ingredient on optical properties. The greatest differences compared to the control are observed in the range of 650–750 nm. Such effects confirm observations regarding the pigment components of *Aronia melanocarpa* (Lv et al., 2024), which are capable of increasing reflectance in the red part of the spectrum due to the presence of anthocyanins.

Comparison of the two experimental samples with the addition of 0.7% and 0.9% chokeberry demonstrates a concentration-dependent effect of pigment components. In the blue-green part of the spectrum (λ ≈ 480–560 nm), changes in reflectance between the samples are practically absent, which indicates the stability of this region and the low influence of pigments on short- and mid-wavelength ranges (Król et al., 2025).

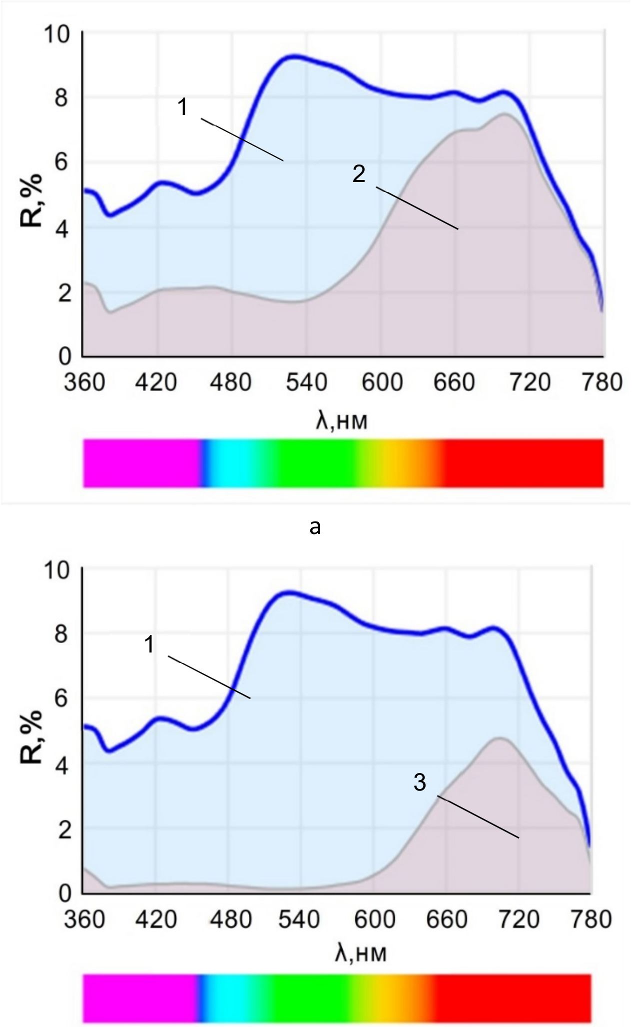
In the red region of the spectrum (λ ≈ 650–750 nm), a noticeable decrease in reflectance is observed with an increase in the concentration of *Aronia melanocarpa* from 0.7% to 0.9%. The sample with 0.9% *Aronia melanocarpa* has lower reflectance values, which indicates more pronounced light absorption by pigment components in the long-wavelength region of the spectrum (Lv et al., 2024). In particular, the maximum R value in the near-IR region decreases from approximately 59.8% (a) to 35% (b). The shape of the curve demonstrates a concentration-dependent rise at long wavelengths, while the blue-green region remains almost unchanged, confirming the specific action of chokeberry anthocyanins, directed mainly at the red part of the visible spectrum (Zhang et al., 2025). This indicates increased light absorption and intensification of the coloration of concentrates with increasing *Aronia melanocarpa* content. Such results are advisable to use for assessing the quality and visual attractiveness of dairy products with plant ingredients.

The reflectance spectra of whey samples (control) and whey obtained during the separation of the dairy-plant concentrate depending on wavelength are shown in Figure 5.

The control whey sample (curve 1) is characterized by a high reflectance coefficient (R ≈ 8–9%) with a pronounced maximum in the blue-green region of the spectrum (λ ≈ 480–560 nm). At the same time, curve 2 and curve 3 (whey with *Aronia melanocarpa*) demonstrate a significant decrease in the reflectance coefficient over the entire visible range. This is due to the presence of dark purple anthocyanin pigments (Wang et al., 2024; Zielińska et al., 2020), which cause intensive light absorption, especially in the green–yellow and red regions (λ ≈ 580–750 nm), where reflectance values decrease to R = 2–4%. The main color is formed predominantly by cyanidin-3-O-galactoside and cyanidin-3-O-arabinoside, which dominate in the composition of *Aronia melanocarpa* (Xu et al., 2024).

Comparison of graphs a and b confirms a dose-dependent effect of *Aronia melanocarpa* concentration on the color properties of the product. An increase in the amount of the plant ingredient to 0.9 % leads to a further decrease in the reflectance coefficient, which is most noticeable in the long-wavelength part of the spectrum (λ ≈ 600–780 nm). This indicates a proportional increase in color intensity and saturation of the product with increasing pigment concentration, which correlates with increased absorbance capacity.

The results are consistent with the general trend of using *Aronia melanocarpa* extracts as natural colorants and functional additives in food products (Artamonova et al., 2025; Wang et al., 2024). Thus, spectrophotometric analysis confirms the effectiveness of using *Aronia melanocarpa* to adjust the color characteristics of whey and to create a functional product with increased biological value (Uzunsoy, 2022).



b

**Figure 5. Reflectance spectra of samples depending on wavelength:
1 – whey (control); 2 – whey obtained during the production of dairy–plant concentrates with the addition of 0.7 % Aronia melanocarpa powder (a); 3 – whey obtained during the production of a dairy-plant concentrate with the addition of 0.9 % Aronia melanocarpa powder (b).**

The results of the instrumental study of the color characteristics of dairy–plant concentrates and whey are presented in Table 4.

The study of the color characteristics of dairy-plant concentrate samples with the addition of *Aronia melanocarpa* powder in amounts of 0.7% and 0.9%, as well as two types of whey after dehydration of the concentrates, confirmed a significant effect of the plant additive on the color characteristics of the system according to CIELAB parameters. The system consists of three coordinates that describe color in a three-dimensional space

Table 4

Color characteristics of dairy–plant concentrates and whey

Experimental samples	Color characteristics						
	L	a	b	ΔL	Δa	Δb	ΔE
Protein concentrate with Promilk (control)	93.63^{a}	-0.75^{a}	10.5^{a}	-	-	-	-
Protein concentrate with the addition of 0.7% *Aronia melanocarpa*	58.53^{b}	7.85^{b}	0.03^{b}	-35.1^{a}	8.61^{a}	-10.47^{a}	37.62^{a}
Protein concentrate with the addition of 0.9% *Aronia melanocarpa*	35.05^{c}	10.6^{c}	-1.36^{c}	-58.58^{b}	11.36^{b}	-11.86^{b}	60.84^{b}
Whey (control)	34.81^{a}	-4.9^{a}	11.81^{a}	-	-	-	-
Whey with 0.7% *Aronia melanocarpa*	15.86^{b}	8.92^{b}	1.74^{b}	-11.69^{a}	13.37^{a}	-5.74^{a}	78.86^{a}
Whey with 0.9% *Aronia melanocarpa*	5.57^{c}	9.27^{c}	1.48^{c}	-21.99^{b}	$13{,}69^{b}$	-6.01^{b}	89.09^{b}

Note: L is lightness; a is red/green coordinate; b is yellow/blue coordinate; ΔL is difference in lightness; Δa is difference in the red/green coordinate; Δb is difference in the yellow/blue coordinate; ΔE is total color difference. Values are means±standard deviation. Means within the same column with different superscripts are significantly different at $p \leq 0.05$.

In the control sample of the dairy protein concentrate, high lightness values ($L = 93.63$) and a positive b value (10.50) were observed, corresponding to a light yellowish shade of the product. The a value was negative (–0.75), indicating the absence of red coloration.

The addition of black chokeberry caused pronounced shifts in all color coordinates compared to the control. In the dairy–plant concentrate with 0.9% *Aronia melanocarpa*, lightness decreased to $L = 35.05$, i.e., $\Delta L = -58.58$, which indicates significant darkening of the product. At the same time, a sharp increase in the a value to 10.60 ($\Delta a = +11.36$) was observed, indicating intense reddish coloration characteristic of chokeberry anthocyanin pigments. The b value became negative (–1.36), and Δb was –11.86, reflecting a shift of color toward the blue-violet region of the spectrum, which is typical for acidic environments involving anthocyanins.

Reducing the black chokeberry dose to 0.7 % in the production of the dairy-plant concentrate caused a less intense, but still significant, shift in color coordinates. L decreased to 58.53 ($\Delta L = -35.10$), while the a and b values changed to 7.85 ($\Delta a = +8.61$) and 0.03 ($\Delta b = -10.47$), respectively. These results indicate a dose-dependent nature of pigmentation, where even minimal concentrations of the plant ingredient (*Aronia melanocarpa*) provide a noticeable visual effect.

In whey, the changes were even more pronounced, which is due to the absence of a protein–fat matrix capable of retaining light and partially masking pigmentation. For whey with 0.9% *Aronia melanocarpa*, the L value was only 5.57 ($\Delta L = -21.99$), which corresponds to a dark purple color. The a coordinate increased to 9.27 (Δa = +13.69), while b decreased to 1.48 (Δb = –6.01), reflecting the dominance of anthocyanin pigments in the dissolved state. Reducing the concentration of Aronia melanocarpa to 0.7 % in whey resulted in slightly higher lightness (L = 15.86; ΔL = –11.69); however, the red hue remained high (a = 8.92; Δa = +13.37), and b remained low (b = 1.74; Δb = –5.74) compared to the control. The ΔE values (37–89) greatly exceed the perceptibility threshold. Therefore, color changes in the experimental samples are very intense and visually perceptible, which should be taken into account in recommendations for the use of colored whey in beverages.

The obtained results are consistent with published data on color formation involving anthocyanin-containing plants. According to studies, anthocyanins of *Aronia melanocarpa* have a high degree of affinity for the aqueous phase and form intense coloration at pH 3–6, which causes an increase in the a parameter and a shift toward purple shades (b – negative values) (Lv et al., 2024; Wang et al., 2024). Protein matrices are capable of partially adsorbing anthocyanins, which leads to less intense coloration compared to whey (Gamage et al., 2024; Robles-García et al., 2025).

Thus, the obtained experimental data correspond to the general patterns of interaction between anthocyanins, milk proteins, and the aqueous phase of the system.

Conclusions

It was established that the addition of Promilk increases the degree of utilization of goat milk dry matter and the yield of the concentrate. This effect is explained by the enrichment of the system with highly functional milk proteins, particularly κ-casein, which contribute to the formation of a denser three-dimensional protein–fat structure. With increasing Promilk concentration, a decrease in free moisture content (from 73.3±0.1% to 48.4±0.1%) and an increase in water-holding capacity (from 64.2±0.1% to 69.8±0.2%) were observed, confirming the influence of dry protein on water-binding capacity. The rational amount of *Aronia melanocarpa* powder added to goat milk with Promilk was determined to be 0.7%. Reducing the chokeberry dose to this level in the production of dairy-plant concentrates caused a less intense but still significant shift in color coordinates: L decreased to 58.53 (ΔL = –35.10), while a and b values changed to 7.85 (Δa = +8.61) and 0.03 (Δb = –10.47), respectively. These results indicate a dose-dependent pigmentation effect, where even minimal concentrations of *Aronia melanocarpa* produce a noticeable visual change. Whey, as the aqueous phase, exhibits the greatest color sensitivity, while the protein concentrate partially adsorbs pigments, forming a more moderate but stable color profile.

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Light brewer’s spent grain as a functional ingredient in sourdough triticale bread production

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Abstract

Introduction. The aim of the research is to determine the effect of light brewer’s spent grain in a sourdough form (BSF) on dough rheological properties and bread quality obtained from three different triticale varieties Ingen 40, Costel and Fanica.

Materials and methods. The rheological properties of triticale dough were evaluated using Mixolab, Alveograph, Falling Number, Rheofermentometer, and a HAAKE MARS 40 rheometer, while bread quality was assessed in terms of acidity, loaf volume, porosity, elasticity, texture, color parameters, and sensory attributes based on a 9-point hedonic scale.

Results and discussion. The incorporation of light brewer’s spent grain in sourdough form into the dough recipe led to a significant decrease in water absorption, dough stability, dough development time, protein weakening (C2), dough storage and loss moduli (G′, G″), dough tenacity, baking strength, and P/L ratio, indicating a weakening of the dough rheological properties. BSF addition significantly increased α-amylase activity, as evidenced by the decrease in falling number values. Mixolab analysis showed that the difference between C3 (starch gelatinization) and C4 (hot gel stability) reflected amylolytic activity and exhibited some fluctuations, likely due to incomplete starch gel formation. BSF also reduced starch gelatinization, as indicated by the decrease in C3 torque. Rheological properties during fermentation improved, with retention coefficients above 90%, indicating the potential to obtain good-quality bread. BSF incorporation resulted in triticale bread of good quality, contributing nutty, earthy, and malty notes that enhanced acceptability. However, it increased acidity and decreased loaf volume, porosity, and elasticity, while modifying textural characteristics.

Conclusion. Bread enriched with light brewer's spent grains in a sourdough form presents a promising avenue for creating value-added, sustainable, and nutritious triticale-based products that appeal to modern consumers seeking healthier and environmentally conscious food choices.

Introduction

Current global challenges due to climate change, rapid population growth, and current consumer preference for healthier foods have led to the search for alternative solutions to traditional cereals used in human nutrition. One such solution is triticale, which is the first cereal obtained from artificial hybridization between tetraploid wheat (*Triticum aestivum*) and rye (*Secale cereale*). In 2022, the top ten triticale producers in the world, by area cultivated and share, were: Poland (34.1%), Belarus (11.2%), France (9.4%), Germany (9%), Spain (7.8%), China (5.5%), Turkey (2.8%), Lithuania (1.7%), Australia (1.7%), and Romania (1.6%) (a5), with more than 90.0% of triticale production being concentrated in Europe (González-Alonso et al., 2024).

By creating this species, the most valuable traits of wheat (functional qualities for food production, early maturity, high grain number per ear, and high grain weight) were combined with the valuable traits of rye (resistance to drought, heat, diseases, pests, and high adaptability to damaged soils and unfriendly areas) (Andraş et al., 2023; Bonea, 2024; Cionca et al., 2024; Vaca-García et al., 2011). Triticale is a highly productive crop even in environments inaccessible to other food crops, with low production costs compared to other crops, and with low sensitivity to biotic and abiotic stress factors (Leonova et al., 2022; Usmanova et al., 2024).

New technologies used in baking or by adding ingredients can produce quality products that are accepted by consumers (Shevchenko, 2022; Shevchenko et al., 2024; Tsykhanovska et al., 2024). Adding potato maltodextrins to triticale flour, mixing triticale flour with 5% and 10% bran or combining several cereals (e.g. wheat, rye and triticale in a ratio of 40:50:10 or 90:10) have shown great promise in producing quality bakery products (Bonea, 2024). Triticale noodles have higher nutritional value and bioactivity compared to conventional wheat noodles, due to the higher content of proteins, minerals, vitamins and phenolic compounds (Liu et al., 2023).

In addition to its high nutritional value, the development of whole grain triticale flour bakery products is also due to the health benefits of these functional products recommended for their antioxidant, anticancer and cardiovascular disease prevention properties (Banu et al., 2020; Li et al., 2024). In general, triticale grains help control diabetes, improve gastrointestinal health, stimulate circulation, increase cell production and stimulate bone growth (Arizmendi-Cotero et al., 2020; Piazza et al., 2023).

Barley spent grain (BSG) is the main by-product of the brewing industry, accounting over 85% of beer manufacturing by-products (Hejna, 2021). Its bioconversion from waste into a useful ingredient aligns with the increasing interest in sustainable and resource-efficient food production (Stabnikova et al., 2023a, b). Barley spent grain is in specially composed from the husk and outer layers of the barley kernel and is rich in dietary fiber, protein, essential amino acids, minerals, and bioactive compounds (Chetrariu and Dabija, 2023; Ivanov et al., 2021). Despite its high nutritional value, approximately 70% of barley spent grain is utilized as animal feed, 10% is converted into biogas and 20% are disposed in landfills (Mitri et al., 2022). The increasing interest in sustainable food systems and circular economy practices had encouraged the incorporation of barley spent grain into food products, such as bakery ones. However, the use of BSG in its current form does not lead to a full valorisation of its nutritional characteristics. It has been reported that BSG presents some antinutritional factors, a low mineral bioavailability and protein digestibility which may be improved by different preliminary treatments such as fermentation ones (Chetrariu and Dabija, 2023; Henkin et al., 2025). Through fermentation complex fibers are partially broken down, bioavailability of nutrients is enhanced, and antinutritional factors are reduced. More, the fermentation process may contribute to the improvement of BSG aromas and dough rheology and bread quality (Ghendov-Mosanu, 2024).

The aim of this work was to establish the possibility of using wholemeal flours from three different varieties of triticale (Ingen 40, Costel, Fanica) cultivated in the Republic of Moldova in breadmaking, and to evaluate the influence of the addition of sourdough from light brewers' spent grain flour on dough rheology and bread quality.

Materials and methods

Materials

Three triticale varieties (Ingen 40, Costel, Fanica) cultivated in the Republic of Moldova (harvest 2023) were used in this study. To obtain integral triticale flour, the grains were milled in a laboratory mill 3100 (Perten Instruments, Hägersten, Sweden) and presented the fallowing data according to the ICC (International Association for Cereal Science and Technology) standard methods: 13.1–14.2% for protein content (ICC 105/2), 1.50–1.70% for ash content (ICC 104/1), and 18.49–25.61% for wet gluten content (ICC 106/2). The brewers' spent grain (BSG) was obtained from the blonde beer process. (Î.M. "Efes Vitanta Moldova Brewery" S.A., Chisinau, the Republic of Moldova). Sourdough from brewers' spent grain flour had been obtained according to the method previously described (Ghendov-Mosanu et al., 2025). The BSG in a fermented form (BSF) were incorporated in 10 and 17.5% levels in triticale flour.

Evaluation of dough rheological properties

Dough mixing and pasting rheological properties were determined using the Mixolab device (KPM, Tripette et Renaud, Paris, France) according to the ICC No. 173 standard method. The Mixolab parameters analysed were water absorption (WA), dough development time (DDT), dough stability (ST) and torques during mixing and heating C1-C5.

Dynamic dough rheological properties have been determined using HAAKE MARS 40 rheometer (Thermo-HAAKE, Karlsruhe, Germany). Frequency sweep tests from 1 to 20 Hz were carried out in which the storage modulus (G') and the loss modulus (G"), were obtained together with the phase change according to the method previously described by Ghendov-Mosanu et al. (2025).

Evaluation of dough rheological properties during extension have been determined using an Alveograph (KPM, Tripette et Renaud, Paris, France) according to ICC 121 standard method on which dough extensibility (L), index of swelling (G), maximum pressure (P), baking strength (W) and configuration ratio (P/L) have been determined.

Rheological properties during fermentation have been determined using a rheofermentometer device (Chopin Rheo, type F4, KPM, Tripette et Renaud, Paris, France) in which the fallowing parameters have been determined: volume of the gas retained in the dough at the end of the test (VR, mL), total CO_2 volume production (VT, mL), retention coefficient (CR, %) and maximum height of gaseous production (H'm, mm) according to the method described by us in our previously study (Ghendov-Mosanu et al., 2025). Also, by using a Falling Number device (FN 1305, Perten Instruments AB, Stockholm, Sweden) the falling number (FN, s) value has been determined according to ICC 107/1 standard method.

Bread-making

For bread-making the dough has been prepared through the direct method. Salt, compressed yeast (*Saccharomyces cerevisiae*), triticale flour, water and sourdough from brewers' spent grain were mixed for 10 – 15 min in a laboratory mixer (Kitchen Aid, Whirlpool Corporation, Benton Harbor, MI, USA). Dough was fermented in a Cooper 72B device (Europe SRL, Malo (VI), Italy) at a temperature of 30±1°C for 120 – 150 min. The dough was divided into 400 g pieces, modelled and finally proofed at a temperature of 38±1°C and a relative humidity of 80 – 85% for 50 min. The dough was steamed and baked in a Cooper 72B oven (Europe SRL, Malo (VI), Italy) at a temperature of 190±10 °C for 50 – 60 min. After baking, the bread samples were cooled to a temperature of 20±1 °C, after which the quality characteristics were analysed.

Bread quality characteristics

The bread was evaluated for its physical, textural, color and sensory characteristics. Loaf volume, porosity, elasticity was determined according to the AACC (2010). The bread acidity was determined according to the method described by Ghendov-Mosanu et al. (2024). The textural characteristics of the bread samples were determined using a Stable Micro Systems TA.HD plus C texture analyzer (United Kingdom) on which the following parameters were determined: firmness, gumminess, cohesiveness, chewiness and resilience. For this purpose, bread slices of 20×20 mm was subjected to a two-compression cycle with a P/75 stainless steel plate, by the following protocol: pre-test speed - 100 m/s; test speed - 5 m/s; post-test speed - 5 m/s and cell load - 5 kg. The color parameters were determined using a Konica Minolta CR-400 colorimeter (Tokyo, Japan). The following parameters were determined: L* (darkness/lightness), a* (red/green tone) and b* (blue/yellow tone). The determinations were based on the CIELab* system. The range in which the absorption of electromagnetic radiation was performed was the UV-vis. Bread sensory characteristics were analyzed using the 9-point hedonic scale with semi-trained panelists. The fallowing sensory characteristics were analyzed: appearance, color, taste, odor, texture, aroma and global acceptability.

Statistical analysis

The data obtained were analysed by using the analysis of variance (ANOVA) and Tukey's test ($p < 0.05$). All calculations were determined using Statgraphics software Centurion XVI 16.1.17 (Statgraphics Technologies, Inc., The Plains, VA, USA).

Results and discussion

Light brewers' spent grain sourdough effect on dough rheological properties

The rheological behavior of the dough during mixing and heating was evaluated using the Mixolab device, which provides key parameters reflecting protein and starch transformations: the C1 value represents the initial dough consistency and water absorption capacity; C2 indicates protein weakening and thus reflects the stability of the gluten network under mechanical and thermal stress; C3 corresponds to starch gelatinization, describing the swelling of starch granules during heating; C4 reflects the stability of the hot starch gel and is associated with enzymatic activity, particularly that of α-amylase; finally, C5 represents starch retrogradation during cooling and is related to bread staling.

The dough mixing and pasting properties of triticale dough with different amounts of sourdough addition are shown in Table 1. As may be seen, water absorption value significantly ($p < 0.05$) decreased by brewers' spent grain addition in a fermented form in dough recipe (BSF), these data being in disagreement with those reported by Aprodu et al. (2017) when BSG was added into wheat flour. Generally, dough stability and dough development time decreased or did not present high variations compared to the control sample. This decrease indicates a weakening of the dough probably due to the gluten dilution since BSF does not contain gluten. The C1 torque is around 1.1 N·m which is the typically adjusted value to Mixolab protocol to determine the proper water absorption value (Golea et al., 2023). The C2 Mixolab torque value indicates the protein weakening which decreased with the increases amount of BSF addition in dough recipe. Because gluten is in a lower amount and also due to the fact that light brewers' spent grain is incorporated in the dough recipe in a sourdough form dough viscosity decreased during heating favouring protein denaturation and an increase of proteolytic enzymes activity fact reflected by a decrease of C2 and an increase of C12 values from the Mixolab curve (Dabija et al., 2017). The C3 values which represent starch gelatinization behaviour decreased with the increase amount of BSF addition in wheat flour. By BSF addition, which is in a high level, the starch amount from the dough system decreased. More, the amount of water is less, which indicates than the starch gelatinisation may not be complete. However, the difference between C3 and C2 torques presented fluctuations probably due to the water availability in the dough system and the ability of starch to gelatinize in a more or less completely form (Codină et al., 2019). The same behaviour may be related to C4 and the difference between C4 and C3 torques (C34). C34 values are related to the amylolytic enzyme activity and therefore the higher the value of this parameter is, the higher the enzymatic activity of amylases are. However, the amylase activity is related to the starch gelling. If the starch gelatinisation is not complete, then the amylolytic activity may be lower. C5 value which is related to the final starch paste viscosity and the difference between C5 and C4 torques (C54) presented some fluctuations probably due to the BSF chemical composition such as non-gluten proteins, nonstarch polysaccharides which modifies three-dimensional network of the dough system (Ghendov-Mosanu et al., 2025).

Frequency sweep behaviour of the dough samples with and without BSF addition are shown in Figure 1. As may be seen, loss (G″) and storage (G′) module increased with frequency. For all samples, the G'' was lower than G' indicating a solid-like behaviour (Tan et al., 2023). Higher amounts of BSF led to a decrease of G′ and G″ values which indicates a decrease of dough viscoelasticity. This may be due to the decrease of gluten content from the dough system which is the mainly responsible for dough viscoelasticity properties. Loss tangent (tan δ) is less than 1 for all dough samples indicating that dough has dominant elastic properties (Li et al., 2023). The BSF addition in dough recipe increases tan δ values which indicates the fact that these samples have a more pronounced viscous component, which can negatively influence bread shape stability and textural properties.

Dough rheological properties during extension are shown in Table 2. As may be seen the alveograph values P, W and the ratio P/L decreased by BSF addition in triticale flour. This indicates a dough weakness probably due to the gluten dilution but also due to the enzymatic activity from light brewers' spent grain sourdough addition which act on gluten and starch. The dough extensibility (L) and index of swelling (G) present some fluctuations for the samples with BSF addition in triticale flour. Generally, at low amounts of BSF addition in dough recipe, these values tend to increase, whereas at high addition levels these values significantly ($p < 0.05$) decreased. This behaviour is due to the enzymatic activity increase in dough system with BSF addition which hydrolyse and increase it extensibility. However, at high amount of BSF addition in triticale flour, it is possible that the dough pH values to reach the isoelectric ones and to modify gluten structure and therefore its extensibility. These data are in agreement with those reported by Codină et al. (2021) when sourdough were added in wheat flour.

Table 1

Mixolab values for triticale dough with light brewers' spent sourdough

Samples	WA (%)	ST (min)	DDT (min)	C1 (N·m)	C2 (N·m)	C3 (N·m)	C4 (N·m)	C5 (N·m)	C12 (N·m)	C32 (N·m)	C34 (N·m)	C54 (N·m)
Ingen 40	57.5±0.3 ^{e}	6.3±0.2 ^{d}	4.78±0.03 ^{f}	1.130±0.0 ^{e}	0.462±0.001 ^{f}	2.027±0.006 ^{d}	0.675±0.003 ^{f}	1.245±0.013 ^{e}	0.668±0.006 ^{a}	1.565±0.021 ^{e}	1.352±0.006 ^{f}	0.570±0.0 ^{f}
Ingen 40_10BSF	47.7±0.01 ^{c}	3.6±0.1 ^{c}	4.08±0.04 ^{e}	1.129±0.001 ^{e}	0.353±0.005 ^{c}	1.855±0.004 ^{c}	0.590±0.006 ^{d}	1.092±0.002 ^{d}	0.776±0.003 ^{f}	1.502±0.013 ^{d}	1.265±0.007 ^{e}	0.502±0.005 ^{e}
Ingen 40_17.5BSF	40.2±0.2 ^{b}	3.6±0.1 ^{c}	3.98±0.01 ^{d}	1.154±0.002 ^{f}	0.353±0.003 ^{c}	1.824±0.008 ^{c}	0.664±0.007 ^{e}	1.096±0.005 ^{d}	0.801±0.002 ^{g}	1.471±0.016 ^{d}	1.160±0.0 ^{d}	0.432±0.005 ^{d}
Costel	60.0±0.3 ^{e,f}	2.8±0.2 ^{b}	2.97±0.03 ^{a}	1.095±0.003 ^{c}	0.379±0.005 ^{d}	1.449±0.010 ^{b}	0.326±0.002 ^{c}	0.599±0.008 ^{c}	0.716±0.005 ^{c}	1.070±0.006 ^{b}	1.123±0.002 ^{d}	0.273±0.002 ^{c}
Costel_10BSF	48.2±0.1 ^{d}	2.3±0.1 ^{a}	3.00±0.02 ^{a}	1.070±0.002 ^{a,b}	0.303±0.004 ^{b}	1.392±0.008 ^{b}	0.245±0.001 ^{a}	0.439±0.013 ^{a}	0.767±0.004 ^{e}	1.089±0.015 ^{b}	1.147±0.007 ^{d}	0.194±0.006 ^{a}
Costel_17.5BSF	37.9±0.1 ^{a}	2.4±0.2 ^{a,b}	2.92±0.01 ^{a}	1.072±0.001 ^{b}	0.266±0.005 ^{a}	1.422±0.006 ^{b}	0.314±0.004 ^{b}	0.588±0.001 ^{b}	0.806±0.001 ^{h}	1.156±0.012 ^{c}	1.108±0.006 ^{b,c}	0.274±0.003 ^{c}
Fanica	60.2±0.1 ^{f}	2.5±0.2 ^{a,b}	3.45±0.04 ^{c}	1.112±0.004 ^{d}	0.421±0.002 ^{e}	1.416±0.011 ^{b}	0.302±0.005 ^{b}	0.552±0.015 ^{b}	0.691±0.001 ^{b}	0.995±0.009 ^{a}	1.114±0.004 ^{d}	0.250±0.007 ^{b}
Fanica_10BSF	48.0±0.2 ^{c,d}	2.6±0.2 ^{a,b}	3.07±0.02 ^{b}	1.120±0.002 ^{d,e}	0.358±0.003 ^{c}	1.375±0.004 ^{a}	0.339±0.012 ^{c}	0.578±0.011 ^{b}	0.762±0.003 ^{e}	1.017±0.012 ^{a}	1.036±0.003 ^{a}	0.239±0.009 ^{b}
Fanica_17.5BSF	39.6±0.2 ^{b}	2.7±0.1 ^{b}	3.07±0.01 ^{b}	1.069±0.001 ^{a}	0.314±0.003 ^{b}	1.386±0.005 ^{b}	0.319±0.001 ^{b}	0.534±0.009 ^{b}	0.755±0.006 ^{d,e}	1.072±0.015 ^{b}	1.067±0.006 ^{b}	0.215±0.004 ^{a}

Mixolab values: WA, water absorption; ST, stability, DDT, dough development time; C1-C5, torques corresponding stages 1-5 of the Mixolab curve; C12, difference of the torques C1 and C2; C32, difference of the torques C3 and C2; C34, difference of the torques C3 and C4; C54, difference of the torques C5 and C4. $^{a–h}$, mean values (n = 3) in the same column followed by different letters are significantly different ($p < 0.05$).

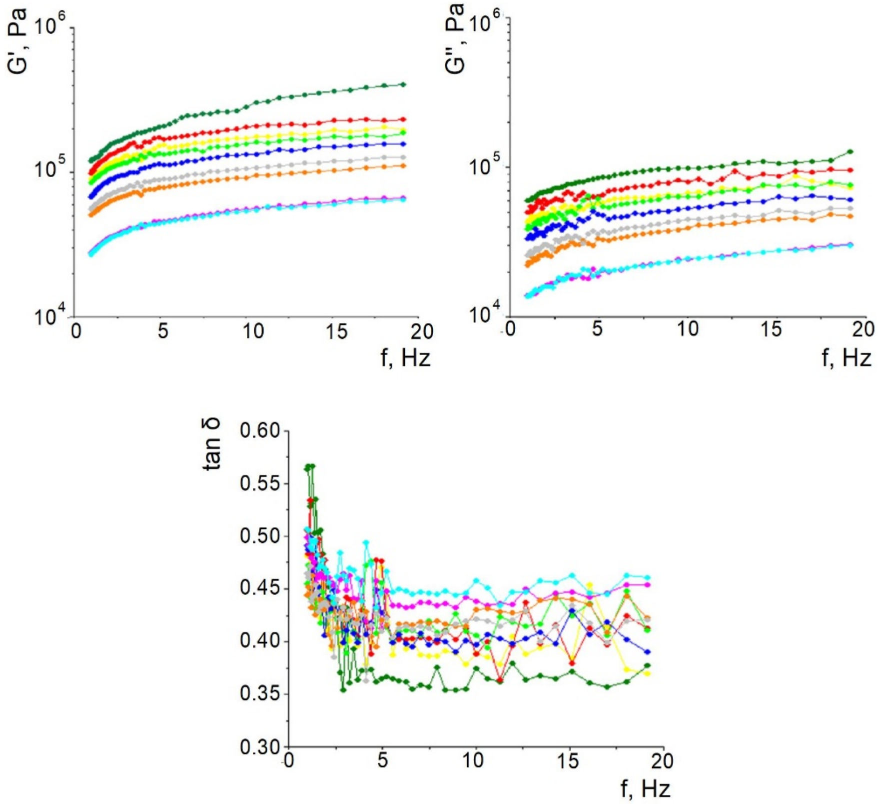


Figure 1. Evaluation with frequency of *G'*, *G''* and tan δ for dough samples (-●-Ingen 40; -●- Ingen40_10BSF; -●- Ingen40_17.5BSF; -●- Costel; -●- Costel 10BSF; -●- Costel 17.5BSF; -●- Fanica; -●- Fanica 10BSF; -●- Fanica 17.5BSF)

Table 2

Alveograph values for triticale dough with light brewers' spent sourdough

Samples	**P (mm)**	**L (mm)**	**G (mm)**	**W (10^{-4} J)**	**P/L**
Ingen 40	110±4 ^{f}	41±0 ^{c}	14.2±0.2 ^{e}	150±7 ^{e}	2.68±0.07 ^{e}
Ingen 40_10 BSF	52±6 ^{c}	52±1 ^{d}	16.0±0.3 ^{f}	64±0 ^{c}	1.00±0.09 ^{a}
Ingen 40_17.5 BSF	26±3 ^{a}	32±1 ^{b}	12.6±0.1 ^{d}	26±5 ^{a}	0.81±0.01 ^{a}
Fanica	125±4 ^{g}	33±0 ^{b}	12.8±0.2 ^{d}	147±3 ^{e}	3.79±0.08 ^{g}
Fanica_10BSF	67±6 d	31±1 ^{b}	12.4±0.1 ^{c,d}	61±2 ^{c}	2.16±0.11 ^{d}
Fanica_17.5BSF	43±1 ^{b}	23±1 ^{a}	10.6±0.1 ^{a}	22±2 ^{a}	1.87±0.09 ^{c}
Costel	99±7 ^{e}	32±1 ^{b}	12.6±0.1 ^{d}	104±5 ^{d}	3.09±0.06 ^{f}
Costel_10BSF	41±3 ^{b}	30±2 ^{b}	12.2± 0.1 ^{c}	44±3 ^{b}	1.37±0.13 ^{b}
Costel_17.5BSF	23±4 ^{a}	24±1 ^{a}	10.9±0.1 ^{b}	20±2 ^{a}	0.96±0.07 ^{a}

P, maximum pressure; L, dough extensibility; G, index of swelling; W, baking strength; P/L, configuration ratio of the Alveograph curve. $^{a-g}$, mean values ($n = 3$) in the same column followed by different letters are significantly different ($p < 0.05$).

Dough rheological properties during fermentation and the falling number values which expressed the α amylase activity are shown in Table 3. The BSF addition in triticale flour led to a significant ($p < 0.05$) decrease of the falling number values. This indicates an increase of the α amylase activity from the dough system probably due to the increase of amylolytic activity from the sourdough obtained from light brewers' spent grains. This increase will favour starch hydrolysis into maltose which will be consumed by yeast to produce carbon dioxide that increases Rheofermentometer parameters H'm and VT (Codină et al., 2013). As may be seen the VR values has also a similar trend as H'm and VT values even if triticale dough with BSF addition has a low gluten content. The CR coefficient, which is the ratio between VR and VT presents very good values higher than 90% which indicates a high ability of the dough samples to retain the gases formed during the fermentation process. This behaviour can positively influence bread loaf volume, porosity and textural properties according to Ktenioudaki et al. (2011). However, for Ingen 40 and Fanica samples with the highest amount of BSF incorporated in dough recipe the CR values begin to decrease probably due to the gluten dilution effect.

Light brewers' spent grain sourdough effect on bread quality characteristics

The bread samples obtained with different amount of BSF addition in triticale flour are shown in Figure 2.

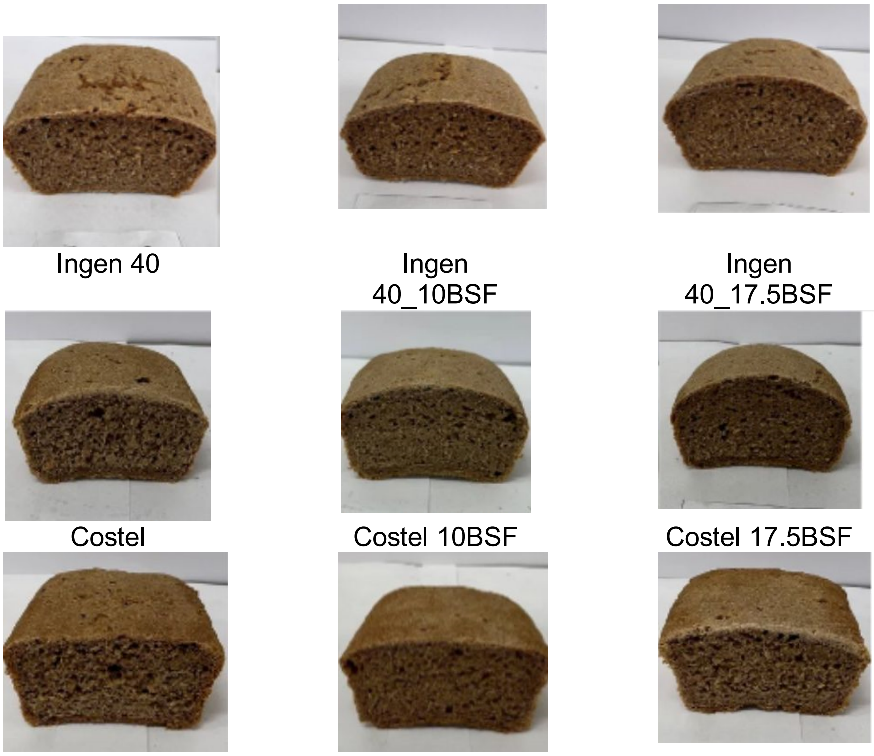
Table 3

Rheofermentometer and falling number values for triticale dough with light brewers' spent sourdough

Samples	H'm (mm)	VT (mL)	VR (mL)	CR (%)	FN
Ingen 40	85.4±0.5 ^{b}	1607±26 ^{b}	1590±12 ^{b}	98.9±0.2 ^{d}	111±2 ^{e}
Ingen 40_10BSF	97.9±0.1 ^{e}	1645±31 ^{b}	1620±18 ^{b}	98.5±0.3 ^{c,d}	90±0 ^{d}
Ingen 40_17.5BSF	91.4±0.3 ^{d}	1865±19 ^{d}	1679±9 ^{b,c}	90.1±0.1 ^{a}	70±1 ^{b}
Costel	68.4±0.8 ^{a}	1328±7 ^{a}	1311±7 ^{a}	98.7±0.02 ^{c,d}	71±1 ^{b}
Costel 10BSF	90.2±0.1 ^{d}	1898±12 ^{d}	1872±11 ^{d}	98.6±0.02 ^{c,d}	67±1 ^{b}
Costel 17.5BSF	101.3±0.1 ^{f}	2229± 8 ^{f}	2212±14 ^{f}	99.2±0.03 ^{d}	64±1 ^{a}
Fanica	88.4±0.3 ^{c}	1648±11 ^{b}	1602±13 ^{b}	97.2±0.1 ^{b}	73±1 ^{c}
Fanica 10BSF	90.2±0.1 ^{d}	1752±14 ^{c}	1746±8 ^{c}	99.7±0.1 ^{d}	67±1 ^{b}
Fanica 17.5BSF	90.3±0.1 ^{d}	1988±9 ^{e}	1963±14 ^{e}	98.7±0.2 ^{c,d}	63±2 ^{a}

H'm, maximum height of gaseous production; VT, total CO_2 volume production; VR, volume of the gas retained in the dough at the end of the test; CR, retention coefficient. $^{a–f}$, mean values (n = 3) in the same column followed by different letters are significantly different (p < 0.05).

The bread physico-chemical characteristics are shown in Table 4. As may be seen, the acidity of the bread samples from triticale flours without the addition of sourdough from light brewers' spent grain flour varies between 4.60–6.46 degrees. At the same time, it can be noticed that the use of sourdough in the preparation of the dough influenced the increase of acidity of the bread samples by an average of 3 and 4.5 degrees when adding sourdough in an amount of 10% and 17.5% of the total flour mass. This increase of the acidity values can be explained by the intake of acids accumulated in the sourdough during the fermentation process (Ghendov-Mosanu et al., 2025).



Ingen 40

Ingen 40_10BSF

Ingen 40_17.5BSF

Costel

Costel 10BSF

Costel 17.5BSF

Fanica

Fanica 10BSF

Fanica 17.5BSF

Figure 2. The triticale bread samples with different amount of BSF addition

Table 4

Physico-chemical characteristics values and global acceptability for triticale bread with light brewers' spent sourdough

Samples	**Acidity (degrees)**	**Porosity (%)**	**Elasticity (%)**	**Loaf volume (cm^3/100g)**	**Global acceptability (dimensionless)**
Ingen 40	4.60±0.02 ^{a}	58.3±0.6 ^{e}	67.3±0.6 ^{g}	239±4 ^{d,e}	8.62±0.05 ^{e}
Ingen 40_10BSF	8.08±0.01 ^{d}	53.0±0.3 ^{d}	62.1±0.1 ^{d}	217±3 ^{a,b}	8.55±0.03 ^{d,e}
Ingen 40_17.5BSF	9.84±0.02 ^{f}	52.9±0.1 ^{d}	58.7±0.2 ^{c}	216±5 ^{a,b}	8.00±0.0 ^{c}
Costel	5.70±0.03 ^{b}	51.3±0.2 ^{c}	64.8±0.1 ^{e}	260±4 ^{f}	8.43±0.03 ^{d}
Costel 10BSF	8.68±0.02 ^{e}	45.6±0.4 ^{b}	55.9±0.1 ^{b}	236±6 ^{c,d}	8.37±0.01 ^{d}
Costel 17.5BSF	10.70±0.04 ^{g}	44.3±0.2 ^{a}	53.7±0.2 ^{a}	216±3 ^{a,b}	7.18±0.04 ^{a}
Fanica	6.46±0.01 ^{c}	52.4±0.1 ^{c}	65.9±0.2 ^{f}	242±4 ^{d,e}	8.76±0.06 ^{e,f}
Fanica 10BSF	8.20±0.0 ^{d}	51.7±0.3 ^{c}	58.2±0.3 ^{c}	235±2 ^{c,d}	7.98±0.02 ^{c}
Fanica 17.5BSF	10.80±0.03 ^{g}	45.0±0.2 ^{b}	54.6±0.1 ^{b}	232±5 ^{c,d}	7.31±0.02 ^{b}

$^{a–g}$, mean values (n = 3) in the same column followed by different letters are significantly different (p < 0.05).

The porosity and elasticity of the bread samples without the addition of sourdough from the brewers' spent grain flour varies between 51.3–58.3%, and 64.8–67.3% respectively, the highest value being obtained for the bread sample obtained from the Ingen 40 triticale flour variety. The variation in the porosity and elasticity of the bread samples can be explained by the variation in the chemical composition of triticale varieties (Codina et al., 2025), in particular by the variation in gluten proteins content, which plays the main role in the gluten formation process and influences the dough's ability to retain the gases formed during fermentation process. At the same time, the addition of sourdough from light brewers' spent grain flour in the bread recipe, significantly ($p < 0.05$) decreased the porosity and elasticity values. The loaf volume of bread samples without sourdough from light brewers' spent grain flour addition varied between 239–242 cm^3/100g. As may be seen the light brewers' spent sourdough addition decreased the values of bread samples. The explanation is similar to those for the porosity and elasticity decrease and namely doughs lose it ability to retain the gases formed during fermentation, as Rheofermentometer data has previously indicated.

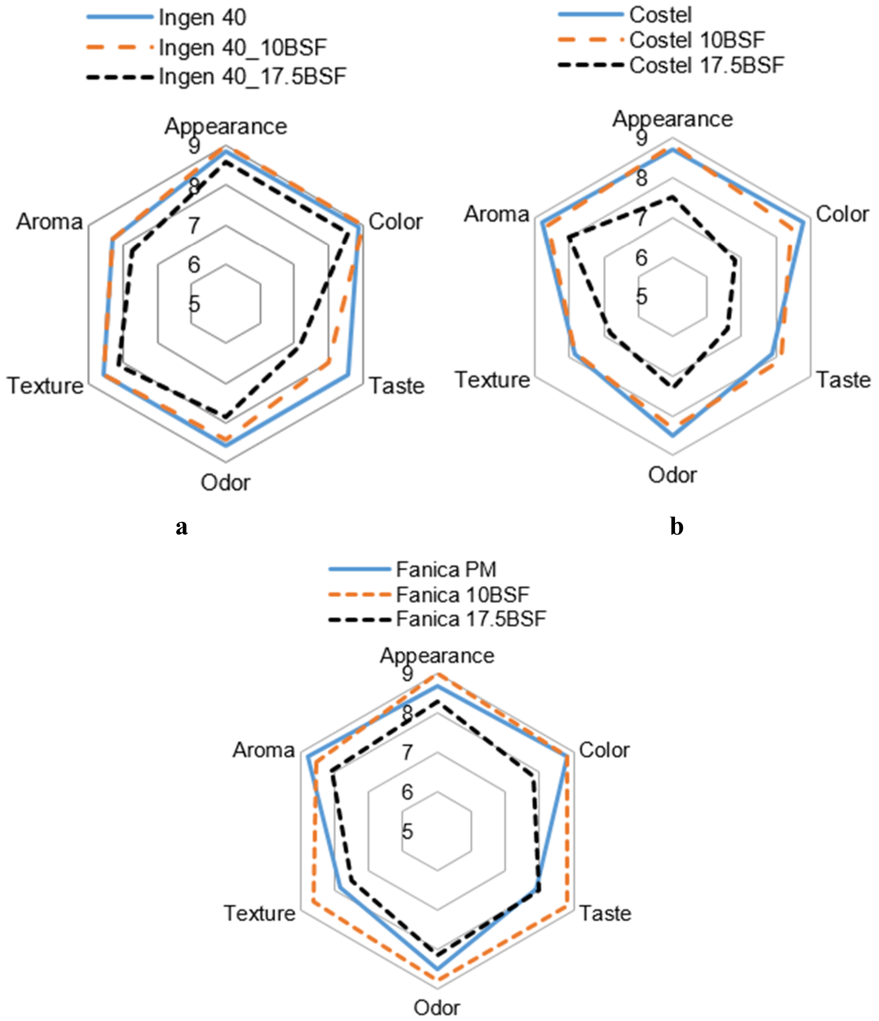
In the examined samples, the highest global acceptability values were recorded for the triticale bread samples without BSF addition (Table 4). The global acceptability scores ranged from 8.43 (Costel) to 8.76 (Fanica), while Ingen 40 showed an intermediate value of 8.62. Bread samples containing the lowest level of BSF addition (10%) exhibited global acceptability scores that did not differ significantly ($p > 0.05$) from those of the control samples (without BSF) in the case of Ingen 40 and Costel. For Fanica, however, BSF addition significantly affected global acceptability, resulting in a score of 7.98 ($p < 0.05$). Increasing the BSF concentration to 17.5% led to a decrease in global acceptability across all analysed samples, as illustrated by the sensory profile presented in Figure 3.

Sensory evaluation of the triticale bread samples indicated that the appearance of breads without BSF and with 10% BSF addition did not differ significantly (Figure 3).

In contrast, samples containing 17.5% BSF received lower appearance scores, falling below 8.0 in the case of the Costel and Fanica varieties. The addition of 17.5% BSF significantly affected crust color in the Costel and Fanica samples, with recorded values of 6.83 and 7.30, respectively. This effect can be attributed to crust browning caused by non-enzymatic reactions, including caramelization and the Maillard reaction (Pulis, 2010). Regarding crumb texture, the highest scores were obtained for all Ingen 40 bread samples. In contrast, Costel and Fanica samples containing 17.5% BSF received scores below 7.0, which may be explained by the high dietary fiber content of BSF and its impact on crumb structure.

Taste, odor, and aroma were influenced by both the dough preparation process and the ingredients used. Triticale bread enriched with BSF exhibited higher acidity, likely due to lactic acid formation during fermentation. The use of *Saccharomyces cerevisiae* contributed to a pleasant sensory profile, characterized by a mildly sweet taste and malt-like aroma, accompanied by typical cereal notes. Overall, sensory analysis demonstrated that triticale bread supplemented with 10% BSF was of very good quality.

The bread colour values are shown in Table 5. The colour of the bread samples was dark brown, characteristic to bakery products that are obtained from wholemeal flours. Positive values of parameters a* and b* indicate a shade of colour towards red and yellow. Also, a decrease in the lightness of the bread was found in the samples with the addition of sourdough from the light brewers' spent grain flour. The dark colour of the bread samples, can be explained, by the presence of flavone pigments from the alleuronic layer and grain shell particles, and also, due to the increasing intensity of the enzymatic hydrolysis of the starch and protein with the formation of simple carbohydrates and amino acids during bread-making for the samples with sourdough addition in bread recipe (Ghendov-Mosanu et al., 2024). This favours the Maillard reaction in the bread baking process which will increase the darkness of the bread samples.



a

b

c

Figure 3. Sensory characteristics of bread samples with different varieties of triticale with light brewers' spent sourdough:
a – Ingen 40; b – Costel; c – Fanica.

The bread textural characteristics are shown in Table 6. As may be seen, the hardness values vary between 21.25–53.14 N. By light brewers' spent sourdough addition in bread recipe the hardness value increased. This may be due to the increase in the content of bran particles into the bread due to the sourdough addition from the light brewers' spent grain flour.

Table 5

Colour values for triticale bread with light brewers' spent sourdough

Samples	CIELab color parameters		
	L*	a*	b*
Ingen 40	51.80±0.0^{e}	6.19±0.11^{c}	16.31±0.09^{a}
Ingen 40_10BSF	46.68±0.19^{d}	4.73±0.18^{a}	17.85±0.04^{b}
Ingen 40_17.5BSF	43.99±0.23^{c}	5.44±0.15^{b}	17.67±0.12^{b}
Costel	57.59±0.56^{f}	6.78±0.02^{c}	23.88±0.08^{c}
Costel 10BSF	46.22±0.31^{d}	5.15±0.04^{a}	33.21±0.11^{f}
Costel 17.5BSF	36.37±0.11^{a}	7.67±0.06^{d}	28.66±0.05^{d}
Fanica	50.44±0.22^{e}	10.72±0.08^{e}	35.00±0.10^{g}
Fanica 10BSF	41.83±0.17^{b}	5.72±0.11^{b}	33.15±0.13^{f}
Fanica 17.5BSF	37.19±0.11^{a}	5.77±0.04^{b}	30.16±0.09^{e}

L*, darkness/brightness; a*, shade of red/green; b*, shade of blue/yellow.
$^{a–g}$, mean values ($n = 3$) in the same column followed by different letters are significantly different ($p < 0.05$).

Table 6

Textural values for triticale bread with light brewers' spent sourdough

Samples	Hardness (N)	Adhesivity (N·s)	Coeshiviness (dimensionless)	Resilience (dimensionless)	Chewiness (N)
Ingen 40	21.25± 0.14^{a}	14.61± 0.04^{a}	0.719± 0.013^{f}	0.380± 0.002^{e}	15.28± 0.08^{a}
Ingen 40_10BSF	41.74± 0.21^{c}	32.94± 0.05^{c}	0.719± 0.009^{f}	0.403± 0.001^{f}	29.99± 0.01^{e}
Ingen 40_17.5BSF	49.53± 0.16^{e}	43.57± 0.08^{f}	0.733± 0.015^{g}	0.294± 0.002^{c}	36.30± 0.06^{h}
Costel	35.55± 0.14^{b}	31.61± 0.07^{c}	0.575± 0.005^{a}	0.276± 0.005^{b}	20.44± 0.11^{b}
Costel 10BSF	44.83± 0.09^{d}	34.85± 0.11^{d}	0.580± 0.0^{a}	0.290± 0.007^{c}	26.01± 0.01^{d}
Costel 17.5BSF	52.43± 0.18^{f}	44.25± 0.08^{f}	0.680± 0.0^{e}	0.381± 0.002^{e}	35.69± 0.04^{h}
Fanica	35.95± 0.12^{b}	25.64± 0.13^{f}	0.613± 0.005^{b}	0.306± 0.001^{c,d}	22.05± 0.09^{c}
Fanica 10BSF	50.98± 0.01^{e}	39.66± 0.07^{e}	0.633± 0.004^{c}	0.329± 0.003^{d}	32.27± 0.07^{f}
Fanica 17.5BSF	53.14± 0.07^{f}	38.43± 0.09^{e}	0.654± 0.005^{d}	0.221± 0.003^{a}	34.73± 0.04^{g}

$^{a–h}$, mean values ($n = 3$) in the same column followed by different letters are significantly different ($p < 0.05$).

The adhesivity values increase for the bread samples in which light brewers' spent sourdough has been incorporated. This effect may be due to the reduction of the water absorption capacity in the dough recipe, which may contribute to a stickier bread crumb.

Cohesiveness is a measure of the bread ability to maintain its structural integrity when it is subjected to deformation or breaking forces (e.g. during chewing). A decrease of these values by BSF addition in triticale flour indicates that bread sample become more crumbly or fragile probably due to a less gluten content from the samples (Curti et al., 2014). At high amount of BSF addition in triticale flour the resilience values decreased for Ingen 40 and Fanica variety. However, for the Costel variety the resilience value increase. An increase in bread resilience indicate that the bread is bouncier and more elastic and a decrease indicates that bread samples will slowly recovers when is subject to a small deformation. This may be related to the gluten content from the samples which may be in a higher or a less amount depending on the triticale variety used in bread recipe. Chewiness values increase for bread samples with BSF incorporated in their recipe. This indicates that the bread will requires more effort to bite and swallow probably due to the denser crumb by BSF addition in dough recipe (Huang et al., 2024).

Conclusions

Using triticale wholemeal flour can lead to the production of good-quality bread. The addition of sourdough from light brewer's spent grain flour contributes to increasing the biological value of bakery products due to its rich composition, including amino acids, dietary fiber, mineral substances, and bioactive compounds, while also valorising agro-food waste from the beer industry.

Light brewer's spent grain sourdough (BSF) significantly affects the rheological properties of triticale dough during mixing and pasting, leading to decreased water absorption, dough stability, dough development time, and Mixolab torques C2 and C3, while C4 and C5 show variability depending on the triticale variety and the level of BSF incorporation. The addition of brewer's spent grain sourdough reduces dough viscoelasticity by lowering G′ and G″ values, although elastic properties remain dominant, as indicated by tan δ values below 1 for all samples, and it weakens dough during extension by decreasing tenacity and baking strength.

During fermentation, brewer's spent grain sourdough has a positive effect on dough rheology by increasing H'm (maximum height of gaseous production), VT (total CO_2 volume production), and VR (volume of the gas retained in the dough at the end of the test) values, while CR (retention coefficient) values above 90% indicate the potential to obtain good-quality bread. Additionally, the significant decrease in falling number values suggests increased α-amylase In terms of bread quality, BSF addition significantly increases acidity and textural parameters such as hardness, adhesiveness, cohesiveness, and chewiness, while reducing loaf volume, porosity, and elasticity. It also results in a darker crumb and crust color. Sensory characteristics are influenced by both formulation and processing, leading to a slightly sweet taste, malt-like aroma, and characteristic cereal notes. Overall, the results indicate that supplementation with 10% brewer's spent grain sourdough produces triticale bread with very good sensory quality.

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Influence of surface treatment with high oleic sunflower oil and thyme essential oil on Kashkaval cheese quality

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Abstract

Introduction. The aim of this study is to investigate the incorporation of high-oleic sunflower oil and thyme essential oil as surface treatments for Kashkaval cheese to improve product quality, enhance the aroma–flavor profile, and extend shelf life.

Materials and methods. A surface coating of Kashkaval cheese was prepared using a mixture of high-oleic sunflower oil and thyme essential oil (0.1% and 0.2%). The samples were monitored at 45 days, 3 months, and 6 months of storage. Chemical, microbiological, and sensory analyses were conducted according to standard ISO and BDS methods, while fatty acids, organic acids, and amino acids were determined by GC/MS.

Results and discussion. The results show that surface treatment with high-oleic sunflower oil and thyme essential oil improves the stability and quality of Kashkaval cheese during storage. The acid value increased from 0.13 to 0.30 mg KOH/g in the control (untreated Kashkaval cheese, without surface coating), while lower values were observed in treated samples (up to 0.28 and 0.19 mg KOH/g for 0.1% and 0.2%, respectively). Peroxide values remained lower in coated samples (0.15–0.19 meq O_2/kg) compared to the control (0.24 meq O_2/kg). A decrease in pH (to 4.80) and an increase in titratable acidity (up to 208 °T in control and 230 °T in 0.2% sample) were observed. Fat content decreased to 27.00% in the control, while higher values were retained in treated samples (28.20–28.50%). No significant changes were found in fatty acid composition; linoleic acid remained stable (5.53–6.81 g/100 g), while linolenic acid slightly increased (up to 1.17 g/100 g). Microbiological results showed better preservation of lactic acid bacteria in treated samples (7.8–8.0 log CFU/g) compared to the control (6.2 log CFU/g), and lower yeast and mold counts (5.9–6.1 vs. 6.5 log CFU/g). Sensory evaluation indicated the highest stability at 0.1% essential oil (score 8 after 6 months), while the control decreased to 7. These findings suggest that the combination of high-oleic sunflower oil and thyme essential oil acts as an effective natural preservative, reducing lipid oxidation and microbial spoilage.

Conclusions. Surface coating with high-oleic sunflower oil and thyme essential oil enhances the quality and storage stability of Kashkaval cheese, with 0.1% thyme essential oil identified as the most effective treatment.

Keywords:

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Introduction

Interest in the incorporation of functional additives into food products, including dairy, has increased due to their potential to improve quality, flavor, aroma, and extend shelf life (Adinepour et al., 2022; Paredes-López et al., 2022; Stabnikova et al., 2023a). Essential oils and plant extracts (Dimov et al., 2023; Granato et al., 2018; Ibrahim and El Deen, 2011; Ivanova et al., 2017, 2020a, b; Mohanad et al., 2020; Salama et al., 2021; Vasheka and Petrusha, 2022), glyceride oils (Granato et al., 2018; Ivanova et al., 2017, 2020b; Juric et al., 2022), and fruit-derived products such as juices and purees (Bashta et al., 2021; Cușmenco and Bulgaru, 2020; Gouda and Hamed, 2020; Ivanova et al., 2023b; Karasawa and Mohan, 2018; Roy et al., 2015; Sahingil and Hayaloglu, 2022; Stabnikova et al., 2023, 2024, 2025; Zahid et al., 2022) are commonly used as functional additives in food production. Essential and glyceride oils, either individually or in combination, are frequently applied as surface coatings on dairy products, including cheeses such as Kashkaval, to extend shelf life (Kavas and Kavas, 2014; Klisovic et al., 2022; Tomar et al., 2020).

The essential oil of common thyme (*Thymus common* L.) has a specific odor and aroma (Baser and Buchbauer, 2010), has pronounced antimicrobial (Boruga et al., 2014; Dababneh, 2007; De Marino et al., 2009; Iftikhar and Batool, 2020; Imelouane et al., 2009; Rota et al., 2008; Tural and Turhan, 2007) and antioxidant properties (Grosso et al., 2010; Iftikhar and Batool, 2020; Kulisic et al., 2005; Tural and Turhan. 2007), which is due to the phenols thymol and carvacrol contained in its composition (Kawalszyk et al., 2020; Lopez-Cordova, 2021). These biological properties of the essential oil are a prerequisite for its use as a functional additive to various food products (Kumar et al., 2009; Sacchetti et al., 2005; Zhekova, 2012). The chemical composition of the essential oil of common thyme, variety “German Winter,” was established previously (Dobreva et al., 2024), with the main components being thymol (53.55%), γ-terpinene (11.13%), p-cymene (14.96%), borneol (2.75%), β-linalool (2.07%), and carvacrol (2.00%). The oil exhibited antioxidant activity as determined by the DPPH (2,2-diphenyl-1-picrylhydrazyl) assay (165.91 mM TE/g) and the ABTS [2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid)] assay (127.65 mM TE/g). Although data are available on the use of essential oils in Kashkaval production, no studies have examined the effect of thyme essential oil, applied as a surface coating, on the chemical, microbiological, and sensory characteristics of the cheese.

This study aimed to assess the impact of thyme essential oil used as a surface-applied functional additive on the chemical, sensory, and microbiological parameters of Kashkaval cheese during storage at 45 days, 3 months, and 6 months.

Materials and methods

Materials

High-oleic sunflower oil was supplied by Papas Oil AD (Bulgaria) with the following characteristics: acidity (as oleic acid) ≤ 0.1%; peroxide value ≤ 1 meq O_2/kg; iodine value 77–88; saponification value 182–194; and fatty acid composition as follows: palmitic acid 5.5–6.5%, stearic acid 3.0–5.5%, oleic acid 75–82%, linoleic acid 8–19%, and linolenic acid 0.5–1.5%. Common thyme essential oil was used (Dobreva et al., 2024).

Raw cow's milk was supplied by the Academic Technological Complex of Thracian University - Stara Zagora, Bulgaria with indicators: dry non-fat residue 9.60±0.33%, proteins 3.33±0.08%, fat 3.60±0.10%, density 1.032±0.010 g/mL, freezing point 0.580±0.030 °C, pH

6.5±0.1. The raw milk meets the organoleptic, chemical and microbiological parameters of EU Regulation 853 (2004).

The starter culture used for Kashkaval production was YO-Autiv RSF-736® direct vat set (DVS) (Chr. Hansen, Denmark), containing mesophilic and thermophilic microflora (*Lactobacillus helveticus, Lactococcus lactis* subsp. *cremoris*, *Lactococcus lactis* subsp. *lactis*, and *Streptococcus thermophilus*). It was added at a rate of 1.5 g per 10 L of milk in accordance with the manufacturer's instructions.

Rennet enzyme used was chymosin CHY-MAX® Supreme (Chr. Hansen, Denmark), added at 0.25 mL per 10 L of milk according to the manufacturer's instructions. Calcium chloride (initial concentration 33%) was purchased from Biokom Trendafilov EOOD, Bulgaria.

Production of Kashkaval cheese

Kashkaval cheese was produced according to Kozhev (2006). After grading, raw cow's milk was thermized at 63–65 °C for 15–20 s. The milk was then cooled to 32–34 °C, and the DVS starter culture was added at 1.5 g/10 L of milk. Calcium chloride (3 mL/10 L), previously diluted 1:10 with water, and milk-coagulating enzyme (strength 1:70,000; 0.25 mL/10 L), previously diluted 1:1 with water, were also added. Coagulation was allowed to proceed at curdling temperature for 20–30 min until a firm curd formed.

The curd was cut, baked, and the whey drained. The curd pieces underwent chederization at 35–40 °C for 60–90 min, followed by evaporation, kneading, and salting in 10–12% brine (titratable acidity up to 15 °T) at 70–72 °C. The cheese was then molded into 0.2 kg portions, removed from the molds, and cooled at 10 °C for 2–3 days. A surface coating of high-oleic sunflower oil with thyme essential oil was applied by spraying, followed by vacuum treatment.The coated Kashkaval samples were ripened at 10–12 °C for 45 days and subsequently stored at 4 ± 2 °C for up to 6 months.

The control sample was untreated Kashkaval cheese, without the application of any surface coating.

Determination of chemical and physical parameters of Kashkaval cheese

Chemical and physical parameters of Kashkaval cheese were determined using standard methods. The acid value was measured by titration according to BSS EN ISO 660 (2020), while the peroxide value was determined using the iodometric method (BSS EN ISO 3960, 2017). Fat content was assessed volumetrically using the Gerber method (ISO 19662, 2018), and dry matter was analyzed gravimetrically (ISO 5534, 2004). Active acidity (pH) was measured potentiometrically with a 7110 WTW pH meter (Germany), and titratable acidity was determined by titration according to BSS 1111 (1980).

Determination of metabolites in Kashkaval cheese

The content of polar and non-polar metabolites, including fermentation products and flavor/aroma compounds, in Kashkaval cheese was determined by GC/MS. Retention times of the detected compounds were compared with reference libraries, including the Golm Metabolome Database (GMD) (Hummel et al., 2010) and the NIST 08 library (Manion et al., 2015).

Microbiological analysis

Sample preparation for microbiological analysis was carried out according to BSS EN ISO 6887-5 (2020). *Lactobacillus* spp. counts were determined following BSS ISO 9232

(2005), while *Lactococcus* spp. and *Streptococcus thermophilus* were enumerated according to BSS EN ISO 7889 (2005). Viable counts were obtained using selective synthetic nutrient media, MRS (Merck) for *Lactobacillus* spp. and M17 (Merck) for *Lactococcus* spp. Yeasts and molds were enumerated on YCG medium (Merck) in accordance with BSS EN ISO 6611 (2006).

Sensory analysis

Sensory analysis of Kashkaval cheese was conducted according to BSS 15612 (1983).

Statistical analysis

All experiments were performed in triplicate. Values presented in the tables represent the mean±standard deviation (SD). Statistical analysis was conducted using analysis of variance (ANOVA) with Statgraphics Centurion XVI, Version 16.2.04 (StatPoint Technologies, Inc., USA), and differences were considered significant at $p < 0.05$ (Ivanova, 2025).

Results and discussion

Physical and chemical parameters of a mixture of sunflower and essential oil

Data on the changes in acid and peroxide values of the mixture of high-oleic sunflower oil and thyme essential oil are presented in Table 1. The acid and peroxide values of both pure high-oleic sunflower oil and oil blends containing 0.1% and 0.2% thyme essential oil remained low even after 6 months of storage. This indicates the high quality of the vegetable oil and supports the long shelf life of the oil blends (Farhoosh and Hoseini-Yazdi, 2014; Wasowics et al., 2004).

Table 1

Change in the acid and peroxide value of oil mixtures

Indicators	**Control after storage**			**Mixture with 0.1% thyme essential oil, after**			**Mixture with 0.2% thyme essential oil, after**		
	45 days	3 months	6 months	45 days	3 months	6 months	45 days	3 months	6 months
Acid value, mg KOH/g	$0.13^{a}\pm 0.01$	$0.25^{b}\pm 0.01$	$0.30^{c}\pm 0.01$	$0.21^{b}\pm 0.01$	$0.25^{b}\pm 0.01$	$0.28^{c}\pm 0.01$	$0.15^{a}\pm 0.01$	$0.18^{d}\pm 0.01$	$0.19^{d}\pm 0.01$
Peroxide value, meq O_2/kg	$0.22^{a}\pm 0.01$	$0.23^{a}\pm 0.01$	$0.24^{ab}\pm 0.01$	$0.16^{c}\pm 0.01$	$0.19^{d}\pm 0.01$	$0.19^{d}\pm 0.01$	$0.11^{e}\pm 0.01$	$0.14^{cf}\pm 0.01$	$0.15^{c}\pm 0.01$

Note: $^{a, b, c, d, e, f}$ - indices showing significant differences ($p < 0.05$) between the mean values in the rows.

Data presented in Table 2 show a decrease in pH and an increase in titratable acidity in all samples during storage, reflecting ongoing lactic acid fermentation and lactose decomposition. Samples treated with thyme essential oil (0.1% and 0.2%) exhibited higher titratable acidity, reaching up to 230 °T for the 0.2% treatment after 6 months, compared to 208 °T in the control. These results indicate that surface treatment influences the intensity of biochemical processes during storage.

According to Guillermo et al. (2016) the low concentrations of essential oils (EOs) from thyme (*Thymus vulgaris*) and oregano (*Origanum vulgare*), suppress pathogenic microorganisms, without them showing significant negative effects on development on acidity and starters cultures (lactic acid bacteria) in the cheese.

Table 2

Change in physical and chemical parameters of Kashkaval cheese

Indicators	After 45 days of storage			After 3 months storage			After 6 months storage		
	Cheese with EO, %			Cheese with EO, %			Cheese with EO, %		
	0	**0.1**	**0.2**	**0**	**0.1**	**0.2**	**0**	**0.1**	**0.2**
OC, %	30.10^{a}± 0.2	30.50^{a}± 0.2	30.40^{a}± 0.1	28.60^{b}± 0.1	28.80^{b}± 0.1	27.60^{c}± 0.2	27.00^{c}± 0.3	28.20^{b}± 0.2	28.50$_{b}$± 0.2
DM, %	62.52^{a}± 0.3	57.35^{b}± 0.1	59.10^{c}± 0.3	60.54^{c}± 0.3	59.08^{c}± 0.3	60.05^{c}± 0.2	60.8^{c}± 0.1	59.38^{c}± 0.3	60.23^{c}± 0.1
pH	5.68^{a}± 0.1	5.46^{b}± 0.1	5.28^{c} ± 0.2	5.36^{d}± 0.1	5.38^{d}± 0.2	5.22^{c}± 0.2	4.80^{e}± 0.2	5.10^{c}± 0.2	5.10^{c}± 0.2
TA, °T	160^{a}± 1.2	160^{a}± 1.0	164^{b}± 1.1	180^{c}± 1.3	184^{c}± 1.3	186^{c}± 1.3	208^{d}± 2.0	220^{e}± 2.1	230^{e}± 2.2

Note: EO – essential oil; OC – oil content; TA – titratable acidity; $^{a, b, c, d, e}$ - indices showing significant differences ($p < 0.05$) between the mean values in the rows.

In the control sample, the oil content (OC) decreased from 30.10% to 27.00% over the 6-month period. The samples treated with essential oil showed higher oil content retention at the end of the period (28.20% and 28.50%) compared to the control. This is probably due to the protective role of the oil coating, which acts as a barrier.

In the control, a slight decrease in dry matter (DM) content was observed, while in the treated samples (especially with 0.1% essential oil) the initial values were lower, but stabilized or slightly increased by the 6th month. This can be explained by the different rate of moisture release in the presence of a surface film.

Surface treatment of Kashkaval cheese with a mixture of high-oleic sunflower oil and thyme essential oil not only modifies the metabolic profile of the product, but also contributes to better preservation of fatty acid content, influences the development of acidity, and acts as an antioxidant barrier. Our results are fully consistent with findings reported by Souha et al. (2025).

Chemical composition of Kashkaval cheese

Fatty acids

The change in the fatty acid composition of Kashkaval cheese during storage (45 days, 3 and 6 months) at different concentrations of thyme essential oil in the oil coating is presented in Table 3.

The results show that saturated fatty acids have the largest relative share in the fatty acid profile of the studied samples, which is typical for dairy products (Paszczyk et al., 2020). Similar results have been found in other types of hard cheeses, including Kashkaval, Cheddar and Tulum cheeses, in which palmitic, myristic and stearic acids are the main components of the lipid fraction (Asif et al., 2023; Çakir, 2026; Ivanova et al., 2020c). Among the short and medium chain fatty acids, changes are observed during storage, with the amounts of butyric, caproic and capric acids varying depending on the duration of ripening and the presence of essential oil. These fatty acids are formed mainly as a result of lipolytic processes and play an important role in the formation of the characteristic aroma of ripening cheeses.

Table 3

Change in the amount of fatty acids in Kashkaval cheese, g/100 g

Fatty acids	after 45 days storage			after 3 months storage			after 6 months storage		
	Cheese with EO, %			Cheese with EO, %			Cheese with EO, %		
	0	0.1	0.2	0	0.1	0.2	0	0.1	0.2
Short and medium chain fatty acids									
Butyric	$1.87^{a}\pm0.15$	$1.68^{b}\pm0.13$	$1.76^{c}\pm0.14$	$1.70^{c}\pm0.15$	$1.99^{d}\pm0.13$	$1.80^{c}\pm0.14$	$1.53^{b}\pm0.12$	$1.63^{b}\pm0.13$	$2.13^{e}\pm0.17$
Caproic	$1.26^{a}\pm0.10$	$1.13^{b}\pm0.09$	$1.18^{b}\pm0.09$	$1.20^{a}\pm0.10$	$1.34^{c}\pm0.11$	$1.21^{a}\pm0.10$	$1.03^{d}\pm0.08$	$1.10^{b}\pm0.09$	$1.43^{e}\pm0.11$
Capric	$0.74^{a}\pm0.01$	$0.66^{b}\pm0.01$	$0.69^{b}\pm0.01$	$0.73^{a}\pm0.01$	$0.79^{c}\pm0.01$	$0.71^{b}\pm0.01$	$0.60^{d}\pm0.01$	$0.65^{b}\pm0.01$	$0.84^{e}\pm0.01$
Caprinic	$1.31^{a}\pm0.10$	$1.18^{b}\pm0.09$	$1.23^{c}\pm0.10$	$1.28^{c}\pm0.10$	$1.39^{d}\pm0.11$	$1.26^{c}\pm0.10$	$1.07^{d}\pm0.08$	$1.14^{b}\pm0.09$	$1.49^{e}\pm0.12$
Lauric	$1.50^{a}\pm0.12$	$1.35^{b}\pm0.11$	$1.41^{c}\pm0.11$	$1.45^{c}\pm0.11$	$1.60^{d}\pm0.13$	$1.44^{c}\pm0.11$	$1.22^{e}\pm0.10$	$1.31^{e}\pm0.01$	$1.71^{d}\pm0.13$
Long chain fatty acids									
Myristic	$6.14^{a}\pm0.48$	$5.34^{b}\pm0.42$	$5.77^{c}\pm0.46$	$5.90^{c}\pm0.50$	$6.55^{e}\pm0.52$	$5.70^{c}\pm0.45$	$4.84^{c}\pm0.38$	$5.36^{b}\pm0.42$	$6.99^{e}\pm0.55$
Palmitic	$12.77^{a}\pm1.01$	$11.11^{b}\pm0.88$	$12.01^{c}\pm0.95$	$12.00^{c}\pm1.00$	$13.63^{d}\pm1.08$	$11.86^{c}\pm0.94$	$10.08^{e}\pm0.08$	$11.17^{b}\pm0.88$	$14.55^{d}\pm1.15$
Stearic	$5.11^{a}\pm0.40$	$4.45^{b}\pm0.35$	$4.80^{c}\pm0.31$	$4.60^{c}\pm0.40$	$5.45^{b}\pm0.43$	$4.74^{c}\pm0.37$	$4.03^{d}\pm0.32$	$4.17^{e}\pm0.35$	$5.82^{c}\pm0.46$
Arachidonic	$0.78^{a}\pm0.01$	$0.89^{b}\pm0.01$	$0.84^{b}\pm0.01$	$0.80^{a}\pm0.01$	$0.88^{b}\pm0.01$	$0.92^{c}\pm0.01$	$0.94^{c}\pm0.01$	$0.96^{c}\pm0.01$	$0.90^{b}\pm0.01$
Behenic	$0.12^{a}\pm0.01$	$0.14^{b}\pm0.01$	$0.13^{a}\pm0.01$	$0.11^{a}\pm0.01$	$0.14^{b}\pm0.01$	$0.14^{b}\pm0.01$	$0.15^{b}\pm0.01$	$0.15^{b}\pm0.01$	$0.14^{b}\pm0.01$
Monounsaturated fatty acids									
Oleic	$0.15^{a}\pm0.01$	$0.13^{b}\pm0.01$	$0.14^{b}\pm0.01$	$0.14^{b}\pm0.01$	$0.16^{a}\pm0.01$	$0.14^{b}\pm0.01$	$0.12^{b}\pm0.01$	$0.13^{b}\pm0.01$	$0.17^{a}\pm0.01$
Vaccenic	$0.13^{a}\pm0.01$	$0.11^{a}\pm0.01$	$0.12^{a}\pm0.01$	$0.12^{a}\pm0.01$	$0.13^{a}\pm0.01$	$0.12^{a}\pm0.01$	$0.10^{b}\pm0.01$	$0.11^{b}\pm0.01$	$0.14^{a}\pm0.01$
Polyunsaturated fatty acids									
Linoleic	$5.53^{a}\pm0.44$	$6.34^{b}\pm0.50$	$5.97^{c}\pm0.47$	$5.70^{c}\pm0.51$	$6.24^{b}\pm0.49$	$6.52^{b}\pm0.51$	$6.72^{d}\pm0.53$	$6.81^{d}\pm0.54$	$6.43^{b}\pm0.51$
Linolenic	$0.62^{a}\pm0.01$	$0.71^{a}\pm0.01$	$0.81^{a}\pm0.01$	$0.70^{a}\pm0.01$	$0.93^{b}\pm0.01$	$1.0^{b}\pm0.08$	$0.91^{b}\pm0.01$	$1.17^{b}\pm0.09$	$1.03^{b}\pm0.08$

Note: *EO – essential oil; $^{a, b, c, d, e}$ - indices showing significant differences ($p < 0.05$) between the mean values in the rows.

Similar changes have been described in other studies on hard cheeses, in which lipolysis leads to a gradual increase in free fatty acids during ripening (Ioannidou et al., 2022; Tardiolo et al., 2025). Long-chain saturated fatty acids (myristic, palmitic and stearic acids) also show fluctuations with storage time. These fatty acids are major structural components of milk fat and their relative proportion usually changes to a lesser extent during ripening, which is typical for hard cheeses (Paszczyk et al., 2020). The amount of monounsaturated vaccinic acid increases throughout the storage period, which is favorable for surface-treated cheeses, as it is a precursor of conjugated linoleic acid, which is characterized by a number of benefits for human health (Ivanova, 2021). The amount of oleic acid also shows a tendency to slightly increase during storage. Similar dynamics were observed in Tulum cheese during ripening, where changes in monounsaturated fatty acids are associated with ongoing lipolytic processes and the metabolic activity of the microflora (Luna-Fox et al., 2025). The data obtained on the change in the amount of these fatty acids indicate that the surface treatment acts as a barrier to oxygen, which leads to a modification of the microbiological activity, and hence the metabolic profile of the product.

The amount of polyunsaturated fatty acids is preserved during storage. When storing cheese in olive oil, Klisović et al. (2022) found that the amount of these fatty acids decreased, while in samples of Tulum increases (Rençber et al., 2025). The differences found can be explained by the difference in the composition and technology of dairy products (Nájera et al., 2021). The higher relative proportion of saturated fatty acids at the low concentration of thyme (0.1%) can be explained by partial lipolysis or insufficient antioxidant capacity to stabilize reactive unsaturated bonds at this dose. Conversely, at a concentration of 0.2% thyme essential oil, the ratio changes, which is also confirmed by the data of Alloh et al. (2024). Studies by many authors indicate the effect of essential oils on the fermentation and biohydrogenation of fatty acids, since many of their main components are active antioxidants (Abd El-Aziz et al., 2023; Busetta et al., 2022).

The change in cholesterol during storage is presented in Figure 1. The amounts of cholesterol are close to those found for different types of cheeses in central and southern Europe (Czerwonka et al., 2024; Di Trana et al., 2022). It has been established that its amount is influenced by the type of milk used, but also depends on the technology applied (Manuelian et al., 2017).

Kinik et al. (2025) reported that cholesterol content in hard cheeses decreases during ripening and storage. Currently, diets including low-cholesterol dairy products are recommended to mitigate adverse effects on human health (Lordan et al., 2018). Plant-derived antioxidants are often incorporated into dairy products to help reduce cholesterol levels (Güney, 2020). As shown in Figure 1, the presence of 0.1% thyme essential oil resulted in a decrease in cholesterol content in Kashkaval cheese during 6 months of storage.

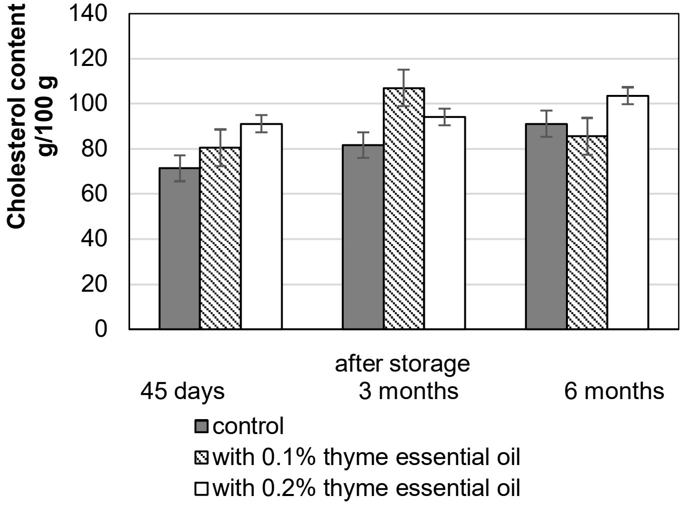

Figure 1. Change in cholesterol content in Kashkaval cheese during storage

Organic acids

The change in organic acid content in Kashkaval cheese during storage (45 days, 3 and 6 months) at different concentrations of thyme essential oil in the oil coating is presented in Table 4. Various organic acids are present in fermented dairy products, some of which are formed during lactic acid fermentation, while others are present from subsequent biochemical transformations during ripening and storage processes (Ahmed et al., 2025). The data shows that the amount of lactic acid is in the highest values, followed by oxalic, formic and acetic acids, also found by other authors in the production of different cheese types (McMahon et al., 2014). During storage, the amount of lactic acid decreases, which is also claimed by Fox et al. (2017). The amount of pyruvic acid remains unchanged during storage, which indicates a slow rate of biochemical transformations (McSweeney, 2004). Citric acid is essential in the formation of the taste and aroma of cheese, and the tendency of its amounts to vary during storage is not the same for all samples. A change is detected in the sample with 0.2% essential oil addition.

This is probably due to thymol, a major component of the essential oil, which has pronounced antimicrobial and antioxidant properties (Yidiz et al., 2021). The presence of hippuric and uric acids may originate from the use of the cheese-specific starter culture, which is also traditionally used to make yogurt, and their amounts do not vary during storage (Güler, 2014).

Table 4

Change in the amount of organic acids in Kashkaval, mg/100 g

Organic acids	after 45 days storage			after 3 months storage			after 6 months storage		
	Cheese with EO, %			Cheese with EO, %			Cheese with EO, %		
	0	0.1	0.2	0	0.1	0.2	0	0.1	0.2
Pyruvic	20.36^{a} ±1.6	22.40^{b} ±1.8	21.28^{c} ±1.7	21.20^{c} ±1.5	20.77^{a} ±1.6	22.84^{b} ±1.8	23.30^{b} ±1.8	21.70c ±1.7	21.18c ±1.7
Lactic	605.49^{a} ±47.8	553.08^{b}c ±43.6	483.81^{c} ±38.2	550.05^{b} ±50.0	476.11^{c} ±37.6	461.89^{c} ±36.4	415.02^{c} ±32.7	393.60^{d} ±31.1	490.15^{c} ±38.7
Oxalic	107.23^{a} ±8.5	117.95^{b} ±9.3	112.05^{c} ±8.8	110.01^{c} ±8.5	109.37^{c} ±8.6	112.05^{c} ±8.8	122.72^{b} ±9.7	111.29^{c} ±9.0	111.56^{c} ±8.8
Uric	4.78^{a}± 0.4	5.25^{b}± 0.4	4.99^{c}± 0.4	4.88^{c}± 0.4	4.87^{c}± 0.4	5.36^{b}± 0.4	5.09^{a}± 0.4	4.97^{a}± 0.4	5.47^{b}± 0.4
Acetic	65.82^{a}± 5.2	60.52^{b}± 4.8	50.36^{c}± 4.0	40.50^{d}± 0.4	32.66^{e}± 2.6	36.04^{e}± 2.8	27.11^{e}± 2.1	25.68^{e}± 2.0	22.16^{e}± 1.8
Formic	78.72^{a}± 6.2	86.60^{b}± 6.8	82.27^{c}± 6.5	79.50^{a}± 7.0	80.30^{d}± 6.3	88.33^{e}± 7.0	90.10^{e}± 7.1	83.91^{c}± 6.6	81.91^{c}± 6.5
Hippuric	14.83^{a}± 1.2	16.31^{b}± 1.3	15.50^{c}± 1.2	15.00^{d}± 1.3	15.12^{d}± 1.2	16.64^{b}± 1.3	16.97^{e}± 1.3	15.81^{c}± 1.3	15.43^{c}± 1.2
Citric	58.90^{a}± 4.6	64.79^{b}± 5.1	61.55^{c}± 4.9	59.50^{a}± 5.0	60.08^{a}± 4.7	66.08^{b}± 5.2	62.78^{c}± 5.0	61.28^{c}± 4.8	67.41^{b}± 5.3

Note: *EO – essential oil; $^{a, b, c, d, e}$ - indices showing significant differences ($p < 0.05$) between the mean values in the rows.

Amino acid

The change in amino acids in Kashkaval during storage (45 days, 3 and 6 months) at different concentrations of thyme essential oil in the oil coating is presented in Table 5.

Table 5

Change in the amount of amino acids in Kashkaval cheese, g/100 g

Amino acids	**After 45 days of storage**			**After 3 months storage**			**After 6 months storage**		
	Cheese with EO, %			**Cheese with EO, %**			**Cheese with EO, %**		
	0	**0.1**	**0.2**	**0**	**0.1**	**0.2**	**0**	**0.1**	**0.2**
Essential amino acids									
Valine	2.72^{a}± 0.21	3.00^{b}± 0.24	2.85^{b}± 0.22	2.75^{a}± 0.23	2.78^{a}± 0.22	3.05^{b}± 0.24	3.12^{c}± 0.25	2.90^{b}± 0.23	2.83^{b}± 0.22
Leucine	4.19^{a}± 0.33	4.61^{b}± 0.36	4.37c± 0.35	4.22^{a}± 0.31	4.27^{a}± 0.34	4.70±^{b} 0.37	4.79^{b}± 0.38	4.46^{c}± 0.35	4.36^{c}± 0.34
Isoleucine	1.71^{a}± 0.13	1.88^{b}± 0.15	1.78^{c}± 0.14	1.73^{a}± 0.12	1.74^{a}± 0.14	1.91^{b}± 0.15	1.95^{b}± 0.15	1.82^{b}± 0.14	1.78^{c}± 0.14
Threonine	2.00^{a}± 0.16	2.20^{b}± 0.17	2.09^{c}± 0.16	2.01^{a}± 0.17	2.04^{a}± 0.16	2.24^{b}± 0.18	2.29^{b}± 0.18	2.13^{c}± 0.17	2.0^{a}± 0.16
Methionine	0.13^{a}± 0.01	0.15^{a}± 0.01	0.14^{a}± 0.01	0.13^{a}± 0.01	0.13^{a}± 0.01	0.15^{a}± 0.01	0.15^{a}± 0.01	0.14^{a}± 0.01	0.1^{a}± 0.01
Phenylalanine	4.82^{a}± 0.38	5.30^{b}± 0.42	5.03^{c}± 0.40	4.85^{a}± 0.35	4.91^{c}± 0.39	5.40^{b}± 0.43	5.51^{b}± 0.43	5.13^{c}± 0.40	5.01^{c}± 0.40
Lysine	8.13^{a}± 0.64	8.95^{b}± 0.71	8.50^{c}± 0.67	8.20^{a}± 0.74	8.30^{a}± 0.65	9.12^{b}± 0.72	9.31^{b}± 0.73	8.67^{c}± 0.68	8.46^{c}± 0.67
Tyrosine	0.21^{a}± 0.01	0.24^{a}± 0.01	0.22^{a}± 0.01	0.20^{a}± 0.01	0.22^{a}± 0.01	0.24^{a}± 0.01	0.24^{a}± 0.01	0.23^{a}± 0.01	0.22^{a}± 0.01
Non-essential amino acids									
Alanine	5.01^{a}± 0.39	5.51^{b}± 0.43	5.23^{c}± 0.41	5.05^{a}± 0.45	5.11^{a}± 0.40	5.62^{b}± 0.44	5.73^{b}± 0.45	5.34± 0.44	5.21^{c}± 0.41
Glycine	0.87^{a}± 0.01	0.96^{b}± 0.01	0.91^{a}± 0.01	0.90^{a}± 0.01	0.89^{a}± 0.01	0.98^{b}± 0.01	0.99^{b}± 0.01	0.93^{a}± 0.01	0.90^{a}± 0.01
Tryptophan	0.27^{a}± 0.01	0.30^{a}± 0.01	0.29^{a}± 0.01	0.27^{a}± 0.01	0.28^{a}± 0.01	0.31^{a}± 0.01	0.31^{a}± 0.01	0.29^{a}± 0.01	0.28^{a}± 0.01
Serine	3.03^{a}± 0.24	3.34^{b}± 0.26	3.17^{c}± 0.25	3.04^{a}± 0.24	3.09^{a}± 0.24	3.40^{b}± 0.27	3.47^{b}± 0.27	3.23^{c}± 0.25	3.15^{c}± 0.25
Aspartic acid	0.15a± 0.01	0.17^{a}± 0.01	0.16^{a}± 0.01	0.15^{a}± 0.01	0.16^{a}± 0.01	0.17^{a}± 0.01	0.18^{a}± 0.01	0.16^{a}± 0.01	0.16^{a}± 0.01
Arginine	1.90^{a}± 0.15	2.09^{b}± 0.17	1.99^{a}± 0.16	1.91^{a}± 0.13	1.94^{a}± 0.15	2.13^{b}± 0.17	2.18^{b}± 0.17	2.03^{a}± 0.16	1.98^{a}± 0.16
Ornithine	0.18^{a}± 0.01	0.20^{a}± 0.01	0.19^{a}± 0.01	0.19^{a}± 0.01	0.18^{a}± 0.01	0.20^{a}± 0.01	0.20^{a}± 0.01	0.19^{a}± 0.01	0.19^{a}± 0.01
Glutamine	0.30^{a}± 0.01	0.33^{a}± 0.01	0.31^{a}± 0.01	0.30^{a}± 0.01	0.30^{a}± 0.01	0.33^{a}± 0.01	0.34^{a}± 0.01	0.32^{a}± .010	0.3^{a}± 0.01
Glutamic acid	4.23^{a}± 0.33	4.66^{b}± 0.37	4.42^{c}± 0.35	4.27^{a}± 0.31	4.32^{a}± 0.34	4.75^{b}± 0.37	4.84^{b}± 0.38	4.51^{c}± 0.36	4.40^{c}± 0.35

Note: *EO – essential oil; $^{a, b, c}$ - indices showing significant differences ($p < 0.05$) between the mean values in the rows.

Data show variation in their amounts during storage, which can be explained by the microbiological and enzymatic processes occurring in the samples during ripening, including decarboxylation, deamination and use of amino acids as a substrate for secondary metabolic reactions (Atasever and Mazlum. 2024). The effect of the essential oil, especially its main component thymol, which probably exerts a specific effect on the proteolytic activity of the microflora and limits the degradation and subsequent metabolization of amino acids (Makhal et al., 2012).

The higher degree of preservation of essential amino acids in the essential oil samples is an indicator of a higher biological value of the final product, explained by the antioxidant effect of thymol (Miguel, 2010).

Sensory profile

The change in the sensory parameters of Kashkaval during storage is presented in Figure 2.

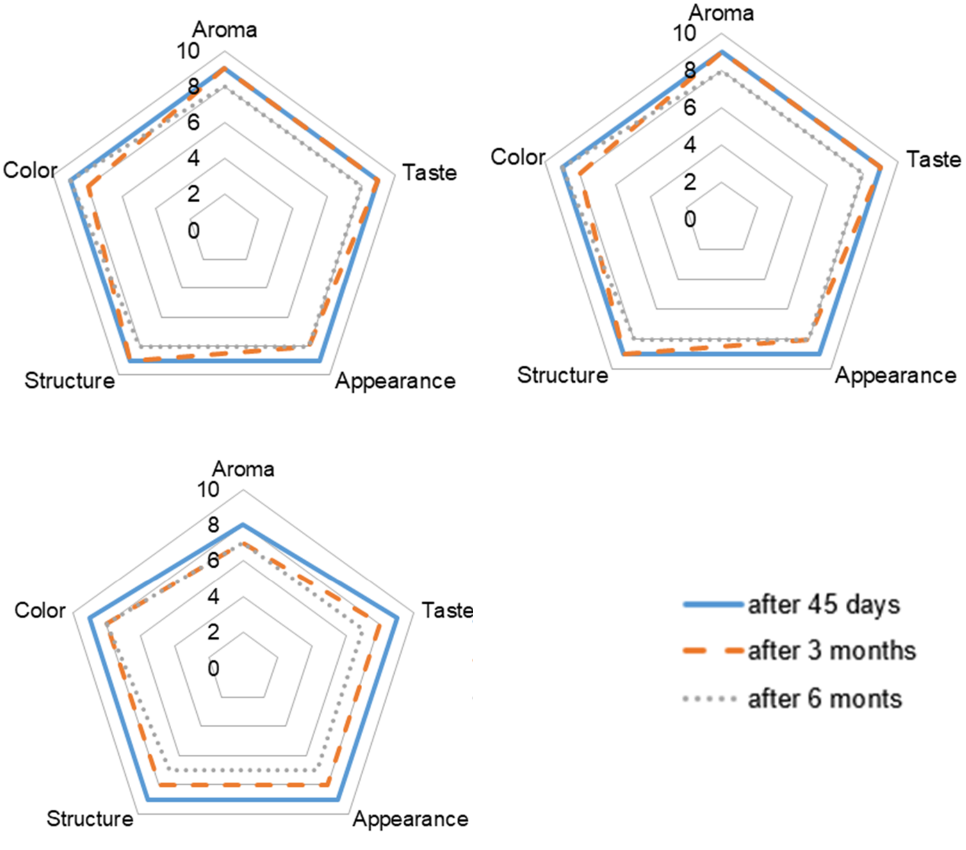

Figure 2. Changes in sensory parameters of Kashkaval cheese during storage with thyme essential oil, %: 0 - a (control); 0.1 - b ; 0.2 – c.

The data from the sensory analysis revealed specific dynamic changes in the quality of the Kashkaval samples containing thyme essential oil. The appearance, taste and aroma of the innovative product are fully preserved compared to the classical technology, while being enriched by the specific organoleptic profile of the added oil.

The control sample initially demonstrated excellent characteristics in terms of appearance, taste and aroma (score 9), but after the third month of storage a natural decline in the intensity of these indicators was observed, reaching an average score of 7 at the sixth month. This regression is related to the normal proteolytic and lipolytic processes during the ripening of Kashkaval.

The sample with 0.1% thyme concentration demonstrated the highest resistance and stability of sensory parameters. At the 6th month of storage, it retained high aroma and taste ratings (8), and did not show the typical deterioration of the organoleptic profile of old cheeses. However, the higher concentration (0.2%) led to a slight decrease in aroma ratings at the end of the period (6th month). The reason for this was the overly intrusive and intense smell of thyme essential oil, which began to dominate the milk profile and changed the typical character of the product.

Similar relationships were reported by Martial et al. (2016), who studied the influence of oregano essential oil on traditional cheeses and found that the balance between oil concentration and starter cultures was critical for the final flavor.

During storage, there was a tendency towards a uniform decrease in the scores for all sensory parameters in the control. In the samples with a higher concentration of thyme, slight deviations in color and surface were detected at the end of the 6th month, as well as a transformation of the aroma-taste complex. These findings correspond to the results of Metwalli (2011), who when enriching “Karish” cheese with natural preservatives (garlic and propolis), noted that high concentrations can suppress the specific milky aroma of the product.

Due to the low content of polyunsaturated fatty acids (linoleic acid) in the high-oleic sunflower oil , the risk of fat oxidation is reduced. This directly correlates with the high taste and aroma ratings in the samples with 0.1% thyme up to 6 months, where there are no off-flavors of oxidation.

Although present in lower amounts, sulfur-containing amino acids play a key role in the development of the characteristic aroma. Thyme essential oil (main components thymol and carvacrol) acts synergistically with amino acid degradation products, preventing the formation of undesirable bitter peptides, which was observed in the control sample after 3 months. These findings are consistent with the results reported by Olmedo et al. (2013), who evaluated the oxidative and fermentation stability of cream cheese flavored with oregano and rosemary essential oils.

Microbiological analysis

The results on the dynamics of the viable cell count of lactic acid bacteria in Kashkaval cheese are presented in Figures 3 and 4.

From the results shown in Figure 3, it can be seen that the control sample exhibited a slight increase in the count of lactobacilli from 6.5 $\log_{10}$ CFU/g at the beginning of the storage period to 6.7 $\log_{10}$ CFU/g after 3 months. The viable cells count decreased to 6.2 $\log_{10}$ CFU/g at the end of the storage period.

The initial count of lactobacilli was higher in the samples treated with a mixture of sunflower oil and thyme essential oil (0.1 and 0.2%) than in the control sample. Sample with 0.1% showed values of approximately 7.9 $\log_{10}$ CFU/g at the beginning of storage, which remained relatively stable throughout the storage period (8.0 $\log_{10}$ CFU/g at the 3rd month and 7.8 $\log_{10}$CFU/g at the 6th month). In the sample with 0.2% essential oil, an increase was recorded from about 7.1 $\log_{10}$ CFU/g at the beginning of storage to approximately 7.8 $\log_{10}$ CFU/g at the 6th month.

The obtained results indicate that the surface treatment does not exert an inhibitory effect on *Lactobacillus* spp. Samples with 0.1 and 0.2% essential oils were characterized by better preservation and/or stimulation of the viable cell counts of lactobacilli compared to the control sample. Thyme essential oil, despite its known antimicrobial properties, probably exhibits a selective effect in the concentrations used, without significantly inhibiting the lactic acid microflora from the starter culture.

The count of *Lactococcus* spp. and *Streptococcus* spp. (Figure 4) in the Kashkaval cheese samples also varied depending on the applied treatment and the storage duration (45 days, 3 and 6 months), with significant differences between the control sample and treated samples with 0.1% and with 0.2% thyme essential oils.

In the control sample the values were the lowest throughout the storage period – about 6.8 $\log_{10}$ CFU/g at the beginning, with a slight increase to 6.9 $\log_{10}$ CFU/g at the 3rd month, followed by a decrease to 6.6 $\log_{10}$ CFU/g at the 6th month. At the beginning of the storage period, samples wit 0.1% and with 0.2% were characterized by a significantly higher count of lactococci compared to the control sample. In the sample wit 0.1% essential oil relatively stable values were recorded - about 8.1 $\log_{10}$ CFU/g at the beginning and at the 3rd month, with a slight decrease to 7.8 $\log_{10}$ CFU/g at the 6th month. In the sample with 0.2%, a more significant decrease was observed – from 8.1 $\log_{10}$ CFU/g at the beginning to approximately 7.7 $\log_{10}$ CFU/g at the 3rd and 6th months.

The applied surface treatment contributes to better preservation of lactococci during the 6-month storage period, with the effect being more pronounced in the sample with 0.1% essential oil. The results obtained show that the applied technology does not compromise the development and survival of the starter microflora, which is essential for the biochemical processes during Kashkaval cheese ripening and for the formation of its sensory characteristics.

The application of plant extracts and essential oils in dairy products is commonly used as a natural antimicrobial approach against major pathogens and spoilage microorganisms such as *Listeria monocytogenes*, *Staphylococcus aureus*, *Escherichia coli*, *Salmonella* spp., as well as yeasts and molds (Riota and Manzi, 2020).

In the present study, the surface treatment of Kashkaval cheese with a mixture of high-oleic sunflower oil and thyme essential oil showed an inhibitory effect on the development of yeasts and molds (Figure 5). In the control sample (Figure 5), a gradual increase in the count was observed from 5.0 $\log_{10}$ CFU/g at the beginning of the storage period to about 5.3 $\log_{10}$ CFU/g at the 3rd month, reaching approximately 6.5 $\log_{10}$ CFU/g at the 6th month. This increase is expected during prolonged storage and reflects the development of secondary microflora.

At the beginning of the storage period, lower values were observed in the sample with 0.2% essential oil (4.7 $\log_{10}$ CFU/g) compared to the control sample (5.0 $\log_{10}$ CFU/g) and sample with 0.1% theme essential oil (5.3 $\log_{10}$ CFU/g). At the third month of storage, the values for samples with 0.1% and 0.2% thyme essential oils remained relatively stable, whereas at the end of the storage period they increased to 5.9 $\log_{10}$ CFU/g and 6.1 $\log_{10}$ CFU/g, respectively. However, in both treated variants the final values (at the 6th month) were lower than those observed in the control sample, indicating the antimicrobial effect of the essential oil. These results are in agreement with findings in studies (Bukwicki et al., 2018; Kholy et al., 2017).

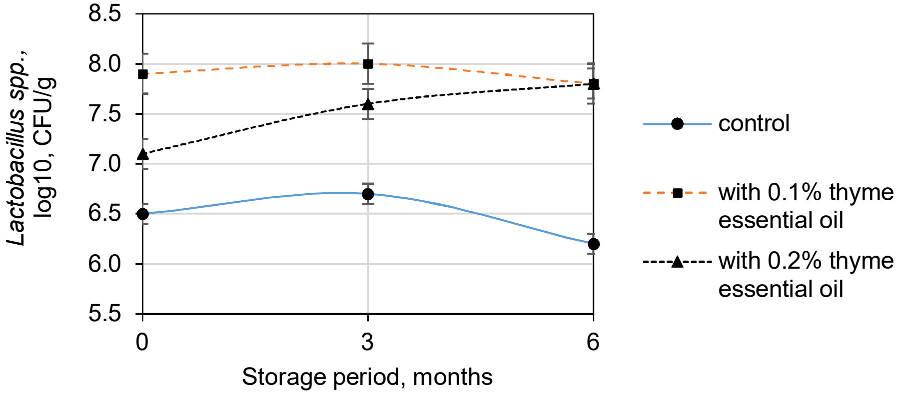

Figure 3. Viable cell count of *Lactobacillus* spp. in Kashkaval cheese coated with high-oleic sunflower oil and thyme essential oil during the storage

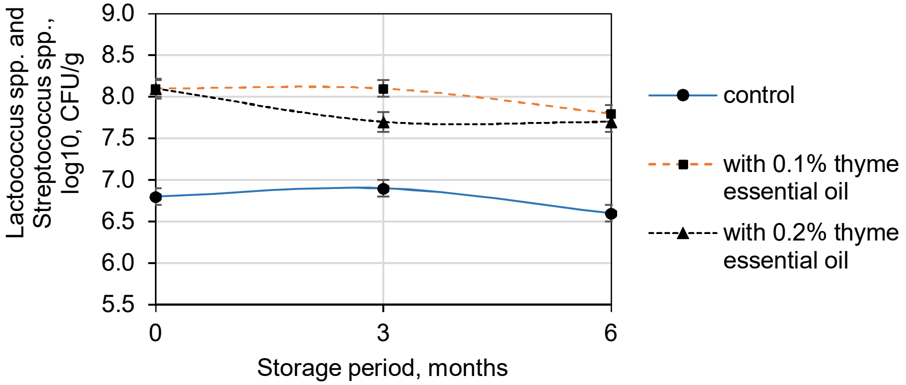

Figure 4. Viable cell count of *Lactococcus* spp. and *Streptococcus* spp. in Kashkaval cheese coated with high-oleic sunflower oil and thyme essential oil during the storage

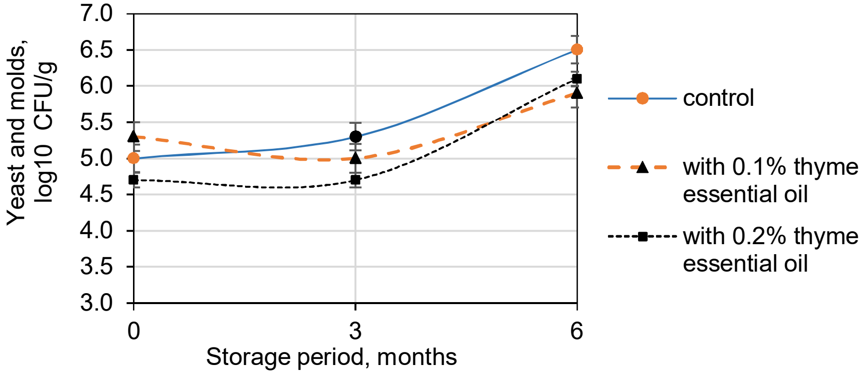

Figure 5. Viable count of yeasts and molds in Kashkaval coated with high-oleic sunflower oil and thyme essential oil during storage

Conclusions

The application of a mixture of high-oleic sunflower oil and thyme essential oil to the surface of Kashkaval cheese can serve as a functional ingredient. During storage for 45 days, 3 months, and 6 months, no significant changes were observed in the fatty acid profile, organic acids, or amino acid content. This surface treatment also supports potential health benefits by contributing to a reduction in cholesterol levels during storage. Additionally, surface treatment enhances the aromatic and flavor profile of the cheese while helping to control microbiological activity. Coating with 0.1% thyme essential oil in high-oleic sunflower oil slows down amino acid transformations during storage and helps maintain the nutritional value of the dairy product.

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Properties of extruded breakfast cereals made from fermented sorghum flour and tigernut pomace blends

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Abstract

Introduction. In recent years, there has been a growing demand for functional and gluten-free foods driven by increasing consumer interest in healthier dietary choices. Therefore, this study evaluated selected quality attributes of extruded breakfast cereals produced from blends of fermented sorghum flour (FSF) and tigernut pomace (TNP).

Materials and methods. D-optimal design was used in the preparation of the flour blends, resulting in ten experimental runs. Extruded breakfast meals were manufactured from the blends through a hot extrusion process and were analysed for functional, colour, textural, and sensory properties using standard methods.

Results and discussion. The water absorption capacity, oil absorption capacity, swelling power, solubility index and dispersibility ranged from 402.29–611.11%, 193.48–426.88%, 3.41–4.65, 27.40–48.75%, 48.50–66.00%, respectively. Lightness, redness and yellowness of the extruded breakfast meal ranged from 36.59–40.27, 0.82–2.28 and 11.69–13.57, respectively. Hardness, springiness, adhesiveness, cohesiveness, fracturability, gumminess and chewiness ranged from 117.05–311.59 N, 0.11–0.16, 0.47–14.26, 0.12–0.17, 30.61–84.49, 14.20–36.88 and 1.81–4.35, respectively.

The results obtained from the textural properties showed that chewiness, energy to peak, gumminess, fracturability, hardness increased significantly ($p<0.05$) with increase in tigernut pomace blends. Taste, aroma, colour, texture and appearance ranged from 5.77–7.53, 5.77–6.67, 6.25–7.93, 5.59–7.30 and 5.90–7.03, respectively.

Sensory quality attributes showed that 88.75% of FSF and 11.25% of TNP had the highest overall acceptability. Addition of tigernut pomace to fermented sorghum flour had a significant ($p<0.05$) effect on the colour and functional properties of the extruded breakfast meal while there was no significant ($p>0.05$) effect in springiness, cohesiveness, energy to peak and springiness.

Conclusions. The study showed that extruded breakfast cereal containing 88.75% fermented sorghum flour and 11.25% tigernut pomace achieving the highest overall acceptability (7.30).

Introduction

Breakfast is the day's nutritional foundation or first meal. Breakfast meals for adults and babies in underdeveloped nations, notably Sub-Saharan Africa, are centred on a local staple diet of grains, legumes, and a few tubers (Okache et al., 2020). They are foods that must be reconstituted before being consumed. Corn, rice, wheat, oats, and barley are the most common grains used in the production of breakfast meal (Gazza et al., 2024). However, in a bid to fortify breakfast meal with other essential nutrients, cereals are combined with other raw materials. In order to combat with the rising cost of conventional raw materials, underutilized, unconventional and indigenous raw materials are used (Ene et al., 2022, Ojo et al., 2025a). Considering the well documented health benefits of tigernut and sorghum, combination of fermented sorghum flour and tigernut pomace flour for extruded breakfast meal production is advocated.

Sorghum (*Sorghum bicolor* (L.) Moench) is a whole-grain cereal more commonly used as animal feed than as a food source in Western countries (Stefoska-Needham et al., 2015). It is known as Guinea corn in West Africa and ranks as the fourth most important cereal crop worldwide after rice, wheat, and maize. Sorghum grain is an excellent alternative food source due to its gluten-free nature, high resistant starch content, and richness in minerals and bioactive phenolic compounds (Batariuc et al., 2024; González-Victoriano et al., 2025; Punia et al., 2021). Compared to other major cereal crops, sorghum contains higher levels of phenolic compounds, mainly including simple phenolic acids, tannins, and flavonoids (Shen et al., 2018). These bioactive compounds contribute to the modulation of gut microbiota and exhibit various biological activities, such as anti-inflammatory, antioxidant, antithrombotic, and antidiabetic effects (Romeilah et al., 2021). However, sorghum starch shows relatively low digestibility compared to other cereals, partly due to the presence of anti-nutritional factors such as tannins, which hinder starch digestion and nutrient absorption (Ge et al., 2020).

Fermentation is a primary technique of boosting food flavour, broadening the application scope of cereals, and increasing their usage ratio (Mykhonik and Hetman, 2022). Fermentation of cereals enhances the shelf stability, texture, aroma, flavor, nutritional quality, and digestibility of their products, while significantly reducing antinutrient content (Ojo et al., 2025b). Fermentation has been shown to be an effective biochemical method for reducing anti-nutritional components in cereals, as well as improving the digestibility of starch and protein, balancing of amino acid, and nutritive density (Ge et al., 2020). Researches have shown that fermentation increases the concentration of sorghum amylose and its retrogradation value (Ge et al., 2018). Furthermore, Osman et al. (2004) found that spontaneous fermentation of sorghum boosted the levels of lysine, leucine, isoleucine, and methionine significantly.

Tigernut (*Cyperus esculentus*) is a monocotyledonous plant in the Cyperaceae family, which comprises over 4,000 species. It is a perennial grass that grows in damp areas and is commonly regarded as a weed, particularly in vegetable fields (Suleiman et al., 2018). Tigernut is a spherical rhizome crop that can be consumed raw, dried, or processed (Bazine and Arslanoglu, 2020). Major producers include Nigeria, Togo, Niger, Benin, the United States, Iraq and Morocco. In Nigeria, it is locally known as Aya in Hausa, Ofio in Yoruba, and Akiausa in Igbo, with three cultivated types: black, yellow, and brown. Of these, only the yellow and brown varieties are widely available in the market. When chewed raw, tigernut produces a sweet-nutty flavour (Maduka and Ire, 2018).

Tigernut tubers are rich in protein, vitamins, minerals, and other phytonutrients. They are particularly abundant in insoluble fibre, which aids digestion and helps prevent constipation. The tubers also contain arginine and fibre, both of which may contribute to lowering blood sugar levels (Bilikis and Olarenwaju, 2015). Tigernut by-products include oil, flour, and pomace.

Tigernut pomace has been applied in various food products such as biscuits, cakes, cookies, beverage powders, pasta, ice cream, complementary foods, and as a soup thickener (Ayodele et al., 2019; Gambo and Da’u, 2014; Oke et al., 2022; Sánchez-Zapata et al., 2009). It is commonly used as a functional food component, serving as a fat substitute, fat-reducing agent during frying, binder, volume enhancer, stabilizer, and bulking agent, and responds to the current trend of bioconversion of plant processing waste into value-added products (Stabnikova et al., 2023a, b).

It is well established that combining cereal flours with plant-based materials can enhance the nutritional value of food products. For example, the addition of seedless grape pomace has been shown to improve the nutritional quality of extruded maize (Ungureanu-Iuga et al., 2024) and corn-based snacks (Mironeasa et al., 2024).

In a similar context, agro-industrial by-products such as tigernut pomace have attracted increasing attention as functional food ingredients. Although often discarded as waste, tigernut pomace is a valuable source of dietary fibre (Sánchez-Zapata et al., 2012). Its inclusion in food formulations can provide nutritious products at low cost while promoting the utilization of underutilized crops (Ene et al., 2022). Consequently, combining tigernut pomace with sorghum in food products may enhance nutritional quality and contribute to addressing food insecurity.

Therefore, the aim of this study was to evaluate the functional, physical, textural, and sensory properties of an extruded breakfast product made from blends of fermented sorghum flour and tigernut pomace.

Materials and methods

Materials

Sorghum, tigernut, vegetable oil, vanilla flavour and salt were purchased from Eleweran market in Ogun state.

Preparation of fermented sorghum flour

The method described by Ojo et al. (2025b) was used in the preparation of fermented sorghum flour (FSF). Sorghum grains were sorted to remove foreign materials. It was rinsed with clean water and steeped in water for 72 h for fermentation. It was drained and was dried for 24 h at 70°C. Milling was done using laboratory hammer mill (Fritsch, D-55743, Idar-Oberstein-Germany).

Preparation of tigernut pomace

Tigernut pomace flour was prepared according to the method described by Oke et al. (2022). Brown tigernuts (*Cyperus esculentus*) were sorted to remove foreign materials such as pebbles and stones and then washed under running water. The cleaned samples were soaked for 8 h and subsequently wet-milled using a laboratory hammer mill (Fritsch, D-55743, Idar-Oberstein, Germany). The resulting slurry was filtered through muslin cloth to obtain the extract, leaving tigernut pomace (TNP) as a residue. The pomace was then dried in a cabinet dryer at 60 °C for 24 h.

The formulation of the flour blends is presented in Table 1 based on a D-optimal mixture design. A total of ten experimental runs were generated, with 100% fermented sorghum flour serving as the control sample. The flour blends were packaged and stored until use for breakfast meal production.

Table 1

Formulations of extruded breakfast cereals from fermented sorghum flour-tigernut pomace blends

Sample	FSF (%)	TNP (%)	Sample	FSF (%)	TNP (%)
1	85.00	15.00	6	90.00	10.00
2	88.75	11.25	7	85.00	15.00
3	85.00	15.00	8	87.50	12.50
4	86.25	13.75	9	90.00	10.00
5	87.50	12.50	10	100.00	00.00

FSF - fermented sorghum flour; TNP - tigernut pomace.

Preparation of extruded breakfast meal

The experimental design for the composition of the breakfast meal is presented in Table 1. Breakfast meal samples were prepared according to the method described by Ojo et al. (2025a). The formulation consisted of composite flour (100 g), water (300 mL), margarine (150 g), salt (2 g), and vanilla flavor (2.85 g). The mixtures were processed using a twin-screw extruder (Quitong-Kneader, Model JS-60D).

The extrusion process was carried out using the different feed compositions and process parameters. After 20 min of mixing, a homogeneous flour mixture was obtained. The extruded samples were collected at the die end of the extruder, cooled to ambient temperature, and stored in high-density polyethylene bags for further analy

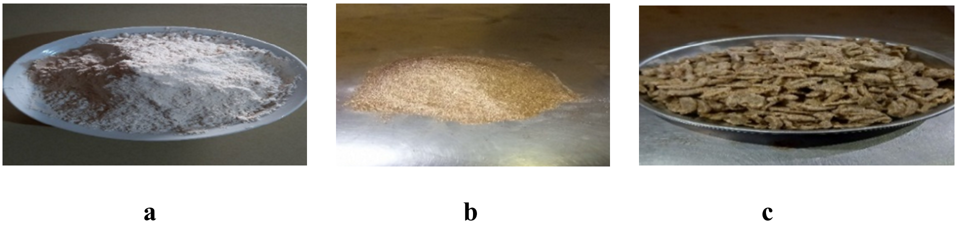

Figure 1. Fermented sorghum flour (a), tigernut pomace (b), and extruded breakfast meal (c)

Evaluation of functional properties of extruded breakfast cereals

Determination of water absorption capacity. Water absorption capacity was determined according to the method of Adebayo-Oyetoro et al. (2016). One gram of each sample was weighed into a clean, graduated conical centrifuge tube and thoroughly mixed with 10 mL of distilled water using a platform tube rocker for 30 s. The mixture was allowed to stand at room temperature for 30 mins and then centrifuged at 3500 rpm for 30 min. The volume of the supernatant was recorded directly from the graduated centrifuge tube. The absorbed water was converted to weight (g) by multiplying the volume by the density of water (1 g/mL). Water absorption capacity was expressed as grams of water absorbed per gram of flour sample.

Determination of oil absorption capacity. Oil absorption capacity was determined according to the method described by Aburime et al. (2020). Approximately 1 g of the sample was weighed into a clean, dry centrifuge tube, and the weight of the empty tube was recorded. Ten millilitres (10 mL) of refined corn oil was added and thoroughly mixed with the sample. The mixture was centrifuged at 3500 rpm for 15 min. The supernatant was carefully decanted, and the tube with its contents was reweighed. The increase in mass was recorded as the oil absorption capacity of the sample.

Determination of swelling power and solubility index. Swelling power and solubility index were determined using the method described by Takashi and Seibel (1988). One gram of flour was weighed into a 50 mL centrifuge tube, and 50 mL of distilled water was added and gently mixed. The mixture was heated in a water bath at 90 °C for 15 min, with gentle stirring to prevent clumping. The tube was then centrifuged at 3,000 rpm for 10 mins. The supernatant was carefully decanted, and the weight of the sediment was recorded. The moisture content of the sediment gel was subsequently determined to calculate the dry matter content of the gel.

$$Swelling\ power = \frac{Weight\ of\ wet\ mass\ sediment\ (g)}{Weight\ of\ dry\ matter\ in\ the\ gel\ (g)}$$

$$Solubility\ index = \frac{Weight\ of\ dry\ solids\ after\ drying\ (g)}{Weight\ of\ sample\ (g)} \times 100$$

Determination of colour properties of extruded breakfast cereals. Colour of the samples were determined using a colorimeter (model - Minolta Chromameter (CR-310, Japan)). The colour values are expressed as L* (lightness), a* (redness / greenness) and b* (yellowness/blueness).

Determination of textural properties extruded breakfast cereal. Texture Profile Analysis (TPA) was used to analyse the texture of the extruded breakfast cereals. Probe diameter was set at 38mm, deflection (mm) was set at 50% strain, speed was set at 102mm/min, preload speed was set at 60mm/min and preload was set at 0.500N. The following parameters were measured hardness (N), springiness, adhesiveness (N.s), cohesiveness, chewiness (N), fracturability (N), gumminess (N), energy to peak (N.m) and stringiness (mm).

Determination of sensory properties of extruded breakfast cereal. This study received ethical approval from the Ethics Committee of the Department of Food Science and Technology, Federal University of Agriculture, Abeokuta. Ethical approval was necessary because semi-trained panellists were involved in the sensory evaluation of the extruded breakfast cereals. Sensory evaluation was conducted following the procedure described by Gagneten et al. (2021). A total of 100 panellists were selected from the university community and asked to evaluate the extruded breakfast cereals for taste, aroma, colour, appearance, and overall acceptability using a 9-point hedonic scale (9 = like extremely; 5 = neither like nor dislike; 1 = dislike extremely).

Statistical Analysis

Data obtained from all analyses were subjected to analysis of variance (ANOVA). Mean separation was performed using Duncan's multiple range test in the Statistical Package for the Social Sciences (SPSS) for Windows, version 23.0 (SPSS Inc., Chicago, USA). Differences were considered statistically significant at $p < 0.05$.

Results and discussion

Functional properties of extruded breakfast meal from blends of fermented sorghum flour (FSF) and tigernut pomace (TNP)

The functional properties of extruded breakfast cereals produced from fermented sorghum flour and tigernut pomace are presented in Table 2.

Table 2
Functional properties of extruded breakfast cereal from blends of fermented sorghum flour and tigernut pomace

FSF (%)	TNP (%)	BD (g/cm^3)	WAC (%)	OAC (%)	SP (g/g)	SI (%)
85.00	15.00	0.91±0.71^a	402.32±0.49^a	4.26±0.60^f	4.65±0.03^e	48.75±1.98^c
88.75	11.25	0.93±0.00^a	503.25±1.19^c	4.12±1.19^c	4.51±0.02^d	29.80±0.14^b
85.00	15.00	0.94±0.71^a	402.30±0.49^a	4.16±0.60^a	4.57±0.03^d	47.48±1.98^c
86.25	13.75	0.94±1.41^a	559.00±0.24^d	2.91±0.76^d	4.41±0.02^d	29.84±0.37^b
87.50	12.50	0.97±0.71^a	457.17±1.20^b	2.35±0.19^b	4.12±0.02^c	28.72±0.39^a
90.00	10.00	0.97±1.41^a	562.68±1.47^e	3.42±0.07^e	4.01±0.03^b	27.40±0.57^a
85.00	15.00	0.99±0.71^b	402.29±0.49^a	4.03±0.60^a	3.43±0.03^{ab}	47.44±1.98^d
87.50	12.50	0.99±0.71^b	457.20±1.20^b	2.36±0.19^b	4.15±0.02^c	28.75±0.39^a
90.00	10.00	1.12±1.41^b	562.70±1.47^e	3.42±0.07^e	4.05±0.03^b	27.43±0.57^a
100.00	0.00	1.15±1.41^b	611.11±0.31^f	1.93±0.23^a	3.41±0.02^a	27.40±0.70^a

Values are mean±standard deviation of duplicate determinations. Mean values along the same column with different superscripts are significantly different ($p<0.05$); (FSF - Fermented sorghum flour: TNP - Tigernut pomace). BD: Bulk density, WAC: Water absorption capacity, OAC: Oil absorption capacity, SP: Swelling power; SI: Solubility index; DISP: Dispersibility

These properties are critical indicators of ingredient behaviour during processing and their potential applications in food systems. The bulk density of the samples ranged from 0.91 to 1.15 g/cm^3, with significant differences ($p < 0.05$) observed among samples. The sample containing 85% FSF and 15% TNP exhibited the lowest bulk density (0.91 g/cm^3), while 100% FSF demonstrated the highest value (1.15 g/cm^3). This inverse relationship between TNP incorporation and bulk density suggests that the addition of tigernut pomace created a more porous structure in the extruded products. Bulk density is a fundamental parameter that influences packaging requirements, storage stability, and handling characteristics during processing operations (Ramashia et al., 2018). The observed decrease in bulk density with increasing TNP content may be attributed to the fibre content of tigernut pomace, which can modify the expansion characteristics during extrusion. Products with higher bulk density are generally preferred for adult food formulations, whereas lower bulk density is advantageous for infant weaning foods as it facilitates easier reconstitution and improved energy density per unit volume (Ramashia et al*.,* 2018).

Water absorption capacity varied significantly ($p < 0.05$) across all samples, ranging from 402.29% to 611.11%. The sample with 100% FSF and 0% TNP exhibited the highest WAC (611.11%), while the blend containing 85% FSF and 15% TNP showed the lowest value (402.29%). This declining trend in WAC with increasing TNP incorporation can be attributed to the displacement of starch by fibre-rich tigernut pomace. The starch granules in fermented sorghum flour possess hydrophilic characteristics that promote water binding through hydrogen bonding with hydroxyl groups. According to Lakshmi et al. (2014), starch components in composite flour blends significantly influence water absorption capacity through their inherent hydrophilic nature. The variation in WAC among samples reflects differences in their hydrophilic constituents, particularly carbohydrates, which demonstrate greater water retention capacity compared to proteins or lipids (Adepeju et al., 2014). Extruded breakfast meals with elevated WAC likely contain substantial amounts of hydrophilic components, including starches and polar amino acid residues, which enhance gelation properties and water-binding capabilities (Kaur et al., 2005). Conversely, formulations with lower WAC are more suitable for infant foods, as they yield thinner gruels upon reconstitution, facilitating easier consumption and digestion for young children (Onweluzo et al., 2009).

The oil absorption capacity ranged from 1.93% to 4.26%, with significant differences ($p < 0.05$) observed among all samples. The control sample (100% FSF with 0% TNP) recorded the lowest OAC (1.93%), while the blend with 85% FSF and 15% TNP achieved the highest value (4.26%). This progressive increase in OAC with rising TNP content can be attributed to the fibre matrix and surface characteristics of tigernut pomace. The porous structure and surface polarity of TNP facilitate enhanced lipid binding through both physical entrapment and hydrophobic interactions (Jose et al., 2022). Oil absorption capacity is a valuable functional property that influences sensory attributes and product stability. Enhanced OAC contributes to improved palatability, superior flavour retention, and extended shelf life in food products (Chandra et al., 2013). The elevated OAC observed in TNP-enriched samples suggests their potential utility in applications where fat retention and mouthfeel are important quality parameters.

Swelling power values ranged from 3.41 to 4.65 g/g, showing significant increases ($p < 0.05$) with TNP incorporation. The highest swelling power (4.65 g/g) was observed in the sample containing 85% FSF and 15% TNP, while the control sample (100% FSF) exhibited the lowest value (3.41 g/g). This enhancement in swelling power with increasing TNP content aligns with the findings of Ayodele et al. (2019) in wheat-TNP pomace blends. The increased swelling capacity may be attributed to the combined effects of the starch fraction from FSF and the fibre components from TNP, which collectively enhance the hydration capacity of the flour blend (Yu et al., 2022). During heating, the starch granules absorb water, swell, and eventually rupture, with the presence of dietary fibre potentially facilitating greater water uptake and retention within the matrix structure.

The solubility index values ranged from 27.40% to 48.75%, demonstrating significant variation ($p<0.05$) among samples. A positive correlation was observed between TNP content and solubility index, with values increasing as TNP incorporation rose. This trend corroborates the findings of Ayodele et al. (2019), who reported that wheat flour substituted with 4% tigernut pomace had the lowest solubility index, while 10% substitution yielded the highest values. The enhanced solubility with increasing TNP content may result from multiple factors, including amylase activity during fermentation and the presence of soluble sugars and solubilized fibre components. Fermentation processes are known to break down complex carbohydrates into simpler, more soluble forms, thereby increasing the overall solubility index of the flour blend. The higher solubility index in TNP enriched samples suggests improved dispersibility and faster reconstitution, which are desirable characteristics for instant breakfast cereal products.

Colour properties of extruded breakfast cereal from blends of fermented sorghum flour (FSF) and tigernut pomace (TNP)

Colour is a critical quality parameter that significantly influences consumer acceptability and purchasing decisions. The colour properties of extruded breakfast cereals, expressed in terms of L* (lightness), a* (redness), and b* (yellowness) values, are presented in Table 3.

Table 3

Colour properties of extruded breakfast cereal from blends of fermented sorghum flour and tigernut pomace

FSF (%)	TNP (%)	L*	a*	b*
85.00	15.00	36.59±0.02^{a}	2.74±0.05^{c}	11.84±0.02^{a}
88.75	11.25	37.42±0.08^{a}	1.97±0.04^{b}	11.69±0.04^{a}
85.00	15.00	38.62±0.02^{ab}	2.82±0.05^{c}	11.84±0.02^{a}
86.25	13.75	37.3±0.02^{ab}	1.86±0.01^{b}	12.48±0.06^{b}
87.50	12.50	38.40±0.04^{b}	1.96±0.02^{b}	12.64±0.01^{b}
90.00	10.00	39.97±0.02^{b}	1.69±0.01^{b}	13.57±0.06^{c}
85.00	15.00	38.19±0.02^{b}	2.79±0.05^{c}	11.86±0.02^{a}
87.50	12.50	38.38±0.04^{b}	1.88±0.02^{b}	12.61±0.01^{b}
90.00	10.00	39.2±0.02^{bc}	1.67±0.01^{b}	13.53±0.06^{c}
100.00	0.00	40.27±0.02^{c}	0.82±0.03^{a}	11.80±0.06^{a}

Values are mean±standard deviation of duplicate determinations. Mean values along the same column with different superscripts are significantly different ($p<0.05$)**;** (FSF - Fermented sorghum flour: TNP - Tigernut pomace

All three colour parameters showed significant differences ($p < 0.05$) among samples. The lightness values ranged from 36.59 to 40.27, with the control sample (100% FSF) exhibiting the highest lightness (40.27) and the blend containing 85% FSF and 15% TNP showing the lowest value (36.59). The progressive decrease in lightness with increasing TNP incorporation can be attributed to non-enzymatic browning reactions occurring during extrusion processing. The elevated temperatures and pressures during extrusion promote both Maillard reactions (between reducing sugars and amino acids) and caramelization of sugars, resulting in the formation of brown melanoidin pigments (Usman et al., 2020). The inherent colour of tigernut pomace, which contains natural pigments, also contributes to the darker appearance of TNP-enriched samples. Redness values ranged from 0.82 to 2.82, with the control sample (100% FSF) recording the lowest value (0.82) and the sample with 85% FSF and 15% TNP exhibiting the highest redness (2.82). These values fall within the range (0.89–2.47) reported by Olugbuyi et al. (2024) for sorghum-date extruded breakfast cereals. The increase in redness with TNP addition reflects the presence of natural colour pigments in tigernut pomace. This chromatic shift toward red indicates the contribution of phenolic compounds and other pigmented substances present in the pomace, which remain stable during extrusion processing. The yellowness values ranged from 11.69 to 13.57, with the control sample (100% FSF) showing the lowest value (11.69) and the blend containing 90% FSF and 10% TNP displaying the highest yellowness (13.57). This variation in yellowness can be attributed to the pigment composition of tigernut pomace. Phytochemical screening studies by Ihenetu et al. (2021) revealed that tigernut possesses considerable anthocyanin content, along with other pigmented compounds such as carotenoids and flavonoids. The differences in yellowness among extruded breakfast cereals reflect both the pigment profile and the compositional characteristics of the pomace (Eke-Ejiofor et al., 2016). The moderate increase in yellowness may also result from the thermal degradation of certain pigments during extrusion, leading to the formation of yellow-brown compounds.

Textural properties of extruded breakfast cereal from blends of fermented sorghum flour (FSF) and tigernut pomace (TNP)

Texture profile analysis revealed significant changes in the textural properties of extruded breakfast cereals with varying FSF and TNP ratios (Table 4).

Table 2

Textural properties of extruded breakfast cereal from blends of fermented sorghum flour and tigernut pomace

FSF (%)	TNP (%)	HRD (N)	SPG	ADH	COH	FRC	GUM	CHW (N)
85.00	15.00	117.05+20.16^{a}	0.12± 0.01^{a}	8.28± 7.64^{ab}	0.12 ± 0.00^{a}	84.47± 26.04^{b}	36.85 ± 1.28^{b}	1.81± 0.16^{a}
88.75	11.25	247.65± 41.77^{b}	0.12± 0.01^{a}	0.47± 0.63^{a}	0.12 ± 0.02^{a}	39.51± 21.31^{ab}	19.89 ± 7.78^{a}	2.58± 0.79^{b}
85.00	15.00	118.59± 20.16^{a}	0.15± 0.01^{a}	8.30± 7.64^{ab}	0.14 ± 0.00^{a}	84.43 ± 26.04^{b}	36.88 ± 1.28^{b}	1.85± 0.16^{a}
86.25	13.75	268.05± 12.82^{bc}	0.13± 0.01^{a}	0.16± 0.17^{a}	0.12 ± 0.01^{a}	39.13± 18.84^{ab}	14.20 ± 1.54^{a}	2.81± 0.26^{b}
87.50	12.50	297.68± 18.22^{c}	0.11± 0.01^{a}	1.06± 0.09^{a}	0.12 ± 0.01^{a}	42.11± 29.32^{ab}	17.68 ± 0.22^{a}	2.90 ± 0.20^{b}
90.00	10.00	309.51± 16.71^{d}	0.12± 0.00^{a}	2.56± 4.44^{ab}	0.12 ± 0.00^{a}	38.26± 32.04^{ab}	15.24 ± 2.35^{a}	2.89± 0.24^{b}
85.00	15.00	117.03± 20.16^{a}	0.13± 0.01^{a}	8.35± 7.64^{ab}	0.17 ± 0.00^{a}	84.49 ± 26.04^{b}	36.81 ± 1.28^{b}	2.29± 0.16^{b}
87.50	12.50	268.66± 18.22b^{c}	0.13± 0.01^{a}	1.08± 0.09^{a}	0.14 ± 0.01^{a}	42.15± 29.32^{ab}	17.70 ± 0.22^{a}	2.74± 0.20^{b}
90.00	10.00	307.53± 16.71^{d}	0.15± 0.00^{a}	2.58± 4.44^{ab}	0.12 ± 0.00^{a}	38.28± 32.04^{ab}	15.27± 2.35^{a}	2.93± 0.24^{b}
100.00	0.00	311.59± 11.03^{d}	0.16± 0.02^{a}	14.26± 13.36^{b}	0.14 ± 0.01^{a}	30.61± 24.48^{a}	18.28 ± 1.42^{a}	4.35± 0.29^{c}

Values are mean±standard deviation of duplicate determinations. Mean values along the same column with different superscripts are significantly different ($p<0.05$).
FS - fermented sorghum flour: TNP - tigernut pomace; HRD – hardness; SPG – springiness; ADH – adhesiveness; COH – cohesiveness; FRC – fracturability; GUM – gumminess; CHW – chewiness

The textural attributes examined included hardness, springiness, adhesiveness, cohesiveness, fracturability, gumminess, and chewiness, all of which are important indicators of product quality and consumer acceptability. Hardness values ranged from 117.05 N to 311.59 N, with the control sample (100% FSF) exhibiting the highest hardness (311.59 N) and the sample containing 85% FSF and 15% TNP showing the lowest value (117.05 N). There is a significant decrease ($p < 0.05$) in hardness with increasing TNP content represents a substantial textural modification. This reduction can be attributed to several interconnected factors. However, the incorporation of TNP reduces the overall starch content in the formulation, leading to decreased starch gelatinization and network formation during extrusion. Second, the absence of gluten in both sorghum and tigernut results in poor protein network development, which would otherwise contribute to structural rigidity. The elevated hardness in the control sample may be explained by greater amylose-lipid complex formation, which strengthens the structural matrix (Cervantes-Ramirez et al*.,* 2020). Higher starch content facilitates more extensive molecular interactions during cooling, resulting in a firmer texture.

Springiness values ranged from 0.11 to 0.16, while cohesiveness ranged from 0.12 to 0.17. Neither parameter showed statistically significant differences ($p > 0.05$) among samples, suggesting that TNP incorporation does not substantially affect the elastic recovery or internal bonding strength of the extruded products. These consistent values indicate that the structural integrity and bite characteristics remain relatively stable across different formulations, which is favourable for product standardization.

Fracturability increased significantly ($p < 0.05$) from 30.61 N to 84.49 N with rising TNP content. The highest fracturability (84.49 N) was observed in samples with 85% FSF and 15% TNP, while the control sample showed the lowest value (30.61 N). Increased fracturability indicates that TNP-enriched samples require greater force to initiate breakage, suggesting a more brittle or crispy texture. This characteristic is desirable in breakfast cereals, as it contributes to the perception of freshness and crunchiness.

The fibre matrix in TNP may create stress concentration points within the extruded structure, leading to this increased fracture resistance. Gumminess values ranged from 14.20 N to 36.88 N, while chewiness ranged from 1.81 N to 4.35 N. Both parameters showed significant decreases ($p < 0.05$) with increasing TNP content. The control sample exhibited the highest values for both gumminess (18.28 N) and chewiness (4.35 N). These reductions suggest that TNP-enriched samples require less energy for mastication, potentially improving eating quality and consumer acceptance. The decreased gumminess and chewiness with TNP addition may result from the dilution effect on starch content and the interference of fibre with starch network formation.

Adhesiveness values ranged from 0.16 N•s to 14.26 N•s, showing significant variation ($p < 0.05$) among samples. The control sample displayed notably higher adhesiveness (14.26 N•s) compared to TNP-enriched formulations. Lower adhesiveness in TNP-containing samples indicates reduced stickiness to oral surfaces, which is generally preferred in dry breakfast cereals. These textural modifications align with previous research on fibre enriched extruded products. Kareem et al. (2015) reported similar trends in extruded snacks produced from cassava-TNP pomace blends, while Jose et al. (2022) observed comparable textural changes in extruded snacks fortified with pineapple pomace. The consistent pattern across different fibre sources suggests that dietary fibre incorporation fundamentally alters the structural properties of starch-based extrudates by disrupting starch gelatinization and network formation during processing.

Sensory properties of extruded breakfast cereals from blends of fermented sorghum flour (FSF) and tigernut pomace (TNP)

Sensory evaluation results are presented in Table 5 and include taste, aroma, colour, texture, appearance, and overall acceptability

Table 3

Sensory properties of extruded breakfast cereal from blends of fermented sorghum flour and tigernut pomace

FSF (%)	TNF (%)	Taste	Aroma	Colour	Texture	Appearance	Overall Acceptability
85.00	15.00	5.77±1.22^{a}	5.77±1.22^{a}	6.30±1.49^{a}	5.60±1.73^{a}	5.90±1.92^{a}	6.27±1.78^{a}
88.75	11.25	7.07±1.41^{c}	6.67±1.24^{b}	6.93±1.63^{ab}	7.30±1.56^{c}	7.03±1.30^{b}	7.30±1.56^{c}
85.00	15.00	5.78±1.22^{a}	5.79±1.22^{a}	6.28±1.49^{a}	5.59±1.73^{a}	5.92±1.92^{a}	6.28±1.78^{a}
86.25	13.75	7.53±1.29^{c}	6.13±1.17^{ab}	6.33±1.27^{a}	6.87±1.36^{bc}	6.40±1.16^{ab}	6.67±1.16^{ab}
87.50	12.50	6.47±1.28^{ab}	6.43±1.17^{ab}	6.70±1.39^{a}	6.73±1.14^{bc}	6.63±1.19^{ab}	6.80±1.06^{ab}
90.00	10.00	6.57±1.57^{ab}	6.20±1.22^{ab}	6.83±1.37^{a}	6.87±1.59^{bc}	6.73±1.43^{b}	6.94±1.20^{ab}
85.00	15.00	5.83±1.22^{a}	5.76±1.22^{a}	6.25±1.49^{a}	5.63±1.73^{a}	5.90±1.92^{a}	6.28±1.78^{a}
87.50	12.50	6.50±1.28^{ab}	6.44±1.17^{ab}	6.73±1.39^{a}	6.75±1.14^{bc}	6.67±1.19^{ab}	6.81±1.06^{ab}
90.00	10.00	6.54±1.57^{ab}	6.17±1.22^{ab}	6.85±1.37^{ab}	6.89±1.59^{bc}	6.72±1.43^{b}	6.97±1.20^{ab}
100.00	0.00	6.17±1.58^{ab}	6.27±1.53^{ab}	7.93±1.57^{b}	6.33±1.92^{ab}	6.83±1.80^{b}	6.83±1.64^{ab}

Values are mean±standard deviation of duplicate determinations. Mean values along the same column with different superscripts are significantly different ($p<0.05$); FS - fermented sorghum flour, TNP - tigernut pomace.

These attributes collectively determine consumer preference and market potential of the developed products. Taste scores ranged from 5.77 to 7.53 on a 9-point hedonic scale, with significant differences ($p < 0.05$) observed among samples. The sample containing 86.25% fermented sorghum flour and 13.75% tigernut pomace received the highest taste score (7.53), indicating strong consumer preference. This enhancement in taste acceptability with moderate tigernut pomace incorporation may be attributed to the natural sweetness and nutty flavour profile of tigernut, which complements the mild, slightly fermented taste of sorghum flour. The fermentation of sorghum also contributes desirable flavour compounds that enhance overall palatability. Aroma scores ranged from 5.77 to 6.67, with the sample containing 88.75% fermented sorghum flour and 11.25% tigernut pomace achieving the highest rating. The pleasant aroma profile likely results from volatile compounds generated during fermentation and the characteristic nutty aroma of tigernut. Extrusion processing further develops desirable aroma compounds through thermal reactions, contributing to the overall sensory appeal. Colour acceptability ranged from 6.25 to 7.93, with the control sample (100% fermented sorghum flour) receiving the highest score (7.93). Despite the darker appearance of tigernut pomace-enriched samples observed in instrumental colour analysis, consumer acceptance remained relatively high across all formulations, suggesting that the colour changes were within acceptable ranges. The slight preference for lighter-coloured samples may reflect consumer familiarity with traditional cereal products. Texture scores ranged from 5.59 to 7.30, showing improvement with moderate tigernut pomace incorporation. The sample with 88.75% fermented sorghum flour and 11.25% tigernut pomace achieved the highest texture score (7.30), suggesting that this formulation provided the optimal balance between crispness and ease of chewing. The textural improvements observed in sensory evaluation correlate with the instrumental texture analysis, which showed reduced hardness and chewiness in tigernut pomace -enriched samples. Appearance scores ranged from 5.90 to 7.03, with tigernut pomace-enriched samples receiving favourable ratings. The enhanced appearance acceptance may be related to the more attractive brown colour and surface characteristics imparted by tigernut pomace.

Overall acceptability scores ranged from 6.27 to 7.30, with significant differences ($p < 0.05$) among samples. The sample containing 88.75% fermented sorghum flour and 11.25% tigernut pomace demonstrated the highest overall acceptability (7.30), indicating that this formulation achieved the optimal balance among all sensory attributes. This finding suggests that moderate tigernut pomace incorporation (approximately 11-12%) enhances sensory appeal without compromising consumer acceptance. The improvement in sensory attributes with tigernut pomace addition can be attributed to the synergistic effects of fermented sorghum flour and tigernut pomace. The intermolecular interactions within the fibre network structure of tigernut pomace may create a more desirable texture and mouthfeel, while the natural compounds in tigernut contribute pleasant taste and aroma characteristics. These results align with previous studies by Omoba et al. (2021) and Oke et al. (2022), who reported improved sensory acceptance of cakes and noodles, respectively, when fortified with tigernut pomace.

Conclusions

The exploration of food by-products for promoting valorization represents a promising approach to enhancing the concept of a circular economy. This study demonstrated that the incorporation of tigernut pomace as a functional ingredient increased oil absorption capacity, redness, springiness, solubility index, and fracturability, while reducing bulk density, water absorption capacity, lightness, hardness, and chewiness. Similarly, sensory evaluation indicated that taste, aroma, texture, and appearance were improved with the addition of tigernut pomace, with the extruded breakfast cereal containing 88.75% FSF and 11.25% tigernut pomace achieving the highest overall acceptability (7.30). The use of tigernut pomace represents a valuable development in the production of weaning foods for children and for individuals suffering from celiac disease. However, further studies are recommended to assess the microbiological profile and storage stability of these products.

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Physicochemical changes in cream cheese induced by glucono-δ-lactone

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Abstract

Introduction. The aim of this study was to evaluate the effectiveness of glucono-δ-lactone as an acidulant in cream cheese production using acid–rennet coagulation.

Materials and methods. Cream mixtures were fermented with mesophilic lactic acid bacteria and coagulated using chymosin (CHY-MAX® M 1000), followed by acidification with glucono-δ-lactone. The structural and mechanical properties of the curds were analyzed using a Kinexus Pro+ rotational rheometer. The degree of structural recovery was calculated, titratable acidity was determined by potentiometric titration, and water activity was measured using a HygroLab 2 analyzer.

Results and discussion. The influence of milk buffer systems on the rate of glucono-δ-lactone hydrolysis was confirmed, highlighting their role in modulating acidification kinetics during cheese production. During the combined fermentation of cream with a mesophilic starter culture and chymosin, glucono-δ-lactone had sufficient time to fully exert its acidifying function, ensuring a gradual and controlled decrease in pH. A fundamental difference in the dynamics of acidity development was observed between cream fermented by lactic acid bacteria and cream acidified by glucono-δ-lactone. The time required for the cream to reach its isoelectric point was reduced by 3–5 hours under the combined action of glucono-δ-lactone, starter culture, and chymosin compared to the control without this acidulant, indicating a more efficient coagulation process. The yield of cream cheese increased with the addition of 0.4–1.0% glucono-δ-lactone to cream, compared to samples obtained by acid–rennet coagulation without this acidulant and by direct acidification. This improvement was associated with enhanced moisture retention within the protein matrix. Moreover, the use of glucono-δ-lactone significantly reduced protein and fat losses while increasing the fat content in total solids, contributing to improved product quality and economic efficiency. The rheological properties of cream cheese produced with 0.4–0.6% glucono-δ-lactone, including apparent viscosity, yield stress, and thixotropic behavior, were closest to those of the control obtained by traditional acid–rennet coagulation, suggesting its suitability for maintaining desirable textural characteristics and consumer acceptability.

Conclusions. Glucono-δ-lactone at a concentration of 0.4–0.6% reduced the time required for enzymatic coagulation of cream, increased cream cheese yield, and improved the viscosity and thixotropic properties of the final product. Therefore, this acidulant can be recommended as an effective processing aid in cream cheese production.

Introduction

Cream cheese is widely appreciated for its desirable sensory properties and versatility in various food applications. It is produced by enzymatic or thermo-acid coagulation of milk–cream mixtures, followed by whey separation and self-pressing of the resulting curd (Chen et al., 2013). However, traditional enzymatic production is time-consuming due to the slow development of acidity during lactic acid fermentation until a pH of 4.6–4.8 is reached, as well as the extended whey separation from the protein-rich curd (Alan, 2021).

To reduce the duration of the cream cheese manufacturing process, the dosage of the starter culture is typically increased, the fermentation and curd processing temperatures are raised, and self-pressing is replaced with the centrifugation or ultrafiltration of the curd (Ong et al., 2018). To improve quality and increase yield, adding a milk-clotting enzyme to standardized mixtures is recommended (Bartoshak and Polishchuk, 2025a).

The coagulation of milk and cream can also be accelerated through full or combined acidification. This results in cream cheeses with a reduced fat content, yet the sensory properties characteristic of full-fat cheeses remains (Feeneyet al., 2021). In the production of soft cheeses and sour milk cheese, milk is acidified using a naturally derived acidulant called glucono-delta-lactone (GDL, E575). This cyclic ester of gluconic acid ($C_6H_{10}O_6$) gradually converts to gluconic acid in water, slowly lowering the pH. In a milk medium, the acidity changes more slowly under the influence of GDL than in water due to the presence of a buffering system consisting of proteins, phosphates, and citrates (Aydogdu et al., 2023). As the acidity of milk approaches its isoelectric point, casein micelles aggregate and coagulate (Sekiguchi et al., 2013). The ability of GDL to control the acidity of milk systems creates conditions that promote uniform protein coagulation (Rajani et al., 2024), resulting in the formation of a cream-like protein gel with a homogeneous structure (Qu et al., 2025; Takeuchi et al., 2008).

GDL is technologically effective in the production not only of traditional dairy products but also of recombinant products such as cream cheese (Bihola et al., 2025) and cream cheese analogues (Kim et al., 2022). A positive effect of GDL on proteins has also been observed in soy milk, where gel formation occurs more rapidly at higher pH values compared to the action of lactic acid bacteria with varying abilities to produce exopolysaccharides.

Although traditional protein gels have higher water-holding capacity, strength, and spreadability compared to GDL-acidified gels, the latter form a denser structure (Pang et al., 2019). Alginate hydrogel formation can also be promoted under competitive conditions between GDL and alginate for Ca(II) ions (Ben Djemaa et al., 2024).

Pre-acidification of cream using GDL to pH 6.0 accelerates lactic acid fermentation by 3–4 h, while visible curd formation occurs already at pH 5.1 (Bartoshak and Polishchuk, 2025b). However, this acidification approach requires further investigation over a wider pH range. Glucono-δ-lactone enhances the milk-clotting activity of lactic acid bacteria, enables controlled acidification of milk–cream mixtures, and promotes uniform protein coagulation, resulting in a smooth curd texture and increased product yield. Therefore, its combined use with coagulants of different origins warrants further investigation.

Materials and methods

Materials

Cream (10% fat) was used for cream cheese production. To investigate glucono-δ-lactone hydrolysis, cow's milk (3.2% fat) was additionally used. Cream fermentation was carried out using a starter culture of mesophilic lactic acid bacteria *Lactococcus lactis* subsp. *lactis*, *Lactococcus lactis* subsp. *cremoris*, and *Lactococcus lactis* subsp. *diacetylactis* (“Iprovit”, Ukraine) and the chymosin preparation CHY-MAX® M 1000 (Chr. Hansen, now Novonesis). The cream and milk were acidified with glucono-delta-lactone produced by “Primehim Ukraine”.

Preparation of test samples

Model samples of cream cheese were obtained by acid-rennet coagulation of cream, which was pasteurized at 86–88 °C, homogenized at 10±1 MPa using a homogenizer Manton Gaulin 15M-8TA, in accordance with the recommendations of Kim et al. (2022), and cooled to 30 °C. Calcium chloride (40 g/100 kg), as well as lyophilized mesophilic starter culture and chymosin in standard amounts recommended by the manufacturers, were added to the prepared cream under constant stirring. After mixing, GDL was added to the cream mixtures in varying amounts ranging from 0.4 to 1.0%. This GDL dosage range is an average value for performing complex acid-rennet coagulation of milk proteins, according to Lucey et al. (2000). Cream fermentation was carried out at 30 °C until the acidity reached no more than pH 4.8. The obtained curd was cut into cubes with an edge length of 1.0–1.5 cm, held for 30 min, and heated to 45–50 °C with gentle stirring to intensify whey separation. The curd grains were then cooled to 18–20 °C, transferred onto four-layer cheesecloth, and subjected to self-pressing until whey separation ceased and a smooth, matte curd surface was formed. Subsequently, the cream curd was wrapped in polymer film, placed in a refrigeration chamber at 4–6 °C, and held under these conditions for at least 24 h.

Experimental design

In the first stage, the effect of glucono-δ-lactone (GDL) on acidity changes in water, milk, and cream was investigated to elucidate its behavior in systems with different chemical compositions over a concentration range of 0.5–2.0%. GDL hydrolysis was carried out for 240 min at 30 °C, with acidity measured at 60 min intervals, in accordance with conditions reported for similar systems (Lucey et al., 2000; Sekiguchi et al., 2023; Takeuchi and Cunha, 2008). The selected temperature (30 °C) is also commonly used in the production of soft cheeses obtained by acid–rennet coagulation (Sinaga et al., 2017), supporting the relevance of this hydrolysis regime. The point at which the increase in acidity began to slow was considered an indirect indicator of the completion of GDL hydrolysis. These results were subsequently used to determine appropriate conditions for GDL application in cream.

In the second stage, two control cream cheese samples were prepared: one obtained by acid–rennet coagulation of cream without GDL addition (control 1), and another produced by direct acidification of cream with 1.0% GDL (control 2). Experimental samples were produced by acid–rennet coagulation of cream acidified with GDL at concentrations of 0.4%, 0.6%, 0.8%, and 1.0%.

The resulting samples were analyzed for moisture, protein, and fat content, including fat in total solids. In addition, cheese yield, structural and mechanical properties, and water activity were evaluated to assess the impact of GDL on product quality.

Methods

The acidity of the cream and curds was determined potentiometrically using a laboratory meter ADWA AD1200 ATC pH/MV/ISE/Temp at 20±0.5 °C.

The water activity (Aw) of cheese samples was measured at 25 °C using a calibrated water activity meter (HygroLab 2, Rotronic, Switzerland) following ISO standard methodology.

The protein content of cheese and whey samples was determined by the Kjeldahl method (AOAC official method), while the fat content was measured using the Gerber acid-butyrometric method (AOAC official method).

The total solids content of cream, whey and cheese samples was determined by the gravimetric method via oven drying at 102–105 °C until constant mass, in accordance with ISO standard methods.

Cheese yield was determined as the ratio of the mass of the finished product to the mass of processed milk (Fox et al., 2017), expressed as a percentage.

The effective viscosity and yield stress of the cream cheese samples were measured using a Kinexus Pro+ rotational rheometer (Malvern Instruments Ltd, United Kingdom) after storage in a refrigerated chamber at 4±2 °C for at least 24 h. For the measurements, a serrated upper geometry (KNX2031) and a serrated lower geometry (KNX0122) were used. The methodology followed that described by Brighenti et al. (2008).

To determine the ultimate shear stress, samples were prepared using a metal corer with an inner diameter of 25 mm. The formed samples were cut into slices from 2 to 2.5 mm thick and 25 mm in diameter, heated to 13 °C, and held for 5 minutes to allow internal stresses to equilibrate. To determine the effective viscosity, the samples were heated to 13 °C, mixed to obtain a representative sample, placed on the lower geometry, excess product was removed, and the samples were held at the specified temperature for 5 min. The effective viscosity was determined by measuring the change in shear rate during forward sweep in the range from 0.1 to 100 s^{-1} and during backward sweep in the range from 100 to 0.1 s^{-1}. The range of measured viscosity under these conditions was between 1 mPa·s and 10,000 Pa·s, which was sufficient for studying the viscosity characteristics of cream cheese as a non-Newtonian structured system.

The degree of structural recovery in the cheese samples was calculated based on the effective viscosity at the end of the measurement at a shear rate gradient $\gamma = 0.1$ s^{-1} (backward sweep), taking as 100% the effective viscosity of the virtually undamaged structure at the start of the measurement $\gamma = 0.1$ s^{-1} (forward sweep) (Liang et al., 2022).

Statistical analysis

The experiments were conducted in three or more replicates, and the results were expressed as the mean±standard deviation. The data were analyzed using one-way analysis of variance (ANOVA) and Tukey's HSD tests with the SPSS statistical software package. A statistically significant difference was determined at $p \leq 0.05$.

Results and discussion

Acid-forming capacity of glucono-δ-lactone in aqueous media with different compositions

In the first stage, the changes in acidity of water, milk (3.2% fat), and cream (10% fat) were monitored over 240 min following the addition of 0.5–2.0% glucono-δ-lactone (GDL) as a direct acidulant. At the start of the experiment, in the absence of GDL, the pH values were approximately 7.0 for water, 6.67–6.68 for milk, and 6.65–6.67 for cream. The effects of increasing GDL concentrations on the active acidity of water, milk, and cream are summarized in Table 1.

Table 1

Changes in pH of water, milk (3.2% fat) and cream (10% fat) during hydrolysis of glucono-δ-lactone

Time (min)	GDL (%)	Water	Milk (3.2% fat)	Cream (10% fat)
60	0.5	5.91±0.20^{a}	6.43±0.24^{a}	6.49±0.17^{a}
	0.75	5.85±0.22^{b}	6.03±0.21^{b}	6.03±0.20^{b}
	1.0	5.68±0.22^{c}	5.93±0.19^{c}	5.93±0.16^{c}
	1.25	5.54±0.21^{d}	5.76±0.19^{d}	5.76±0.19^{d}
	1.5	5.38±0.21^{e}	5.55±0.18^{e}	5.55±0.20^{e}
	1.75	5.28±0.20^{e}	5.43±0.20^{f}	5.43±0.16^{f}
	2.0	5.22±0.19^{e}	5.38±0.18^{f}	5.24±0.15^{g}
120	0.5	5.26±0.21^{a}	5.82±0.20^{a}	5.99±0.20^{a}
	0.75	5.00±0.18^{b}	5.50±0.18^{b}	5.62±0.17^{b}
	1.0	4.81±0.21^{c}	5.35±0.19^{c}	5.40±0.17^{c}
	1.25	4.68±0.21^{d}	5.12±0.16^{d}	5.28±0.18^{c}
	1.5	4.51±0.22^{e}	4.92±0.18^{e}	5.03±0.16^{d}
	1.75	4.25±0.18^{f}	4.74±0.16^{f}	4.81±0.17^{e}
	2.0	3.96±0.17^{g}	4.62±0.17^{g}	4.71±0.18^{f}
180	0.5	4.61±0.18^{a}	5.68±0.18^{a}	5.71±0.19^{a}
	0.75	4.43±0.19^{b}	5.43±0.19^{b}	5.54±0.17^{b}
	1.0	4.11±0.18^{c}	5.21±0.20^{c}	5.29±0.19^{c}
	1.25	4.01±0.19^{d}	5.01±0.21^{d}	5.13±0.19^{d}
	1.5	3.69±0.18^{e}	4.75±0.16^{e}	4.81±0.15^{e}
	1.75	3.55±0.16^{f}	4.65±0.17^{f}	4.70±0.17^{f}
	2.0	3.27±0.15^{g}	4.60±0.15^{f}	4.62±0.16^{f}
240	0.5	4.48±0.20^{a}	5.55±0.19^{a}	5.63±0.17^{a}
	0.75	4.22±0.17^{b}	5.38±0.17^{b}	5.48±0.18^{b}
	1.0	3.84±0.17^{c}	5.13±0.15^{c}	5.27±0.18^{c}
	1.25	3.65±0.16^{d}	4.96±0.16^{d}	5.11±0.18^{d}
	1.5	3.33±0.15^{e}	4.65±0.16^{e}	4.78±0.16^{e}
	1.75	3.18±0.15^{f}	4.60±0.17^{e}	4.69±0.15^{f}
	2.0	3.01±0.16^{g}	4.55±0.16^{e}	4.60±0.16^{f}

Note: Data are presented as mean±standard deviation (n = 3). Different superscript letters within the same time interval indicate significant differences ($p < 0.05$), according to analysis of variance (ANOVA) followed by Tukey's HSD post hoc test; GDL - glucono-δ-lactone.

The most significant alterations in acidity were observed in aqueous solutions of GDL. Specifically, the pH of water reached 4.68 after 3 h at 1.25% GDL and decreased to a minimum of 3.01 after 4 h at 2% GDL. The buffer system of milk (3.2% fat) slowed the change in acidity slightly. A GDL concentration of 0.5–1.0% did not result in complete milk coagulation within 4 h. Only at concentrations of 1.75–2.0% the acidity of milk reached the isoelectric point. The curd formed by acid coagulation was relatively dense and rich in fat. The acidity of cream decreased more slowly than that of milk, resulting in the formation of a delicate, creamy curd.

The compositional and functional differences between cream and milk are mainly attributed to the higher fat content in cream, which decreases the aqueous phase fraction while increasing the concentration of buffering components such as proteins, phosphates, and citrates (Salaün et al., 2005). Accordingly, at GDL concentrations of 0.5–1.25%, the pH did not decrease below 5.0 even after 240 min. Increasing the GDL concentration to 1.5–2.0% resulted in pH values below 5.0 after 180 min. In cream containing 1.5% GDL, the onset of structure formation was observed after 120 min at pH ~5.0, while a cream-like curd formed after 180 min at pH 4.79. At a GDL concentration of 2.0%, corresponding to pH 4.6–4.7, a dense protein curd formed with pronounced syneresis.

For all studied systems, a slight decrease in the rate of pH decline and subsequent stabilization of pH values were observed after a certain holding time, namely 90 min for water and 120–180 min for milk and cream. This indicates that the rate of GDL hydrolysis depends on both its concentration and the chemical composition of the medium.

The results indicate that during the complex fermentation of cream using mesophilic starter culture and chymosin, GDL can effectively fulfil its role as an acidulant. The GDL dosage should be selected considering the increased buffering capacity of the cream.

Effect of glucono-delta-lactone on pH during acid–rennet coagulation of cream

To carry out complex acid–rennet coagulation of milk proteins (starter culture + rennet + GDL) under the combined influence of multiple factors on acidity development, the GDL dosage was adjusted to 0.4–1.0%, in accordance with the recommendations of Lucey et al. (2000).

The resulting changes in cream acidity during fermentation at different GDL concentrations are presented in Figure 1.

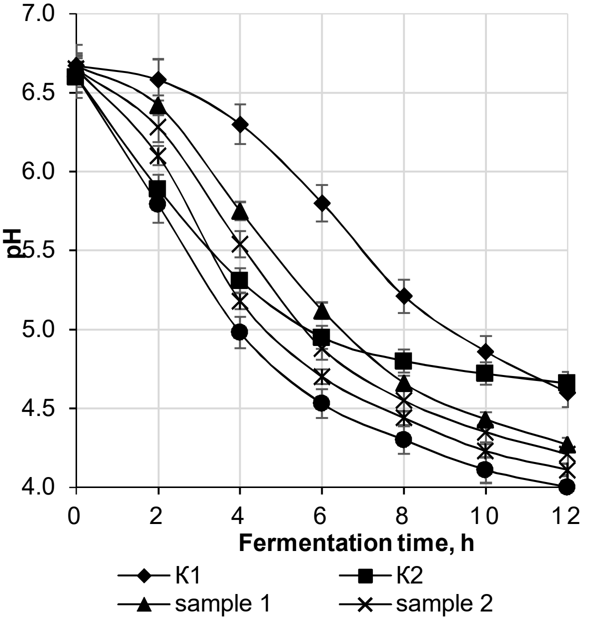

Figure 1. Changes in acidity during acid-rennet coagulation of cream with different glucono-delta-lactone contents

Figure 1 illustrates a fundamental difference in the nature of the acidity changes in the control samples. Specifically, control 1 demonstrated the typical change in the acidity of a milk medium under the action of lactic acid bacteria, with a clearly defined lag phase. The change in cream acidity took a relatively long time as the isoelectric point was reached only after 10–12 h of fermentation. In contrast, GDL acidified the cream more rapidly due to its conversion to gluconic acid, especially during the first 6 h, with a subsequent slowing of this process. By the 12 h of the study, the acidity of both control samples reached nearly the same level (control 1 – pH = 4.66; control 2 – pH = 4.60). However, the curd obtained by direct acidification of cream had a denser consistency and was less greasy than the curd obtained by traditional acid-rennet coagulation of cream.

Samples 1-4, produced by combined cream coagulation using a starter culture, enzyme, and 0.4–1.0% GDL, exhibited intermediate pH values between control 1 and control 2, depending on the GDL dose. The time required to reach the isoelectric point was significantly reduced compared to control 1, amounting to 5–7 h. Thus, the use of GDL to accelerate cream protein coagulation represents a technologically justified approach.

Effect of glucono-delta-lactone on the physicochemical properties of cream cheese

Reducing the duration of the production process is not the sole criterion for selecting the optimal GDL dose, as the physicochemical characteristics and yield of the final product are also critical in determining the conditions for combined coagulation of cream.

The chemical composition of cream cheese samples prepared with different GDL concentrations is presented in Figure 2.

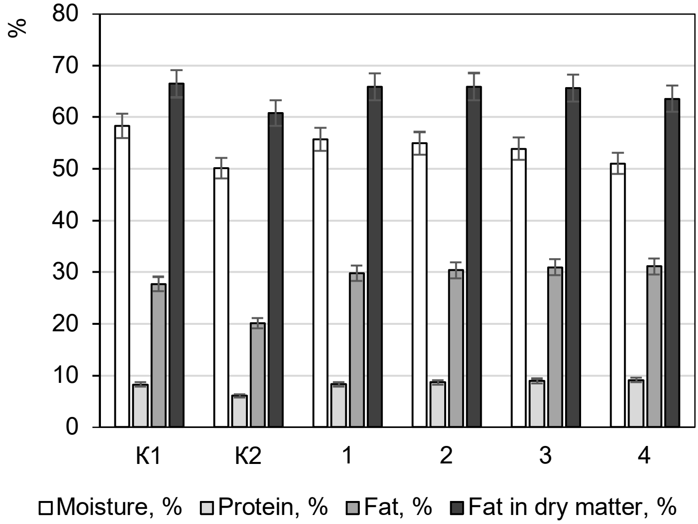

Figure 2. Chemical composition of cream cheese samples obtained with varying glucono-delta-lactone content

The use of GDL alone did not allow the production of cream cheese with a high total solids content, including fat in total solids (control 2). In this sample, the acidity of the cream increased slowly, leading to the formation of a protein curd with lower water- and protein-holding capacity. Consequently, part of the solids, particularly proteins, remained in the whey (Kim et al., 2022).

In contrast, the combination of acid and enzymatic coagulation provided a greater technological effect, and despite a significant reduction in coagulation time, the chemical composition of samples 1-4 was closer to that of control 1. In samples 1-4, a slight decrease in moisture content was observed with increasing GDL dosage, which can be explained by enhanced syneresis of the protein curd due to the formation of a combined gel structure with large pores (>20 µm) (Lucey et al., 1998).

Sample 4, obtained with 1.0% GDL, exhibited a deviation from the trend observed for samples 1-3 (0.4–0.8% GDL). The protein content in soft cream cheese typically reaches 8.0–8.5%. However, due to reduced moisture and intensified syneresis, the protein content in sample 4 increased to 9.11%, indicating densification of the protein matrix. Although sample 4 showed the highest fat content (31.11%), its fat content in total solids was relatively low (63.54%). This can be explained by the elevated total solids content (48.96%), where not only fat but also other components, primarily protein, increased.

The yield of the finished product depends not only on the total solids content but also on the curd's ability to retain fat and moisture (Figure 3).

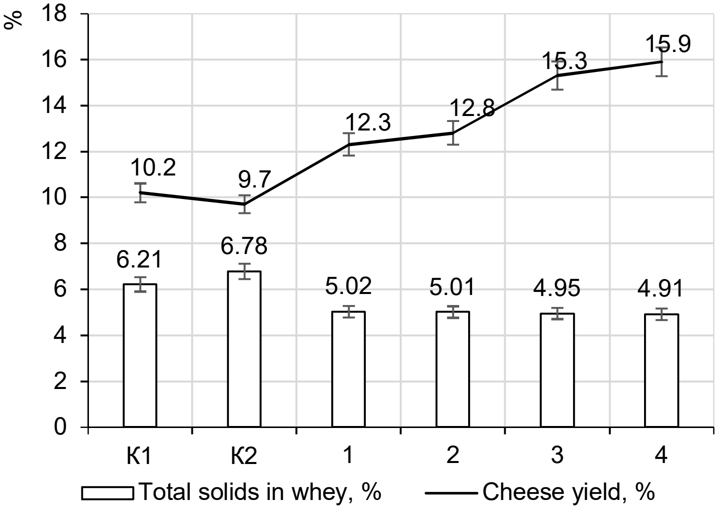

Figure 3. Effect of glucono-δ-lactone concentration on cream cheese yield and total solids content in whey

The use of GDL alone (control 2) resulted in the highest protein losses in the whey and the lowest product yield. Acid coagulation also led to a lower total solids content in the curd. In contrast, the combined use of GDL, starter culture, and rennet provided the greatest technological effect. At the onset of coagulation, chymosin initiated primary aggregation of casein micelles, while subsequent acidification due to lactic acid fermentation and GDL lowered the pH, leading to the formation of a denser protein network (Siqi et al., 2023; Zhao et al., 2028).

The application of a starter culture ensured gradual and controlled pH reduction, while lactic acid bacteria resulted in the production of exopolysaccharides that improved curd texture and water-holding capacity. Simultaneously, the milk-clotting enzyme hydrolyzed casein, reinforcing the protein matrix and enhancing the retention of water and fat, thereby reducing whey losses. Overall, the synergistic effect of GDL, the starter culture, and the enzyme were observed during cream coagulation.

Viscosity characteristics and water activity of cream cheese

According to Ningtyas et al. (2017), cream cheese is classified as a viscoelastic system and a non-Newtonian fluid with a yield stress. The results of the yield shear stress and effective viscosity measurements of cream cheese samples (i.e. the viscosity of the practically undamaged structure during forward sweep at a shear rate $\gamma = 0.1\ s^{-1}$ and during backward sweep at the end of the measurement at a shear rate $\gamma = 0.1\ s^{-1}$) are presented in Table 2. Additionally, the degree of structural recovery, which characterizes the thixotropic ability of the samples, was calculated.

Table 2

Rheological and mechanical properties of cream cheese samples

Samples	Effective viscosity, η, Pa·s·10^3		Yield stress, Pa	Recovery degree, %*
	$\gamma = 0.1\ s^{-1}$ (forward sweep)	$\gamma = 0.1\ s^{-1}$ (backward sweep)		
Control 1	2050.21± 99.04^{a}	454.51±20.74^{a}	518.3±21,37^{a}	22.17
Control 2	4310.02±174.92^{b}	641.30±23.19^{b}	1654.0±74.39^{b}	14.88
1	2260.05± 83.71^{c}	559.20±20.68^{c}	529.3±19.62^{c}	24.74
2	2370.10± 88.89^{c}	566.10±19.59^{c}	587.2±22.04^{d}	23.89
3	2457.32±101.68^{c}	554.41±20.62^{c}	842.9±33.30^{e}	22.56
4	2910.41±103.63^{d}	573.15±22.29^{c}	1482.2±68.14^{f}	19.69

*The degree of recovery was calculated using the arithmetic mean values of the effective viscosity

As shown in Table 2, viscosity (η) decreased with increasing shear rate ($\dot{\gamma}$) because of protein network disruption and alignment of structural elements in the direction of flow. Structural and mechanical properties differed significantly among samples produced with different combinations of milk-clotting agents. The highest shear stress and apparent viscosity were observed in cheese obtained by direct cream coagulation (control 2), indicating a denser structure. Based on this, the samples were ranked as follows (descending values): control 2 → sample 4 → sample 3 → sample 2 → sample 1 → control 1.

The combination of enzymatic and acid coagulation in samples 1–4, obtained using 0.4–1.0% GDL, formed a specific casein gel structure with rheological properties different from those observed in purely enzymatic or acid coagulation, which is consistent with the findings of Lucey et al. (2000). Samples 1–2, obtained using 0.4% and 0.6% GDL, were the closest to traditional cream cheese (control 1) in terms of structural-mechanical characteristics and thixotropic ability.

According to recent studies, the shear stress limit of cream cheese and model acidic gels is determined within a stress range of 1–1,000 Pa, with structural failure occurring at stresses on the order of hundreds of Pascals (Gutiérrez-Méndez et al., 2019). The shear stress of samples 1–2, obtained using 0.4% and 0.6% GDL (529.3–587.2 Pa), was characteristic of systems with good spreadability while retaining the ability to hold their shape. The yield stress of control sample 2 and samples 3 and 4 with 0.8% and 10% GDL content indicates a harder but somewhat brittle structure, consistent with the data (Kim et al., 2022).

Water activity (a_n) in the cream cheese samples showed no significant differences. The highest value ($a_n = 0.922$) was observed in control 2, and the lowest ($a_n = 0.908$) in sample 4. Thus, the coagulation method had no appreciable effect on this parameter. All samples were classified as high-water activity products.

The results of this study confirmed the suitability of using 0.4–0.6% GDL in cream cheese production, as it shortened the production cycle, increased product yield, and enabled the achievement of physicochemical and structural–mechanical properties characteristic of this product.

Conclusions

The rapid hydrolysis of glucono-δ-lactone (GDL) enables effective utilization of its acid-forming capacity during complex cream fermentation for cream cheese production. The dynamics of acidity changes in cream are strongly influenced by the type and combination of protein coagulants. The addition of 0.4–1.0% GDL accelerates the decrease in pH to the isoelectric point by 3–5 hours in cream cheese produced via the acid–rennet method.

When used alone as an acidulant, GDL leads to the greatest protein loss with the whey and the lowest cream cheese yield. In contrast, the combined application of GDL, starter culture, and rennet produces the most favorable technological outcomes. The shear stress and effective viscosity of cream cheese obtained solely by GDL acidification were the highest, reflecting a denser structure, whereas samples exhibiting structural–mechanical properties and thixotropic behavior characteristic of traditional cream cheese were achieved only when GDL was combined with a starter culture and chymosin.

These findings provide a basis for optimizing the cream cheese production process, increasing yield while maintaining its characteristic quality attributes. Future studies will focus on the microbiological and physicochemical changes during storage of cream cheese produced using combined coagulation.

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Fat phase composition and oxidative stability of protein–fat emulsions

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Abstract

Introduction. Protein-fat emulsions are widely used as delivery systems for biologically active fat-soluble compounds; however, their stability is limited by oxidative degradation of the lipid phase, the rate of which is determined by the fatty acid composition of the fat and the effectiveness of antioxidant protection.

Materials and Methods. The stability of the fat phase of protein-fat emulsions differing in their content of saturated, monounsaturated, and polyunsaturated fatty acids was evaluated. Resistance to oxidative degradation was assessed by monitoring changes in peroxide value during storage. Fatty acid composition was determined by gas chromatography of fatty acid methyl esters. Epigallocatechin gallate was used as an antioxidant at a concentration of 200 mg/kg of emulsion.

Results and discussion. Replacing sunflower oil with a blended oil (coconut, sesame, wheat germ, and others) in the protein-fat emulsion increased the induction period to 9 h or longer. This observation is consistent with reported data on the positive effect of a balanced fatty acid composition on the oxidative stability of lipids. Emulsions with a high content of polyunsaturated fatty acids (PUFA > 40% of total fatty acids) exhibited a significantly higher rate of lipid peroxidation compared with samples rich in monounsaturated fatty acids. During storage, control samples without antioxidants showed an increase in peroxide value of 30–60%, depending on the fatty acid profile of the lipid phase.

In samples with a high content of polyunsaturated fatty acids, the peroxide value increased 1.5–2.0 times faster compared with emulsions in which the proportion of monounsaturated fatty acids exceeded 45%. This confirms the higher susceptibility of PUFA-rich lipid systems to oxidative degradation due to the presence of multiple double bonds and a greater number of oxidation-prone sites.

The use of epigallocatechin gallate as a natural antioxidant significantly influenced the course of oxidative processes. The addition of 200 mg/kg of this compound reduced the peroxide value by an average of 45% compared with control samples. The antioxidant effect was maintained throughout the entire storage period, indicating its effective involvement in inhibiting the primary stages of lipid peroxidation. Furthermore, the stabilization effect was more pronounced in emulsions containing higher proportions of unsaturated fatty acids. Overall, these results demonstrate the potential of epigallocatechin gallate for improving the oxidative stability and extending the shelf life of protein-fat emulsions with an optimized fatty acid composition.

Conclusions. Optimization of the fatty acid composition of the lipid phase, in combination with the use of natural antioxidants, is an effective approach to enhancing the oxidative stability of protein-fat emulsions.

Introduction

Protein-fat emulsions (PFE) have recently attracted considerable attention as versatile systems for the encapsulation of fat-soluble components in food, cosmetic, and pharmaceutical products (Ghelichi et al., 2023; McClements and Decker, 2000; Svishchova et al., 2025; Vorontsov et al., 2025). This interest is primarily due to their ability to provide a stable dispersion of the lipid phase, protect oxidation- or degradation-sensitive fat-soluble compounds (e.g., polyunsaturated fatty acids, vitamins, antioxidants), and enable controlled release under specific conditions or at targeted sites.

Despite the widespread use of emulsions, maintaining their stability remains an unresolved challengeIn particular, Aslam et al. (2023) reported a relatively short shelf life at moderate temperatures, whereas commercial products require long-term stability even under temperature fluctuations, pH variations, and in the presence of salts and other components.

The lipid phase in both types of emulsions serves as the main site for the accumulation of unsaturated fatty acids and fat-soluble bioactive compounds, which are highly susceptible to radical-chain oxidation reactions. Studies by Wannasin et al. (2022) have shown that deterioration of sensory properties and nutritional value (bitterness, off-flavors, loss of nutritional quality) is associated with lipid peroxidation and the accumulation of primary and secondary oxidation products. Therefore, protecting the lipid phase from oxidation is a key requirement for maintaining the quality of the final product.

According to Johnson et al. (2015), the oxidative stability of the lipid phase in emulsions is closely related to its fatty acid composition, particularly to the degree of unsaturation of the fats. In emulsified systems, the effect of fatty acid composition on oxidative stability is amplified due to the high specific interfacial area. Aslam et al. (2023) demonstrated that polyunsaturated fatty acids (PUFAs) located in the interfacial layers of lipid droplets more readily interact with pro-oxidants in the aqueous phase, including transition metal ions and reactive oxygen species. Thus, an increased degree of unsaturation in the lipid phase not only enhances its chemical reactivity but also promotes the concentration of oxidative processes at the interface, which represents a critical zone for emulsion stability.

Oxidative degradation of polyunsaturated fatty acids leads to the formation of polar low-molecular-weight products, which can alter the rheological properties of lipid droplets and the interfacial tension (Shizhang et al., 2022). Furthermore, secondary oxidation products, in particular α, β-unsaturated aldehydes, can engage in covalent and non-covalent interactions with protein emulsifiers at the interface, causing structural modifications and reducing their stabilizing capacity (Genot et al., 2013). As a result, lipid oxidation, determined by the fatty acid composition of the lipid phase, indirectly promotes physical destabilization of the emulsion by weakening the interfacial film, leading to an increased tendency toward flocculation, coalescence, and phase separation.

At the same time, the use of lipids predominated by monounsaturated fatty acids (MUFAs) or saturated fatty acids (SFAs), such as oleic or stearic acid, results in emulsions with enhanced oxidative stability (Wei et al., 2025). These systems are characterized by a lower rate of lipid radical formation and fewer reactive oxidation products, which positively affects the integrity of the interfacial layer and the long-term physical stability of the emulsion. Therefore, the fatty acid profile of the lipid phase is one of the key factors that must be considered when designing stable emulsion systems with predictable shelf life (Gubsky et al., 2025).

Thus, current scientific evidence provides a basis for the further development of protein-fat emulsions as effective systems for the encapsulation, delivery, and stabilization of fat-soluble components. At the same time, the practical implementation of such systems in food, cosmetic, and pharmaceutical applications requires further research focused on long-term stability, adaptation to various storage conditions, and scalability of the technologies.

The aim of this study was to investigate the effect of the fatty acid composition of the lipid phase on the oxidative stability of protein-fat emulsions and to evaluate the effectiveness of the natural antioxidant epigallocatechin gallate in slowing lipid peroxidation and enhancing the stability of emulsion systems.

Materials and Methods

Materials

The vegetable oils were purchased from retail outlets in Ukraine. Specifically, the following oils were used: amaranth oil (TM Oberig, Ukraine), grape seed oil (TM Beurre, UK), walnut oil (TM MEH, Ukraine), wheat germ oil (TM Oliynitsya, Ukraine), coconut oil (TM Coco Organic, Sri Lanka), hemp oil (TM Craft Oil, Ukraine), corn oil (TM Kama, Ukraine), sesame oil (TM Korysna Oliya, Ukraine), flaxseed oil (TM Golden Kings of Ukraine, Ukraine), sea buckthorn oil (TM Adverso, Ukraine), olive oil (TM Bertolli, Spain), palm oil (TM Musim Mas, Malaysia), rapeseed oil (TM Korolivskyi Smak, Ukraine), soybean oil (TM Golden Kings of Ukraine, Ukraine), high-oleic sunflower oil (TM Stozhar, Ukraine), and linoleic sunflower oil (TM Stozhar, Ukraine).

To slow the deterioration of the lipid phase in the emulsion, 0.02% epigallocatechin gallate (ItalFresh) was added as an antioxidant (Capasso et al., 2025).

A 14% aqueous collagen solution, prepared by enzymatic hydrolysis according to Topchii et al. (2023), contained 8.26% glycine, 6.96% proline, and 16.99% glutamic acid. Due to the presence of peptide fragments with both hydrophilic and hydrophobic regions, the hydrolyzed collagen exhibits amphiphilic properties, enabling it to adsorb at the lipid–water interface and form stable protein-fat emulsions.

Preparation of the protein-fat emulsion (PFE)

The protein-fat emulsion (PFE) was prepared using a ZUVER Mega Grinder 12 cutter (Germany) from a mixture of 37.5% ice-cold water, 37.5% hydrolyzed collagen, and 25% vegetable oil. First, ice-cold water and hydrolyzed collagen were combined and mixed for 2 minutes to ensure proper hydration. Vegetable oil was then gradually added, and the protein-fat complex was formed by high-speed mixing for 5 minutes. The resulting emulsion was subsequently cooled to 0–4 °C, yielding a stable protein-fat emulsion suitable for further analysis or storage.

Determination of the fatty acid composition of oils. The fatty acid composition of the lipid phase was determined by gas chromatography after prior derivatization of the fatty acids into their corresponding methyl esters, as recommended by Masood et al. (2005) and Ostermann et al. (2014). Methylation was carried out by treating the fat sample with a solution of sodium methoxide and heating until complete conversion of both free and esterified fatty acids into methyl esters. Upon completion of the reaction, the methyl esters were extracted with hexane and separated from the aqueous phase.

The obtained extract was analyzed using a Hewlett-Packard HP6890 gas chromatograph (USA) equipped with a flame ionization detector and a capillary SP-2380 column, specialized for fatty acid separation. The analysis was performed with an injector temperature of 280 °C and a detector temperature of 290 °C, which ensured effective separation of fatty acids with varying chain lengths and degrees of unsaturation.

Individual fatty acids were identified by comparing the retention times of peaks in the samples with those of a standard mixture of fatty acid methyl esters. Quantitative analysis was performed using the peak area normalization method, expressing the content of each fatty acid as a percentage of the total identified fatty acids.

Determination of the peroxide value of the protein-fat emulsion. The peroxide value of the protein-fat emulsion was determined according to ISO 3960:2017. The lipid phase of the emulsion was first isolated by centrifugation, and 1.0–5.0 g of fat (depending on the expected degree of oxidation) was accurately weighed (±0.001 g) into a conical flask with a ground-glass stopper. A mixture of iso-octane and acetic acid (2:3, v/v) was added to dissolve the fat completely. An excess of saturated potassium iodide solution was then added, and the mixture was thoroughly stirred and kept in the dark for 1 minute to prevent photochemical decomposition of iodine.

After the reaction, distilled water was added, and the liberated iodine was titrated with a 0.01 N sodium thiosulfate standard solution until the solution became colorless. Freshly prepared starch solution was used as an indicator at the end of the titration, and the titration was continued until the complete disappearance of the blue color. A blank test was performed in parallel following the same procedure without fat. The peroxide value was calculated using the following formula:

$$\mathrm{PV} = \frac{(\mathrm{V} - \mathrm{V_0}) \cdot \mathrm{c} \cdot 1000}{\mathrm{m}}, \qquad (1)$$

where V is the volume of sodium thiosulfate solution used for titration of the sample, mL; V_0 is the volume of sodium thiosulfate solution used in the blank test, mL; c is the molar concentration of the sodium thiosulfate solution, mol/L; and m is the mass of the fat sample, g.

Accelerated stability test of the protein-fat emulsion

The kinetics of fat oxidation were studied using an accelerated Oxitest method on an Oxitest analyzer (Velp Scientifica, Italy). This method is based on passing air at a constant flow rate through a fat layer at an elevated constant temperature and measuring the degree of fat oxidation at specific time intervals. The instrument allows for the evaluation of fat stability in both solid and liquid products without prior fat extraction. The accelerated process employs elevated temperature and pressure within the reactor chambers, and the induction period of oxidation is calculated automatically. The Oxitest operates by monitoring changes in absolute pressure, which occur due to oxygen consumption during oxidation. The reaction is carried out at 90 °C and 6 atm in an atmosphere of pure oxygen.

The oxidation parameters of the protein-fat emulsion were evaluated using the following indicators.

Change in peroxide value (Δ PV):

$$\Delta \mathrm{PV} = \mathrm{PV_{end}} - \mathrm{PV_0}, \qquad (2)$$

where PV_0 and PV_{end} are the peroxide values at the beginning and at the end of the study, respectively, expressed in mmol $^1/_2O_2$/kg.

Oxidation rate during the storage period (V_{avg}):

$$V_{avg} = (PV_{end} - PV_0)/t, \qquad (3)$$

where t is the duration of the experiment, in hours.

All measurements were performed in triplicate, and the results are presented as mean values.

Results and discussion

The characteristics of the vegetable oils used in this study are presented in Table 1. The iodine value reflects the degree of unsaturation of fatty acids in the oils and has a significant effect on their oxidative stability. The saponification value indicates the average molecular weight of the fatty acids constituting the triglycerides.

Table 1

Physicochemical characteristics of the vegetable oils studied

Name of oil	Iodine value, g I_2/100 g	Saponification value, mg KOH/g
Coconut	6–12	248–265
Walnut	140–160	188–195
Hemp seed	145–165	188–195
Sesame	104–120	186–195
Corn	105–130	187–195
Flaxseed	170–200	185–195
Wheat germ	115–135	185–195
Amaranth	95–110	185–195
Sea buckthorn	120–150	185–195
High-linoleic sunflower	110–145	188–194
Soybean oil	120–140	189–195
Grape seed	125–150	188–195
Rapeseed	95–120	170–185
High-oleic sunflower	80–95	188–194
Olive	75–94	184–196
Palm	50–55	190–205

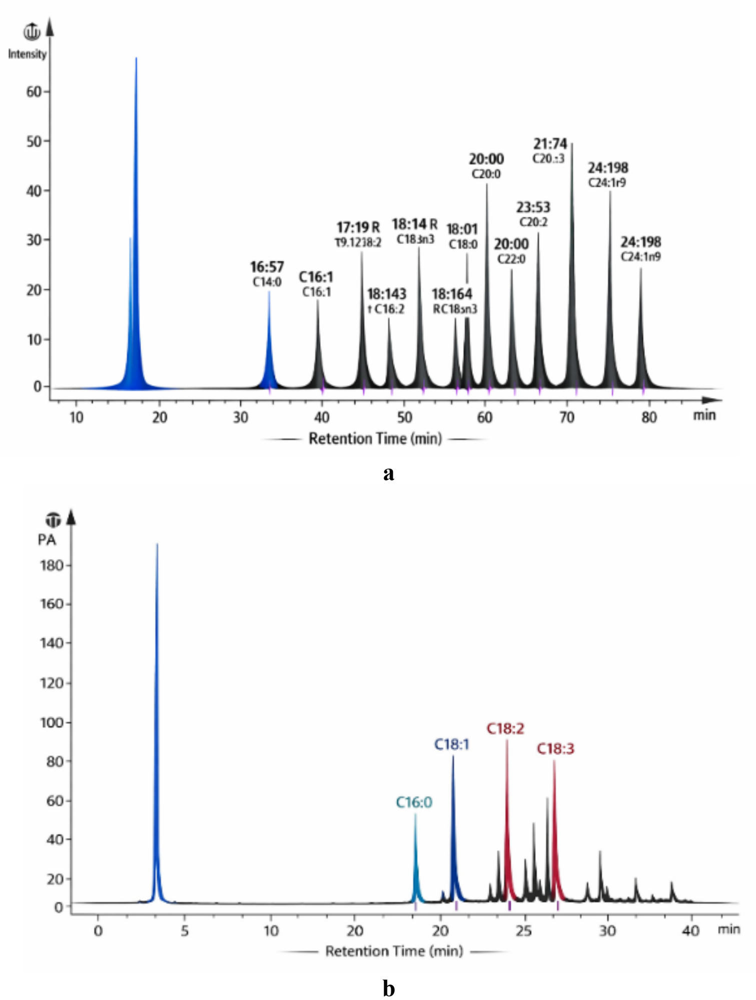
Systematic evaluations (Culler et al., 2021) indicate that diluting complex mixtures of polyunsaturated fatty acids with less unsaturated triglycerides or incorporating oils rich in monounsaturated fatty acids can significantly slow the formation of oxidation products in emulsions. This demonstrates the possibility of formulating the fat phase with a targeted fatty acid profile to enhance its oxidative stability.

The fatty acid profile of the lipid phase is one of the key factors determining the oxidative stability of emulsions (Yun et al., 2012). A high content of polyunsaturated fatty acids (PUFAs) negatively affects emulsion stability due to their high reactivity toward peroxidation, whereas a greater proportion of monounsaturated and saturated fatty acids enhances resistance to oxidation. Accordingly, it is advisable to investigate the possibility of balancing the lipid component in innovative multi-component meat products.

Fatty acid composition of vegetable oils

The biologically active components of vegetable oils are primarily unsaturated fatty acids of the ω-3, ω-6, and ω-9 families, which exhibit pronounced physiological effects (Stabnikova and Paredes-López, 2024; Yaragalla et al., 2025). Unlike animal fats, vegetable oils are a source of physiologically active fatty acids that participate in the construction of cellular membranes. Based on current understanding of the physiological roles of these acids, a distinct branch of nutritional science has emerged, leading to the recognition of the need to regulate and ensure their continuous dietary intake.

Studying the fatty acid composition of vegetable oils used in the domestic food processing industry allows for predicting their potential for further applications. Table 2 presents the fatty acid content of 16 of the most commonly available natural vegetable oils in Ukraine, obtained from the retail market. Chromatograms of the fatty acid composition of two of the investigated oils are shown in Figure 1 as an illustration of the applied method.



a

b

Figure 1. Chromatograms of the fatty acid composition of sea buckthorn and rapeseed oils: sea buckthorn oil (a); rapeseed oil (b)

Table 2

Fatty acid composition of vegetable oils

Name of oil	Content of fatty acid groups, %			Content of individual fatty acids, %		
	SFA	MUFA	PUFA	Oleic ω-9	Linoleic ω-6	Linolenic ω-3
WHO recommendations	33.3	33.3	33.3	-	-	-
Coconut	94.2	4.1	1.7	2.65	0.53	-
Walnut	9.1	18.3	72.6	-	59.36	9.60
Hemp seed	11.9	17.6	70.5	13.83	54.04	15.32
Sesame	16.8	41.2	42.0	38.00	40.72	0.36
Corn	12.4	29.6	58.0	27.77	56.99	0.12
Flaxseed	12.8	16.7	70.5	15.02	12.15	55.53
Wheat germ	20.9	18.7	60.4	17.87	55.03	4.70
Amaranth	19.8	28.9	51.3	23.97	50.34	0.93
Sea buckthorn	31.0	45.6	23.4	5.82	16.83	4.95
High-linoleic sunflower	12.9	27.8	59.3	24.61	58.59	0.10
Soybean	17.2	23.5	59.3	21.34	53.67	5.73
Grape seed	13.1	22.4	64.5	19.60	-	0.45
Rapeseed	8.2	62.4	29.4	59.04	18.67	9.14
High-oleic sunflower	9.20	83.90	6.90	73.74	4.22	1.18
Olive	16.9	71.5	11.6	71.09	7.13	0.60
Palm	66.2	28.1	5.7	25.31	4.78	-

Note: SFA – saturated fatty acids; MUFA – monounsaturated fatty acids; PUFA – polyunsaturated fatty acids; WHO – World Health Organization

It should be noted that the composition of individual oils is unbalanced, as evidenced by the results presented in Table 1. Liquid vegetable oils predominantly contain unsaturated fatty acids, which account for 80–90% of their total fatty acid content. Vegetable oils that are solid at room temperature, namely coconut and palm oils among those studied, are primarily composed of saturated fatty acids. The most balanced oils in terms of composition are wheat germ oil, olive oil, and rapeseed oil. However, the fatty acid profile of none of the individual oils meets the current composition standards, which are provided in the first row of the table and are thoroughly discussed in the following section of the article.

Polyunsaturated fatty acids (PUFAs), which contain two or more double bonds, are characterized by significantly lower allylic C–H bond dissociation energies, making them particularly susceptible to the initiation of radical chain oxidation reactions (Gardner, 1989). As a result, under otherwise equal conditions, emulsions in which the lipid phase is formed from oils with high contents of linoleic, linolenic, or docosahexaenoic acids exhibit a substantially higher rate of accumulation of primary and secondary oxidation products compared with systems based on monounsaturated or saturated fatty acids.

Fatty acid composition of the lipid phase of emulsions

According to current dietary fat guidelines (Schwingshackl et al., 2021), total fat intake should account for 20–35% of daily energy consumption, with consideration of the balance between saturated fatty acids (SFA), monounsaturated fatty acids (MUFA), and polyunsaturated fatty acids (PUFA). The recommended ranges for each class of fatty acids (as % of energy) are as follows: PUFA: 6–11%; MUFA: 10–25%; SFA: ≤7–11%.

According to Hart et al. (2025), a dietary ratio of the aforementioned fatty acid classes of approximately 1.0–1.3 leads to a reduction in LDL cholesterol levels compared with diets with a lower ratio. The World Health Organization (WHO) recommendations on dietary fats emphasize the importance of replacing saturated fatty acids with unsaturated ones to reduce the risk of cardiovascular diseases. As noted by Aranceta et al. (2012), it is important to ensure adequate intake of ω-3 fatty acids alongside a moderate consumption of ω-6 fatty acids, since their effects are always antagonistic to each other.

Considering the recommendations outlined above, the following balanced fatty acid ratio was adopted for the development of food products, representing a physiologically complete fat (g/100 g of total fatty acids): SFA : PUFA ω-6 : PUFA ω-3 : MUFA = 33.5 : 30.0 : 3.0 : 33.5.

Using a calculated approximation method, the composition of three-component vegetable oil blends was determined, with a fatty acid profile corresponding to the established criteria (Table 3).

Table 3

Fatty acid composition of three-component vegetable oil blends

Oil compositions (1:1:1)	Content of fatty acid groups, %			Fatty acid composition ratio		
	MUFA	PUFA	SFA	MUFA: PUFA: SFA	ω-6: ω-9	ω-3 : ω-6
Current composition standards	33.3	33.3	33.3	1:1:1	1:1.8	1:10
Palm oil – amaranth oil – grape seed oil	40.5	26.5	33.0	0.8:0.7:1	1:0.6	1:40
Coconut oil – olive oil – hemp seed oil	31.1	27.9	41.0	1.5:1.1:1	1:1.6	1:3
Coconut oil – sesame oil – wheat germ oil	21.3	34.7	44.0	1.3:0.6:1	1:1.9	1:11

Note: SFA – saturated fatty acids; MUFA – monounsaturated fatty acids; PUFA – polyunsaturated fatty acids.

Table 3 presents a list of oil blends whose calculated composition is close to the recommended standards. The following oil blends are characterized by a fatty acid profileof the main groups approaching the optimal composition (Figure 2).


a b c

Figure 2. Oil mixtures with compositions close to the recommended fatty acid profile: palm oil – amaranth oil – grape seed oil (a); coconut oil – olive oil – hemp seed oil (b); coconut oil – sesame oil – wheat germ oil (c)

It has been shown that the composition of coconut, sesame, and wheat germ oils is characterized by the most balanced fatty acid profile. The ratio of α-linolenic to linoleic acids in this mixture is 1:11 compared to the ideal 1:10, while the ratio of linoleic to oleic acids is 1:1.9 compared to the ideal 1:1.8, indicating its high biological effectiveness. The composition of the other calculated mixtures is inferior to this blend, particularly in terms of biological efficiency. For example, the palm–amaranth–grape seed oil mixture is reasonably balanced regarding the major fatty acid ratios; however, the α-linolenic to linoleic ratio of 1:40 significantly exceeds the recommended level. The coconut–olive–hemp mixture has a linoleic to oleic acid ratio of 1:1.6, which is closer to the recommended value, but it contains insufficient α-linolenic acid. Monitoring the ratios of fatty acids is a necessary condition for simultaneously ensuring the stability, quality, and functional value of protein-fat emulsions.

In the study by Mank and Polonska (2016), a chromatographic analysis of the fatty acid composition of 23 vegetable oils used as components of the fat phase in cosmetic products was carried out. The authors grouped the fatty acids into classes: saturated fatty acids, monounsaturated fatty acids, and polyunsaturated fatty acids. Based on this analysis, they proposed several optimized blends that correspond to the physiological parameters of healthy skin. Among these, the most optimal in terms of monounsaturated and polyunsaturated fatty acid content is the composition containing coconut, sesame, and wheat germ oils. This aligns well with our findings: a three-component mixture of coconut, sesame, and wheat germ oils in a 1:1:1 ratio demonstrates a favorable ratio of linoleic acid (C18:2) to oleic acid (C18:1). It can be concluded that blending different types of vegetable oils allows achieving a favorable fatty acid profile, which is also supported by the results of Mank and Polonska (2016).

Oxidative stability of the lipid phase in emulsions

Oxidative stability is an important indicator of the quality and shelf life of any fat-containing product, as oxidation leads to the formation of low-molecular-weight degradation products with unpleasant odor and taste, rendering the product partially or completely unsuitable for consumption or industrial use. During oxidation, unsaturated fatty acids within triacylglycerol structures are also degraded, leading to the formation of toxic compounds and oxidized polymers. This is important for assessing the sensory and nutritional properties, as well as the potential toxicity, of food products.

One of the simple and effective methods for inhibiting oxidative deterioration of fats is the addition of substances that slow down this process. Such additives include oxidation inhibitors or antioxidants, whose activity has been studied (Tang et al., 2025) and is manifested by an extension of the initial slow phase of oxidation, known as the induction period, which determines the resistance of substances to oxidation.

There are no universal antioxidants that are equally effective for all types of fats; therefore, it is advisable to determine their effectiveness experimentally. The use of a natural antioxidant additive, epigallocatechin gallate, is expected to slow down both hydrolytic and oxidative deterioration of oils within protein-fat emulsions. It is a potent polyphenolic antioxidant, the main catechin of green tea (*Camellia sinensis*), available as a dietary supplement for concentrated intake. In a scientific review (Bartosikova and Necas, 2018), epigallocatechin-3-gallate is described as a powerful natural antioxidant with a high concentration of phenolic hydroxyl groups, which contributes to its radical-scavenging activity and inhibition of lipid peroxidation.

The compound has the formula $C_{22}H_{18}O_{11}$ and a molecular weight of 458.37 g/mol. Due to its unique molecular structure with a large number of phenolic groups (Figure 3), epigallocatechin gallate is capable of neutralizing free radicals that attack the unsaturated components of fats, thereby preventing lipid peroxidation.

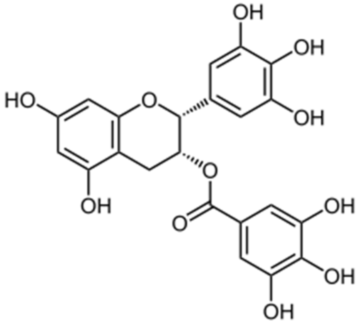


Figure 3. Structural formula of the epigallocatechin gallate molecule

To slow down the deterioration of the lipid phase of the emulsion, a 0.02% epigallocatechin gallate additive was used, as recommended (Capasso et al., 2025).

The oxidation of the protein-fat emulsion was carried out using the accelerated Oxitest method at a temperature of 90 °C. The stability against oxidative degradation was studied by measuring the induction period, which is calculated from the oxidation curve of the tested sample (Figure 4).

The determination of the oxidation induction period is often used for rapid assessment of oxidative stability and for studying aging processes and the effectiveness of stabilizers. A comparison of the Oxitest with other accelerated methods (Chabni, 2024) shows that the induction period is a reliable measure of oxidative stability, effectively characterizing the resistance of oils and fats to the onset of oxidative spoilage. In this method, the induction period is defined as the time required for a sudden change in the intensity of oxidation, during which a pressure drop is recorded in the instrument chamber and a inflection point is observed on the oxidation curve. The induction period is determined by drawing a tangent to the linear portion of the graph.

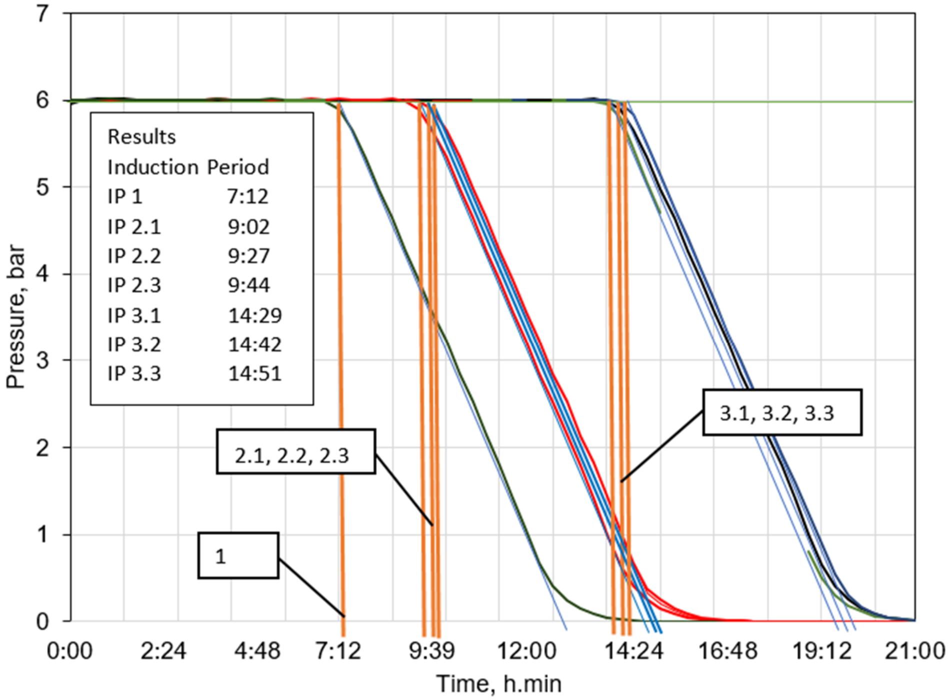


Figure 4. Determination of the induction period by the accelerated method:
1- PFE based on sunflower oil; 2 - PFE based on combined oil; 3 – PFE based on combined oil with antioxidant supplement

Figure 4 shows the oxidation induction time for all the studied fat-containing emulsions, confirming the well-established course of free-radical oxidation in these samples and enabling further analysis of other oxidation patterns using the selected methodology. The induction periods for emulsions prepared with different oils are presented in Table 4.

Table 4

Duration of the induction period for emulsions with different composition

Line No.	Composition of emulsion on oils	Induction time, h:min
1	suflower	7:12
2.1	coconut – sesame – wheat germ	9:02
2.2	coconut - olive - hemp	9:27
2.3	palm - amaranth – grape seed	9:44
3.1	coconut-sesame- wheat germ+0.02% epigallocatechin gallate	14:29
3.2	coconut - olive - hemp +0.02% epigallocatechin gallate	14:42
3.3	palm– amaranth – grape seed+0.02% epigallocatechin gallate	14:51

The duration of the induction period was determined for protein-fat emulsions (PFEs) based on combined oil supplemented with epigallocatechin gallate and ranged from 14 h 29 min to 14 h 51 min, which exceeded the corresponding values for all other studied PFEs. It was found that the PFE prepared with sunflower oil exhibited an induction period of 7 h 12 min during oxidation at 90 °C. The use of combined oil in the PFE formulation increased the induction period to 9 h or more. The observed extension of the induction period (approximately twofold, from 7 h 12 min to 14 h 42 min) in the presence of the antioxidant additive correlates well with established models of hydrocarbon oxidation, in which induction periods can increase significantly in the presence of inhibitors.

Thus, the protein-fat emulsion based on sunflower oil was characterized by the shortest induction period (7 h 12 min), which is consistent with literature data indicating the relatively low oxidative stability of oils with a high content of polyunsaturated fatty acids. For example, Tsao et al. (2021), using the Oxitest method to study expeller-pressed oils, reported a relationship between fatty acid composition and the degree of oil unsaturation. In particular, shelf life was shown to correlate positively with the content of saturated fatty acids and to be independent of the SFA/PUFA ratio. These data, obtained for pressed oils, are consistent with the general trend of oxidation of the fat phase in protein-fat emulsions observed in the present study.

To evaluate antioxidant properties, in addition to assessing the induction period, it is important to determine the kinetic characteristics of the oxidation process. Along with the induction period, key parameters include the average oxidation reaction rate and the time required for complete loss of biological value, which are determined from peroxide value measurements. Variations in peroxide value and the average oxidation rate in the studied protein-fat emulsions during 21 h of accelerated testing are presented in Table 3.

Table 3

Oxidation parameters of protein-fat emulsions

Protein-fat emulsion on	**Peroxide value, mmol $^1/_2O_2$/kg**			**V, mmol $^1/_2O_2$/кг/год**
	PV_0	**PV_{end}**	**ΔPV**	
Suflower oil	1.5	7.8	6.3	0.30
Protein-fat emulsion on combined oils				
Coconut– sesame – wheat germ oil	1.5	6.4	4.9	0.23
Coconut - olive - hemp oil	1.5	6.2	4.7	0.22
palm - amaranth – grape seed oil	1.5	6.1	4.6	0.22
Protein-fat emulsion on combined oils 0.02% epigallocatechin gallate				
Coconut – sesame– wheat germ oil	1.5	4.6	3.1	0.15
Coconut - olive - hemp oil	1.5	4.4	2.9	0.14
Palm - amaranth – grape seed oil	1.5	4.3	2.8	0.13

Note: PFE – protein-fat emulsion; PV – peroxide value; PV_0 - peroxide value at the beginning of storage; PV_{end} - peroxide value at the end of storage; V_{avg} - average oxidation rate during the storage period.

Analysis of the oxidation kinetics of protein-fat emulsions based on peroxide value (PV) revealed the superior stability of samples supplemented with epigallocatechin gallate, as evidenced by significantly lower PVs compared with the other studied emulsions. This finding is supported by the calculated oxidation rates. Specifically, the oxidation rate of the antioxidant-stabilized PFE was approximately twofold lower than that of the control sample. In our opinion, the observed retardation of oxidative susceptibility is associated with an increased concentration of antioxidant compounds in the fat phase of the emulsion. Based on PV measurements, it was established that the addition of epigallocatechin gallate effectively slowed the progression of oxidative spoilage.

It was shown that PFEs based on combined oil were more stable than the control PFE prepared with sunflower oil, even in the absence of additives. The obtained results can be explained by the higher content of saturated and monounsaturated fatty acids, which react with oxygen more slowly than polyunsaturated fatty acids predominating in sunflower oil. In our study, the peroxide value of PFEs containing conventional sunflower oil increased rapidly from 1.5 to 7.8 mmol $½O_2$/kg during 21 h of heating at 90 °C. As shown in the Table 3, peroxide accumulation in the stabilized PFE proceeded more slowly, and the peroxide value at the end of the experiment did not exceed 4.6 mmol $½O_2$/kg.

In a study by Zhou and Ryan (2013), epigallocatechin-3-gallate was shown to reduce both primary and secondary lipid oxidation products in model emulsions, which is consistent with the results obtained in the present work. However, the authors recommend monitoring pH and concentration, as these factors can influence antioxidant or pro-oxidant activity depending on the system.

Beyond chemical stability, oxidative processes also affected the physical state of the emulsions. Samples with a high degree of fat oxidation exhibited increased susceptibility to coalescence and partial phase separation, indicating a relationship between lipid degradation and disruption of interfacial stability. Therefore, optimizing the fatty acid composition in combination with antioxidant protection is a key requirement for maintaining the stability of protein-fat emulsions.

Conclusions

The fatty acid composition of the lipid phase was shown to be a determining factor in the oxidative stability of protein-fat emulsions. Emulsions with a high content of polyunsaturated fatty acids (PUFAs) exhibited a 1.5–2.0-fold faster increase in peroxide value compared with samples dominated by monounsaturated fatty acids. During storage, a gradual accumulation of primary lipid oxidation products was observed; in control samples without antioxidants, peroxide values increased by 30–60%, depending on the fatty acid profile of the fat. Oxidative deterioration of the lipid phase was accompanied by a decline in the physicochemical stability of the emulsions, manifested as increased susceptibility to coalescence and phase separation in samples with high PUFA content.

The addition of epigallocatechin gallate at 200 mg/kg significantly reduced peroxide values by 20–45% compared with control samples, with the antioxidant effect increasing at higher additive concentrations. The highest oxidative stability was observed in emulsions with an optimized fatty acid composition (moderate PUFA content) combined with effective antioxidant protection, highlighting the importance of a comprehensive approach for formulating stable protein-fat emulsions with extended shelf life.

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Rheological and structural properties of fermented coconut beverage as affected by different corn starch types

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Abstract

Introduction. The role of starch in fermented plant-based beverages remains insufficiently understood. This study evaluates the impact of different corn starches on the rheological and structural properties of a coconut matrix.

Materials and methods. Native waxy, native normal, and modified waxy (acetylated distarch adipate) corn starches were added to coconut milk at 2%, 3%, and 4% concentrations. The mixtures were heat-treated at 95 °C for 10 minutes, fermented to a pH of ~4.5, and evaluated for pasting properties, apparent viscosity, macroscopic consistency, water holding capacity (WHC), and microstructure.

Results and discussion. The type and concentration of starch fundamentally determined the spatial organization of the macromolecular network, thereby directly influencing the physical properties of the samples. With increasing concentration, all starches provided higher apparent viscosity in the product. The modified starch did not exhibit pronounced peak viscosity (PV) or breakdown (BD) at any concentration. Under continuous shear, it showed the smallest relative reduction in apparent viscosity among all samples at all levels. Products formulated with modified starch consistently exhibited a homogeneous, smooth system and the highest WHC values. Moreover, at the microstructural level, the modified starch contained swollen granules that retained their intact, rounded shape and were evenly distributed. The native normal corn starch lacked pronounced peaks at 2% and 3%, forming only a moderate PV and a minor BD at 4%. Under continuous shear, its apparent viscosity declined by approximately 50% at 2%, by almost 60% at 3%, and by over 60% at 4%. Macroscopically, normal starch formed noticeably stiffer, gel-like structures with intermediate WHC values; at the maximum 4% concentration, this mass became highly heterogeneous. At the microstructural level, the native normal corn starch showed dense clusters of swollen granules that, with increasing concentration, transformed into a more pronounced, heterogeneous aggregated network attributed to its higher amylose content. Composed almost entirely of amylopectin, the native waxy corn starch demonstrated the highest PV and the most pronounced BD at 3% and 4% concentrations. Under continuous shear, its apparent viscosity underwent a substantial decline, similar to the normal starch. Consequently, it produced a distinctly slimy, cohesive texture and exhibited exceptionally low, nominal WHC values. Correspondingly, the microstructure of the native waxy corn starch was characterised by disintegrated granules and small clusters, lacking a strong gel network.

Conclusions. Starch type plays a key role in determining the rheological behavior and water-holding capacity of fermented coconut beverages. Among the samples studied, modified starch exhibited the highest stabilizing efficiency.

Introduction

Recently, the popularity of plant-based alternatives to dairy products has grown steadily. These products include non-dairy analogues of milk, butter, cheese, yogurt, and ice cream (Fortune Business Insights, 2026). Plant-based beverages are typically produced by aqueous extraction of plant raw materials, often involving size reduction and homogenization to mimic the appearance and texture of bovine milk (Hidalgo-Fuentes et al., 2024; Sethi et al., 2016).

A wide range of plant raw materials is used in the production of plant-based milk alternatives, including cereals (oats, rice, corn, spelt), legumes (soybeans, peanuts, lupins), nuts (almonds, coconuts, hazelnuts, walnuts), seeds (sesame, flax, sunflower), and pseudocereals (quinoa, amaranth) (Sethi et al., 2016). Such diversity results in considerable variation in the nutritional composition and functional properties of the resulting beverages.

In this study, coconut milk was selected as the base substrate due to its distinctive properties (Darsana et al., 2020; Gengan et al., 2025; Matin et al., 2020). Coconut milk is a white, opaque protein–oil–water emulsion with negligible dietary fibre content (Jayanetti et al., 2023). It stands out among plant-based beverages due to its characteristic flavour profile and relatively high nutritional value, associated with its considerable levels of carbohydrates, lipids, proteins, and potassium (Sethi et al., 2016; Shori and Al Zahrani, 2022; Wang et al., 2020).

It is known that fermentation of plant systems improves their nutritional, functional and sensory properties (Montemurro et al., 2021; Mykhonik and Hetman, 2022; Tkesheliadze et al., 2022). However, fermentation is not always sufficient to achieve the desired consistency in a product (Greis et al., 2023), so hydrocolloids (e.g., pectin, locust bean gum, xanthan gum, and starch) are often used to improve it (Boeck et al., 2021). Among them, starch is a highly effective component in food products due to its ability to regulate viscosity and texture and to extend shelf life (Rashwan et al., 2024). Chemically, starch consists of two polysaccharide components — amylose and amylopectin. Their ratio determines the functional properties of starch, which vary significantly depending on the plant origin. Native starch presents certain functional constraints (excessive viscosity at low concentrations, susceptibility to retrogradation, low stability, etc.), which complicate its application in the food industry. To overcome these problems, starch is modified to enhance its functional properties for use in food products (Bashir and Aggarwal, 2019). Among these, acetylated distarch adipate is a widely used industrial ingredient produced by acylation of native starch with adipic and acetic acids. Due to its high resistance to high temperatures, shear stress, and acidic conditions, this modified starch serves as a thickening and stabilising agent in sauces and fermented beverages (Gałkowska et al., 2023; Giacomozzi et al., 2021).

Despite the widespread use of starches in the food industry, the number of scientific studies on their effects on the properties of plant-based fermented beverages remains limited. Some studies focus on the use of other hydrocolloids (El-Sayed et al., 2002; Mauro et al., 2022; Mohd Fazla et al., 2023), often without directly comparing their effectiveness with starches (Baskar et al., 2022). Existing studies on the use of starches have several methodological limitations, making it difficult to systematise their findings. For instance, when corn starch is used in research papers, it is not always specified what type of corn it was derived from (waxy or normal corn) (Baskar et al., 2022; Mohd Fazla et al., 2023). An additional factor of variability is that, across studies, heat-treatment temperatures vary widely, which significantly affects the gelatinisation and final rheological behaviour of starch in the fermented product (Mohd Fazla et al., 2023; Rodríguez-Ruiz et al., 2024; Vitheejongjaroen et al., 2024). Moreover, variations in the botanical origin of the starch (corn, cassava) and its form (native or modified, such as acetylated distarch adipate), combined with the structural and chemical differences among the plant-based matrices (soy, oat, chickpea, coconut), preclude a direct comparison of starch functionality across the available literature (Baskar et al., 2022; Mohd Fazla et al., 2023; Rodríguez-Ruiz et al., 2024; Vitheejongjaroen et al., 2024).

Thus, the current literature does not provide a comprehensive understanding of the role of different starch types in fermented plant-based beverages, largely due to variations in raw material matrices and processing conditions. To address this gap, the present study investigates the effects of various corn starch types on the rheological and structural properties of a fermented coconut beverage, using a unified matrix and strictly standardized processing parameters.

Materials and methods

Materials

Coconut flour, containing approximately 20% plant protein, up to 40% dietary fibre, 7% sugars, and 13% fat (according to the manufacturer's label), was used for coconut milk extraction. Three types of corn starch (*Zea mays*) were evaluated: (i) native waxy corn starch, (ii) native normal corn starch, and (iii) modified waxy corn starch (acetylated distarch adipate). Fermentation was performed using a starter culture formulated for vegan products, containing *Streptococcus thermophilus* and *Lactobacillus delbrueckii* subsp. *bulgaricus*.

Preparation of coconut milk

To obtain coconut milk, the flour was mixed with potable water at a 9:1 (w/w) ratio at 70±5 °C for 10 minutes using a Thermomix TM5 with a butterfly whisk attachment at speed 2. A similar temperature regime for hot extraction has been reported in previous studies (Mauro and Garcia, 2019; Oseni et al., 2017; Suhardiyono et al., 1993). The mixture was filtered through a double layer of gauze and manually squeezed with a twisting motion to maximise the extraction of the liquid phase (Tangsuphoom and Coupland, 2005). The resulting extract was heated and homogenised without prior cooling. At the start of homogenisation, the mixture temperature was 75±2 °C and decreased naturally to 65±2 °C at the end of the process. Homogenisation was carried out at 16,000 rpm for 2 minutes (FROSTY DM-B), processing 350 ml of the mixture in each series. The choice of temperature before homogenisation is consistent with the literature, which states that preheating at 70–90 °C helps reduce the size of fat globules and improves the rheological properties of coconut milk after homogenisation (Peamprasart and Chiewchan, 2006). High homogenisation speeds also increase emulsion stability (Phungamngoen et al., 2016). The resulting coconut milk was cooled to 20±1 °C and stored in sealed glass containers in the dark for no more than 6 hours before further use for the preparation of a fermented beverage.

Coconut milk with starch addition preparation and fermentation

Ten samples were produced, including a control sample based on coconut milk without starch addition and nine experimental samples formulated with three types of starch at varying concentrations. The experimental design and corresponding sample codes are detailed in Table 1. All concentrations were calculated based on the actual moisture content of the starches.

Table 1

Experimental design and sample codes

Starch type	Starch concentration, %	Sample code
None (Control)	0	C
Modified waxy corn starch	2	M2
	3	M3
	4	M4
Native normal corn starch	2	N2
	3	N3
	4	N4
Native waxy corn starch	2	W2
	3	W3
	4	W4

The samples were prepared according to the following procedure: starch and coconut milk were mixed in the specified proportions. The mixture was heat-treated in a Rapid Visco Analyser (RVA) 4800 device by heating to 95 °C and holding for 10 minutes, then cooling to 40 °C. Throughout the process, the paddle rotated continuously at 160 rpm, with the viscosity profile continuously recorded. A similar temperature regime for pasteurising coconut milk is also indicated in another study (Mauro and Garcia, 2019). Next, the starter culture was added according to the manufacturer's recommendations, and fermentation was carried out at 40±1 °C in a static incubator. Fermentation was carried out until the pH reached 4.5. After fermentation, the samples were mixed and stored at 4±2 °C.

Rheological measurements

After 24 hours of storage, the apparent viscosity was measured on an RVA 4800 device at a controlled chamber temperature of 25 °C for 10 minutes, with constant stirring at 160 rpm, and the change in viscosity over time was recorded. For the quantitative analysis of apparent viscosity, the average values for the first 30 seconds (initial apparent viscosity) and the last 30 seconds (final apparent viscosity) were calculated. The percentage decrease in apparent viscosity was calculated using formula (1):

$$\text{Viscosity decrease, \%} = \frac{\eta_0 - \eta_1}{\eta_0} \times 100, \quad (1)$$

where η_0 is the initial apparent viscosity (average value over the first 30 s of measurement), and η_1 is the final apparent viscosity (average value over the last 30 s of measurement).

Visual assessment of consistency

After 24 hours of storage, a preliminary visual assessment of the macroscopic consistency of the thoroughly mixed samples was performed at 20±2 °C. The samples were examined in glass test tubes and described using selected standardised terminology for texture characteristics adapted from ISO (2020). This evaluation was conducted without a trained sensory panel.

Physicochemical analysis

The pH was measured with a digital pH meter previously calibrated with standard buffer solutions; the electrode was immersed directly into the sample. Total solids were determined by the standard oven-drying method (AOAC, 2012).

Determination of water holding capacity

Water-holding capacity (WHC) was determined according to a previously described method (Alvarez-Sabatel et al., 2015), with minor modifications: the sample mass was 9 g, and centrifugation was performed at 3700 × g for 15 min at 10 °C. The separated liquid phase (supernatant) was collected and weighed. WHC was calculated using the following equation (2):

$$\mathrm{WHC}\ (\%) = \frac{m_0 - m_1}{\mathrm{m_0}} \times 100, \tag{2}$$

where m_0 is the sample mass before centrifugation (g), and m_1 is the mass of the separated liquid phase (supernatant) (g).

Microstructure analysis

Microstructural studies of the samples were performed using a ZEISS AX10 light microscope in bright-field mode with a 10× objective lens. A small amount of the product was applied to a microscope slide, evenly distributed in a thin layer. A drop of I_2/KI solution was added, and the slide was covered with a cover glass to ensure diffusion staining of starch granules, allowing visualisation of amylose and amylopectin. Observations were made immediately after the solution was applied. The images were captured with a digital camera integrated with a microscope, and the microphotographs were processed and analysed using ZEN software.

Statistical analysis

Graphs were prepared in Microsoft Excel. Quantitative measurements were carried out in triplicate (n = 3). Physicochemical results are presented as mean±standard deviation (SD). Discrete RVA parameters are given as mean values, and RVA profiles are shown as averaged curves without SD bars for clarity.

Results and discussion

Physicochemical properties of fermented coconut beverage

After 24 hours of storage (at 4±2 °C), the final pH of all samples stabilised at approximately 4.5. Statistical analysis confirmed that neither the type of starch nor its concentration significantly affected the finished product's acidity. Total solids content gradually increased with increasing starch concentration, from 3.5% in the control sample (without starch) to about 7.2% at a 4% starch addition level (M4–W4). Furthermore, within each concentration, no statistically significant differences among starch type were observed (Figure 1).

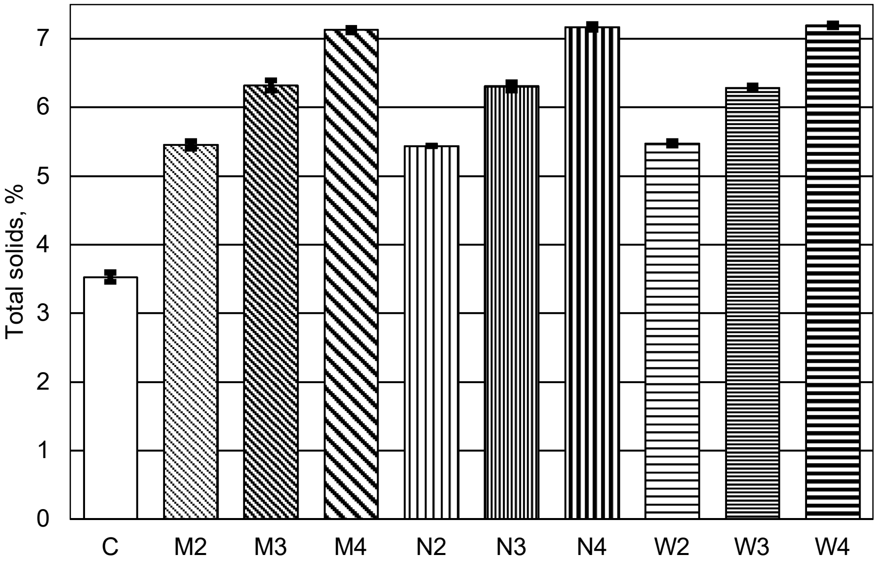

Figure 1. Total solids content of fermented coconut beverages as a function of starch type and concentration (Mean±SD, n = 3)

Given the consistent pH and the similar total solids content among starch types at equal concentrations, these parameters can be excluded as factors explaining textural differences.

Pasting properties of fermented coconut beverages

It is well established that the rheological properties of food products containing starch are largely determined by the botanical origin of starch, the morphology and structural and chemical characteristics of its granules, as well as the type of modification and concentration. This causes differences in granule swelling ability, thermal and mechanical stability, and susceptibility to retrogradation. With this in mind, further analysis was carried out using an RVA, which allowed us to obtain pasting profiles reflecting characteristic changes during heating, holding at 95 °C and subsequent cooling to 40 °C (Figures 2–4). For ease of comparison, a control fermented coconut beverage characterised by consistently low viscosity (≈8–15 cP) was included in all series.

The obtained RVA profiles (Figures 2–4) were analysed using three key rheological parameters: peak viscosity (PV), breakdown (BD), and setback (SV). During the heating phase, PV reflects the maximum swelling capacity of the starch granules before their physical rupture (Leach, 1965). During the holding stage under constant shear, the granules may lose integrity, leading to a decrease in viscosity (Achayuthakan and Suphantharika, 2008). Finally, at the cooling stage, the system's ability to restructure and form a network is evaluated by SV, defined as the difference between the final viscosity at 40 °C and the minimum viscosity (trough viscosity) after the holding stage.

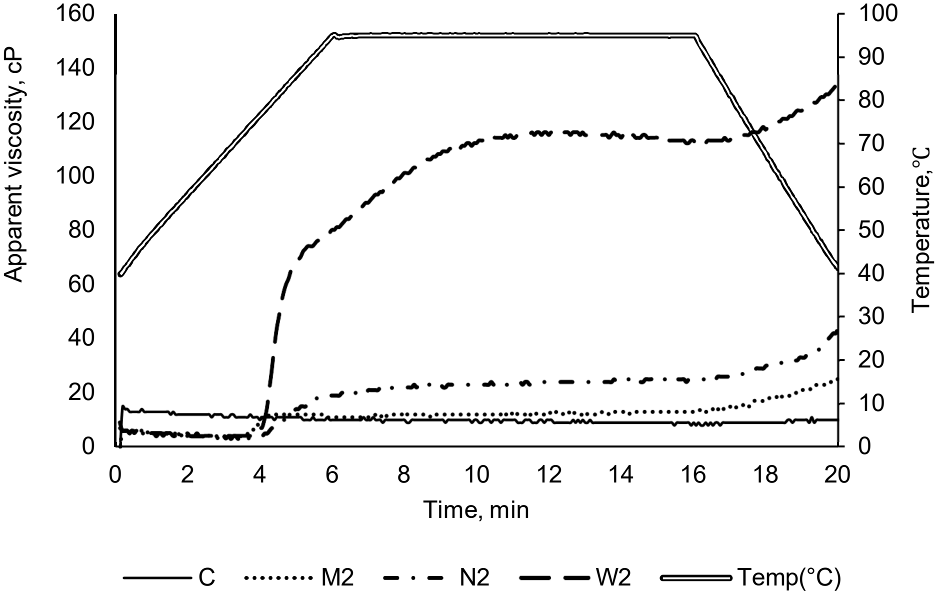

Figure 2. RVA profiles of control and 2% starch-fortified fermented coconut beverage

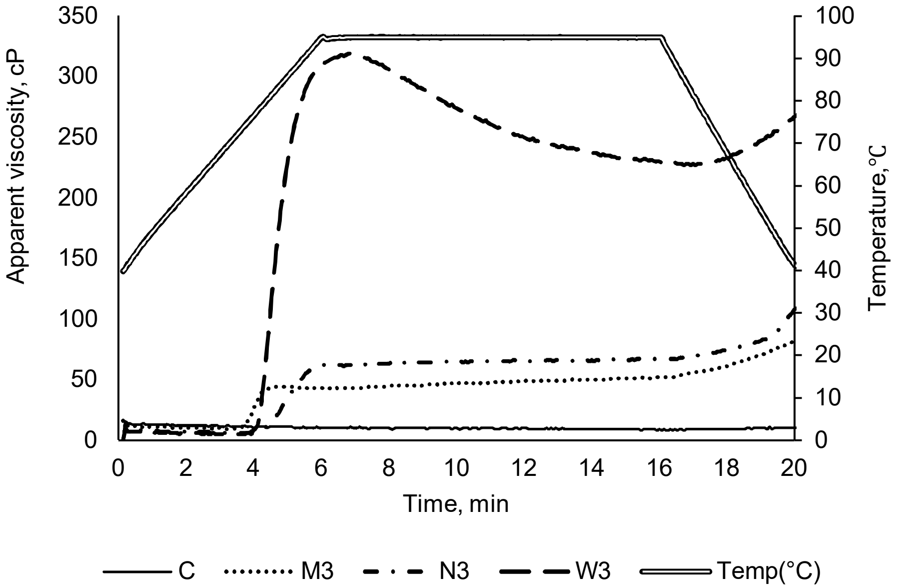

Figure 3. RVA profiles of control and 3% starch-fortified fermented coconut beverage

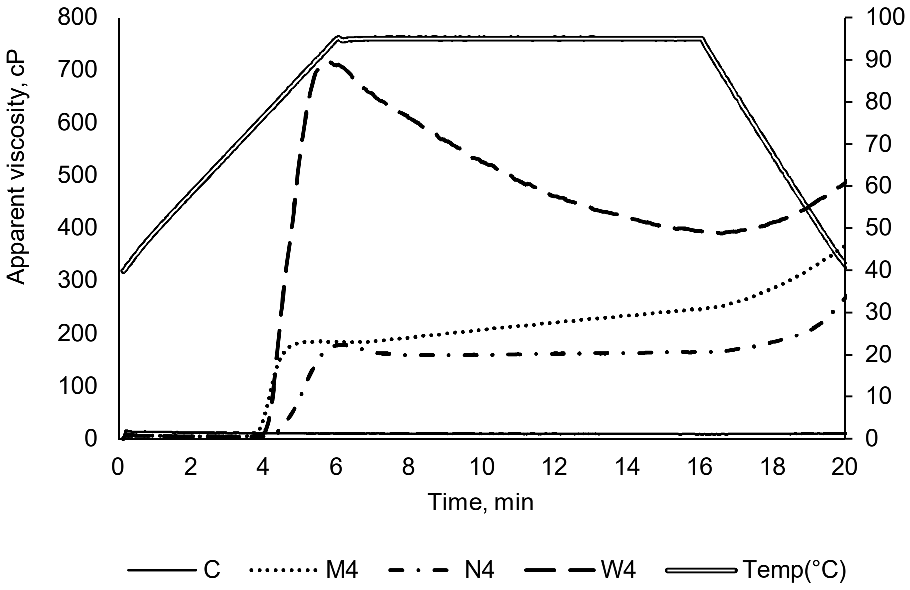

Figure 4. RVA profiles of control and 4% starch-fortified fermented coconut beverage

The analysis of these parameters indicated that the starch type and its concentration determined the rheological behaviour. At 2% and 3% concentrations, neither the modified nor the normal starches formed a pronounced PV. The waxy starch also lacked a PV at 2%, but developed a distinct peak and a BD at 3%. During the cooling phase at 2% and 3% concentrations, W consistently exhibited a lower relative SV than M and N. At 4%, W4 demonstrated the highest PV and the most pronounced BD, while N4 formed a lower PV with minimal BD. M4 showed a continuous increase in viscosity without a peak. Regarding cooling behaviour at this level, the relative SV was highest for N4, intermediate for M4, and lowest for W4.

The changes in rheology with varying concentrations may be explained by the fact that, as starch concentration increases, the granules transition from a 'dilute' suspension to a 'closely packed' and ultimately 'concentrated' state (Steeneken, 1989). The waxy starch is composed almost entirely of amylopectin, with an amylose content of <2% (Fredriksson et al., 1998; Jiranuntakul et al., 2011). The high PV and significant BD typical of waxy starches are directly due to the absence of amylose, which normally acts as a swelling inhibitor (Jiranuntakul et al., 2011). Conversely, the samples containing normal corn starch exhibited a different behaviour, lacking pronounced peaks at 2–3% and forming only a moderate PV with a minor BD at 4%. This can be explained by the fact that normal corn starch contains 23–28% amylose (Cluskey et al., 1980), which actively limits swelling, unlike amylopectin, which is primarily responsible for granule swelling (Tester and Morrison, 1990). An interesting observation was that the normal starch had slightly higher final viscosity values than the modified starch at 2–3%, but was surpassed by M at 4%. It can be assumed that, with increasing concentration, normal starch granules undergo greater destruction, similar to waxy starch, ultimately contributing to a decrease in the final viscosity. The modified starch did not exhibit pronounced PV or BD, indicating its resistance to thermomechanical stress (Gałkowska et al., 2023; Lewandowicz et al., 2022).

Rheological properties of fermented coconut beverages

The apparent viscosity of the fermented coconut beverages after 24 hours of storage at 4 °C exhibited significant concentration- and starch-dependent differences (Figures 5–7). To evaluate the dynamics of structural changes under continuous shear, the initial and final apparent viscosities were defined as the average values of the first and last 30 seconds of the 10-minute measurement, respectively (Figure 8). All samples with starches exhibited a decrease in apparent viscosity from their initial to their final values during the holding period. Previous studies on fermented systems with various thickeners revealed a very similar progressive reduction in viscosity (Mathias et al., 2011).

The control sample yielded very low initial and final values, which are consistent with the liquid nature of the plain coconut base. For the 2% formulations, waxy starch (W2) provided the maximum initial and final values, followed by normal starch (N2) and modified starch (M2). Interestingly, M2 showed the greatest stability with only a minor relative reduction. In contrast, W2 and N2 both lost approximately half of their initial viscosity over the test period. When the starch concentration reached 3%, normal starch (N3) produced the highest viscosity levels, overtaking W3, while M3 remained the lowest. Unlike the 2% variants, every 3% sample suffered a substantial loss of structural integrity. Their viscosity declined by approximately 50% to almost 60% of the initial value. The differences among samples became most pronounced at the 4% level. Normal starch (N4) showed an exceptionally high initial viscosity but experienced the greatest structural breakdown, with a decline of over 60%. This behaviour can be linked to its higher amylose content. Amylose generally forms firm gels (Noda et al., 2003); however, in this specific colloidal system, such structures behave in a brittle manner and are highly vulnerable to continuous shear stress. Waxy starch (W4) also exhibited a substantial initial thickening effect, but its structural integrity broke down considerably, resulting in the lowest final viscosity among the 4% group. Modified starch (M4), on the other hand, began with the lowest initial value but proved highly stable under continuous shear. Due to this minimal relative loss, M4 finished with a significantly higher final viscosity than W4, approaching the levels of N4.

Visual assessment of consistency of the fermented coconut beverages

Along with the rheological data, the visual consistency of the samples was evaluated. As expected, the control remained entirely liquid. Among the thickened formulations, the texture evolved systematically with starch type. The modified starches (M2–M4) consistently produced a homogeneous, smooth system that remained fluid and drinkable even at the maximum 4% concentration. On the other hand, normal corn starch (N2–N4) formed noticeably stiffer, gel-like structures; at 4%, this mass became highly heterogeneous, breaking into large aggregated fragments. By contrast, the waxy variants (W2–W4) developed a distinctly slimy, cohesive texture that became increasingly pronounced at higher concentrations. These macroscopic observations correlate well with the RVA pasting properties and the starch composition—the stable, smooth texture of the modified starch results from its uniform viscosity increase without thermomechanical disruption. Conversely, the slimy nature of the waxy samples correlates with the substantial structural BD during pasting. Because amylose is highly prone to retrogradation upon cooling, it forms a firm gel network, a process that is rheological manifested by a high SV (Noda et al., 2003). Visual observations confirm that these stiff structures fracture readily under continuous shear, explaining the presence of aggregated fragments.

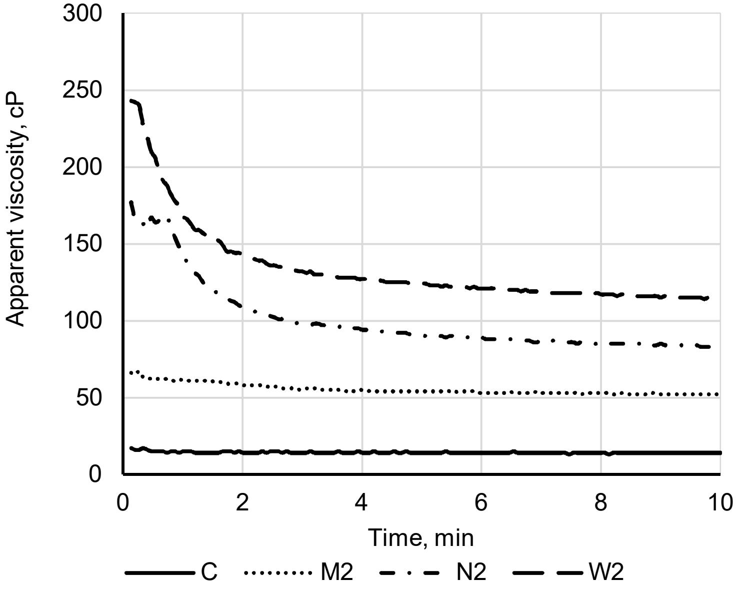

Figure 5. Apparent viscosity of control and 2% starch-fortified fermented coconut beverage at 25 °C over 10 min

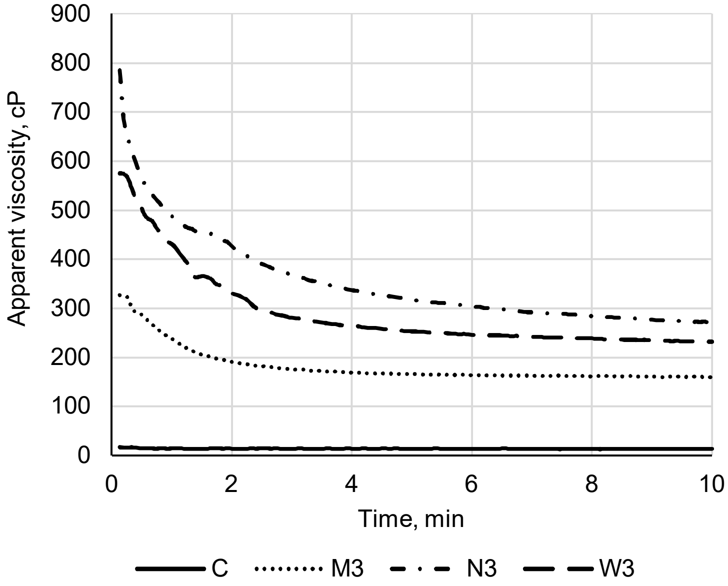

Figure 6. Apparent viscosity of control and 3% starch-fortified fermented coconut beverage at 25 °C over 10 min

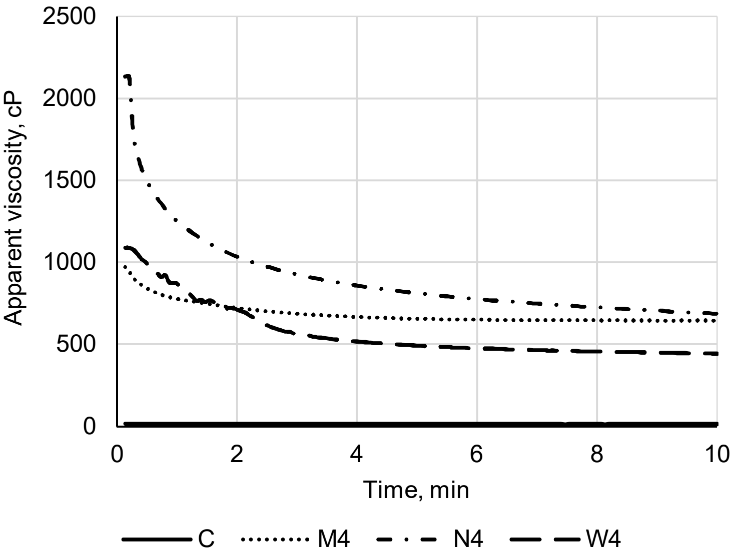

Figure 7. Apparent viscosity of control and 4% starch-fortified fermented coconut beverage at 25 °C over 10 min

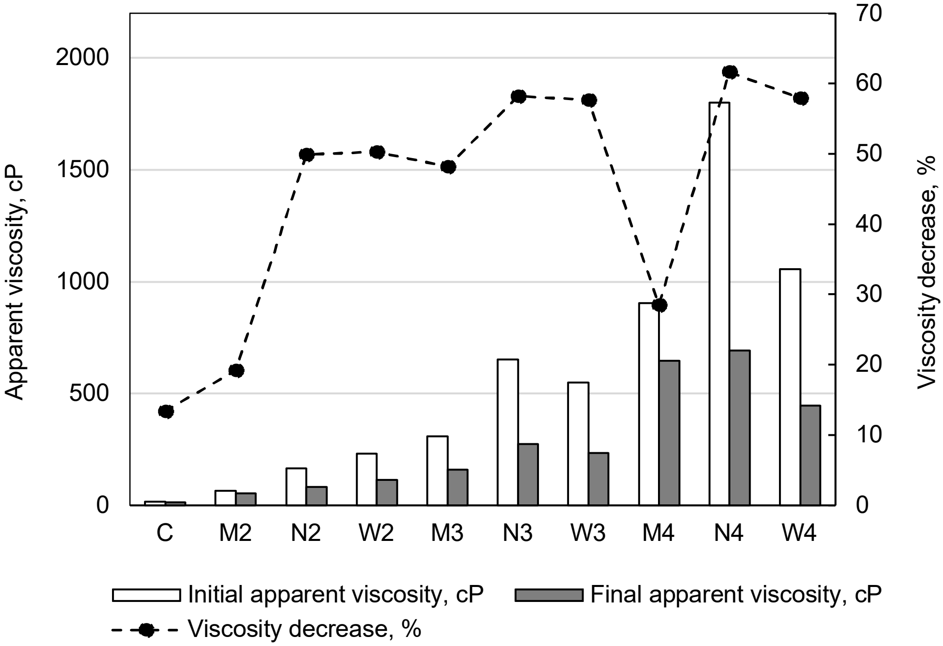

Figure 8. Initial and final apparent viscosity and viscosity decrease of the fermented coconut beverages (25 °C)

Water holding capacity of the fermented coconut beverages

It is well known that one of the key functional properties of starch is its ability to hold water, which directly affects the stability and consistency of fermented systems. The WHC of the samples was evaluated (Table 1).

Table 1

Water holding capacity (WHC) and macroscopic sediment characteristics of the fermented coconut beverages

Sample	WHC, % (Mean±SD)	Visual characteristics of the sediment
C	6.21±0.72	Small sediment caused by solid particles of the coconut base; no starch
M2	40.02±0.68	Dense sediment
N2	36.72±0.56	Dense sediment
W2	14.58±0.93*	No sediment; viscous residue on the tube walls
M3	55.86±0.10	Dense sediment
N3	50.67±0.20	Dense sediment
W3	17.31±0.36*	No sediment; viscous residue on the tube walls
M4	75.28±0.18	Dense sediment
N4	65.76±0.40	Dense sediment
W4	17.19±0.09*	No sediment; viscous residue on the tube walls

Note: * For waxy starch samples (W2–W4), the WHC values are presented as nominal values, as no dense sediment formed after centrifugation; instead, a viscous residue remained on the tube walls.

The control sample exhibited minimal WHC, indicating only sedimentation of small coconut base particles. The incorporation of starch significantly enhanced WHC, a behaviour consistent with its known ability to bind free water and stabilise the gel network in fermented systems (Lobato-Calleros et al., 2014). For both modified (M2–M4) and normal (N2–N4) starches, a concentration-dependent increase in WHC was observed. The modified starch showed the highest WHC, forming a dense, highly stable sediment after centrifugation. The normal corn starch exhibited slightly lower but comparable WHC values, yielding a dense, relatively stable sediment structure. In striking contrast, the waxy starch samples (W2–W4) showed exceptionally low WHC. After centrifugation, they failed to form a typical sediment; instead of a clear separation into sediment and supernatant, the tubes contained a cohesive, sticky mass adhering to the walls.

Consequently, it was impossible to accurately separate and weigh the free liquid phase, as a large portion of the unseparated viscous matrix poured off along with it. As noted in Table 1, the recorded values for the W series are nominal and cannot be interpreted as a classic WHC indicator due to this phase behaviour. This lack of a strong gel network is consistent with the RVA profiles, in which the substantial BD of W samples reflects intense granule destruction during the pasting process.

Overall, the modified starch proved to be the most efficient in retaining water and forming a stable sediment, followed by the normal starch. Since the rheological behaviour and WHC are fundamentally dictated by the spatial organisation of the macromolecular network, the next step was a microstructural analysis of the samples.

Microstructural characteristics of the fermented coconut beverages

The microstructure of the fermented coconut drinks was studied using bright-field microscopy (10×) following diffusion staining with I_2/KI. The control sample (without starch) was not included in the set of microphotographs because it lacked distinct structural elements (presenting as isolated small particles of the coconut base dispersed in a liquid phase); this is consistent with its low viscosity and minimal WHC. Microphotographs of the samples formulated with different types and concentrations of starch are presented in Figure 9.

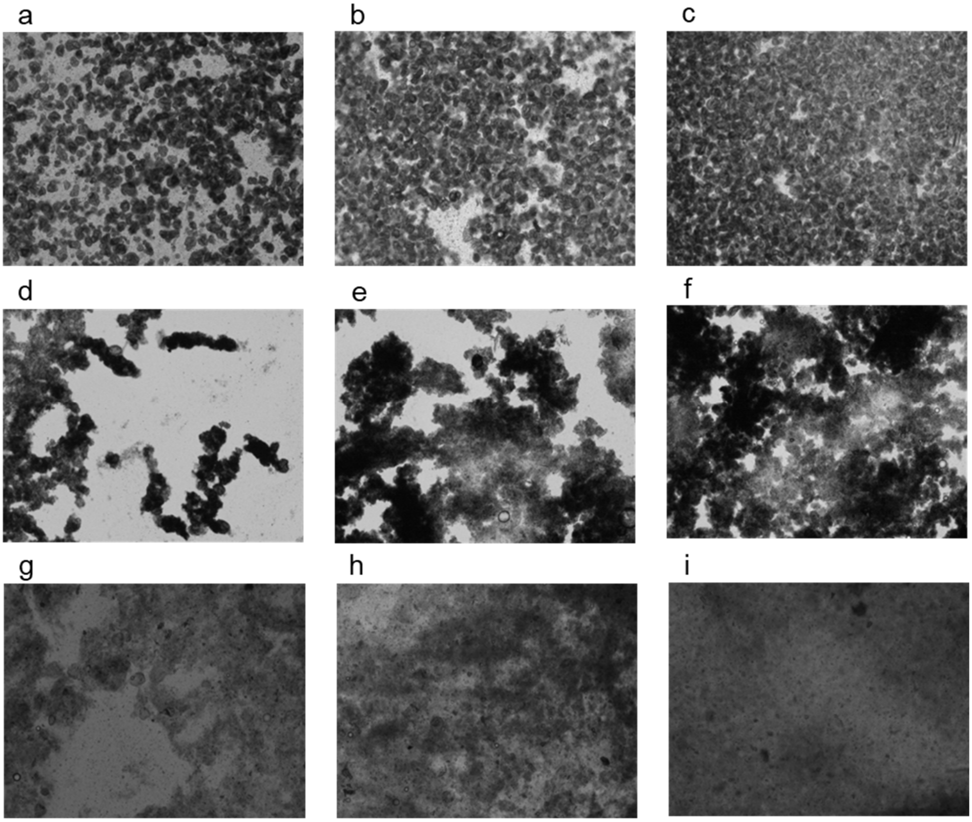

Figure 9. Microphotographs of the fermented coconut beverages with different types and concentrations of starch (bright-field, 10×):
a – M2; b – M3; c – M4; d – N2; e – N3; f – N4; g – W2; h – W3; i – W4

In the samples containing modified starch (M2–M4), the swollen starch granules retained their intact, rounded shape and were evenly distributed throughout the field of view. With increasing concentration, reduced intergranular spaces were observed, and the granules showed no signs of agglomeration or disruption. These microstructural features correlate with stable RVA pasting profiles, the highly stable apparent viscosity under continuous shear, and the maximum WHC values observed in the finished product.

Conversely, the microphotographs of the samples with normal corn starch (N2–N4) showed dense clusters of swollen granules that, with increasing concentration, transformed into a more pronounced, heterogeneous aggregated network. It is postulated that the formation of such clusters is attributed to the higher amylose content of normal corn starch compared to the waxy counterpart. Since leached amylose chains are highly prone to rapid association and partial retrogradation upon cooling, forming a rigid gel network (Fredriksson et al., 1998), this process is likely what manifests visually as the large aggregates.

Consequently, these microstructural features correlate with the high initial apparent viscosity, the subsequent rapid structural breakdown under shear, and the intermediate WHC values found in the finished product. In stark contrast, the microstructure of the samples with waxy corn starch (W2–W4) was characterised by disintegrated granules and small clusters lacking clear granular organisation. This visual evidence of structural disruption directly corresponds to the substantial BD in the RVA profiles, as this rheological parameter specifically indicates the physical rupture of granules. Together, these factors explain the slimy, cohesive texture and the exceptionally low, nominal WHC values observed in these samples.

Ultimately, these microstructural findings confirm that it is the spatial organisation of the starch networks after the heating-cooling cycle that dictates the functional differences in rheological behaviour and WHC among the fermented systems.

Conclusions

This study evaluated the effects of native (waxy and normal) and modified corn starches on the properties of a fermented coconut beverage. At a consistent final pH (~4.5), rheological behaviour and water-holding capacity (WHC) were primarily determined by the spatial organisation of the starch networks.

Modified starch (acetylated distarch adipate) formed a homogeneous microstructure with intact, swollen granules evenly distributed, resulting in a stable RVA pasting profile, the most stable apparent viscosity under shear, a thick and smooth texture, and the highest WHC. Normal corn starch formed dense, heterogeneous clusters, producing a stiffer gel-like consistency with moderate WHC and a decline in viscosity under shear. Waxy corn starch lacked a strong gel network, displaying disintegrated granules and small clusters, leading to a slimy, cohesive texture, low WHC, high peak viscosity, and significant breakdown during shear.

Overall, modified starch was the most effective stabiliser, ensuring optimal texture, rheological stability, and WHC. Future studies should examine the effects of different degrees of cross-linking and other chemical modifications on the stability and texture of fermented plant-based systems using a standardized matrix and processing conditions.

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Metabolic profile and biocontrol potential of endophytic bacteria *Bacillus amyloliquefaciens* E12

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Abstract

Introduction. Endophytic bacteria are promising agents for biotechnological applications in agriculture and food industry. This research presents the results of a comprehensive characterisation of the endophytic strain *B. amyloliquefaciens* E12.

Materials and methods. The isolated endophyte was identified using MALDI-TOF mass spectrometry, classical microbiological methods and VITEK® 2 Compact analyser. Antagonistic activity was tested *in vitro* against phytopathogenic microfungi and bacteria. *In vivo* effects were evaluated on spring wheat seeds. Volatile Organic Compounds (VOCs) profile was analysed by headspace gas chromatography-mass spectrometry on different media.

Results and discussion. Using MALDI-TOF mass spectrometry and VITEK® 2 Compact, endophytic isolate E12 was identified as *Bacillus amyloliquefaciens* with 91.0% confidence. The identified endophyte was differentiated from the phylogenetically closely related species *B. subtilis* by two key characteristics: the ability to form cell chains and the inability to utilise inulin. *B. amyloliquefaciens* E12 demonstrated antagonistic activity against phytopathogenic microfungi *Fusarium oxysporum* GTF1 and *Alternaria alternata* GTA2 (growth inhibition zones 15-18 mm) and phytopathogenic bacteria *Clavibacter michiganensis* subsp. *michiganensis* 10_2 , *Pseudomonas fluorescens* 8573, *Pectobacterium carotovorum* UCM B-1075, *Pseudomonas syringae* UCM B-1027, *Xanthomonas campestris* UCM B-1049 (inhibition zones 22-42 mm). *In vivo* biotesting on wheat seeds showed growth stimulation and complete protection against fungal pathogens. Analysis of the VOCs profile by HS-GC-MS showed that *B. amyloliquefaciens* E12 is an effective producer of acetoin (about 130 ppm) and is characterised by metabolic variability: under conditions of high carbon content, the biosynthesis of growth-stimulating metabolites prevails, and under conditions of enriched nitrogen content, the production of protective compounds is activated. The bioactive metabolite profile of *B. amyloliquefaciens* E12 confirms biotechnological potential for biopreparation development.

Conclusions. Endophytic bacteria *B. amyloliquefaciens* E12 are characterised by effective biocontrol activity and production of growth-stimulating metabolites, providing basis for developing biopreparations for agriculture.

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Introduction

Global food security remains one of the most pressing challenges of the 21st century, exacerbated by rapid population growth (according to UN forecasts, the population will reach 10 billion by 2050), climate change and the progressive degradation of agricultural land (Godfray et al., 2010). According to estimates by the Food and Agriculture Organisation (FAO), agricultural production must increase by 70% over the next two decades to meet global food needs (FAO, 2017). At the same time, one of the biggest challenges for agricultural production is the fight against plant pathogens, which cause an annual decline in the yield of major food and cash crops worldwide by 20–40%, with economic losses of over $470 billion. Phytopathogens cause the loss of at least 47 million tonnes of durable crops and 60 million tonnes of perishable crops, which can have critical consequences for the economy and food security (Cai, 2024).

The intensive use of synthetic pesticides for plant protection increases yields and controls the impact of phytopathogens, but has led to soil and water pollution, ecosystem degradation, the development of pathogen resistance, and the accumulation of toxic residues in crop products, which threatens the safety of the food chain. These challenges highlight the need to develop new biological products – microbial biopesticides as natural agents that can effectively control plant pests and pathogens, offering an environmentally safe and economically viable solution for sustainable food production (Thakur et al., 2020).

One of the most promising biotechnological tools in this area is plant growth-promoting rhizobacteria (PGPR), such as representatives of the genus *Bacillus* (Maslennikova et al., 2023). Biological products based on *Bacillus* spp. make it possible to reduce or completely replace chemical plant protection products, restore the biological activity of soils, increase the resistance of cultivated plants to biotic and abiotic stresses, and improve the quality of crop production (Compant et al., 2005). Endophytic microorganisms, bacteria that colonise the internal tissues of plants without causing visible symptoms of disease and establish symbiotic relationships with the host plant, attract particular attention, providing prolonged protective action throughout the growing season (Hardoim et al., 2015; Santoyo et al., 2016).

Strains belonging to the *Bacillus amyloliquefaciens* group are highly effective in the biocontrol of a wide range of phytopathogens, such as *Fusarium oxysporum* and *Alternaria alternata*, which cause critical diseases in vegetable (tomatoes, cucumbers, cabbage, potatoes) and cereal (wheat, corn, rice) crops: fusarium wilt and alternaria. These microorganisms are characterised by multifactorial mechanisms of protective and growth-stimulating action: production of phytohormones (auxins, cytokinins, gibberellins), improvement of nutrient uptake (nitrogen fixation, phosphate solubilisation), induction of systemic plant resistance (ISR) and direct antagonistic action against phytopathogenic microorganisms (Santoyo et al., 2016). The mechanism of biocontrol activity of *B. amyloliquefaciens* is realised through the production of specific antimicrobial metabolites: lipopeptide antibiotics (bacillomycin D, surfactin, iturin, fengicin), polyketides (difucidin, macrolactin, bacillain), volatile organic compounds (acetoin, 2,3-butanedione, pyrazines) (Borriss et al., 2011; Chen et al., 2009).

Volatile organic compounds (VOCs) play an important role in bacterium-plant interactions, not only stimulating growth and inducing systemic acquired resistance (SAR), but also exerting direct antimicrobial effects against phytopathogens (Kai et al., 2009; Ryu et al., 2004). Acetoin, one of the main VOC metabolites *of Bacillus* spp., induces the expression of plant defence response genes, increases resistance to drought, salt stress and biotic factors, and stimulates growth processes by modulating phytohormonal balance (Xiao et al., 2007). Current approaches to characterising PGPR strains include taxonomic identification, assessment of antagonistic activity *in vitro,* and detailed analysis of the metabolic profile, e.g., VOC production. Metabolic flexibility, i.e., the ability of bacteria to regulate biosynthetic pathways depending on environmental conditions, is an important criterion for biotechnological applications. This allows the production of specific metabolites to be modulated by optimising cultivation conditions (Almeida et al., 2023).

Since the biological efficacy of each strain is individual and specific, it is necessary to conduct a comprehensive assessment of new endophytic isolates to determine their biotechnological potential and develop effective biological products for various agricultural crops (Perez-Garcia et al., 2011).

The aim of this study is to provide a comprehensive characterisation of the endophytic isolate *B. amyloliquefaciens* E12.

Materials and methods

The endophytic isolate was obtained from *Pinus sylvestris* L. seeds collected in the Fastiv district of the Kyiv region, Ukraine. To eliminate epiphytic microbiota and ensure the endophytic origin of the isolated isolate, a method of multi-stage surface sterilisation in a 70% ethanol solution for 5 minutes and a 16.5% hydrogen peroxide solution for 20 minutes was used (Hvozdiak et al., 2001). The cycle of treatment with ethanol and hydrogen peroxide was repeated for complete disinfection of the entire surface of the seeds. Final sterilisation was carried out by briefly immersing the seeds in 96% ethanol, followed by burning over the flame of an alcohol lamp. The sterilised seeds were homogenised aseptically in sterile mortars with the addition of 1 mL of sterile 0.85% NaCl solution for tissue disaggregation, then the resulting suspension was sown on LB (Luria-Bertani) agar. The resulting colonies were selected for further identification and study of functional properties.

Endophytic isolate E12 is stored in the collection of the Department of Phytopathogenic Bacteria of the D.K. Zabolotny Institute of Microbiology and Virology of the National Academy of Sciences of Ukraine.

For routine cultivation, LB agar was used at a temperature of 28-30 °C.

Test cultures were selected to assess the biocontrol potential:

- phytopathogenic microfungi:
  - *Fusarium oxysporum* GTF1;
  - *Alternaria alternata* GTA2.
- phytopathogenic bacteria:
  - *Pseudomonas syringae* (van Hall 1902) UCM B-1027 (NCPPB 281);
  - *Xanthomonas campestris* (Pammel 1895) Dowson 1939 UCM B-1049;
  - *Pseudomonas fluorescens* (Migula 1895) 8573;
  - *Pectobacterium carotovorum* (Jones 1901) Waldee 1945 (Approved Lists 1980) emend. Gardan et al. 2003 UCM B-1075 (NCPPB 312);
  - *Clavibacter michiganensis* subsp. *michiganensis* (Smith 1910) Davis et al. 1984 10_2;
  - *Agrobacterium tumefaciens* (Smith et Townsend 1907) Conn 1942 UCM B-1000 (NCPPB 3787).

Morphological and cultural analysis (description of colonies, cell shape, spore formation, Gram staining, etc.) and physiological and biochemical properties (ability to grow on media with different NaCl concentrations, ability to grow aerobically and anaerobically on glucose, hydrolysis of gelatin and starch, Foges-Proskauer reaction, etc.) of the isolated isolate were performed using classical microbiological methods (Patyka et al., 2017).

The isolated strain was identified using matrix-assisted laser desorption/ionisation (MALDI-TOF MS) with a time-of-flight mass spectrometer (MALDI-TOF MS) on a VITEK

MS device (Biomerieux, France) in accordance with the manufacturer's recommendations. Mass spectra were recorded in the m/z 2000-20000 Da range and compared with the VITEK MS reference database. Taxonomic identification was performed based on a spectral comparison algorithm with the determination of a confidence value. The analysis results were considered reliable at a confidence value of at least 60% (Tsuchida et al., 2020).

For comprehensive phenotypic characterisation of the isolate, a VITEK® 2 Compact automatic microbiological analyser with a VITEK® 2 BCL card (for Gram-positive spore-forming bacteria) was used.

Antagonism against phytopathogenic microfungi *(F. oxysporum*, *A. alternata*) was determined by the delayed antagonism method on Chapek medium. Antagonism to phytopathogenic bacteria was determined using the radial streak method (Klement et al., 1990; Patyka et al., 2017).

To study the effect of the composition of the nutrient medium on the production of VOCs, bacteria were cultivated on three different liquid media:

- synthetic medium (composition: K_2HPO_4 – 1.0 g/L, $(NH_4)_2HPO_4$ – 1.0 g/L, $MgSO_4$ – 5.0 g/L, $CaCl_2$ – 0.1 g/L, glucose – 5.0 g/L);
- Potato Dextrose Broth (Hvozdiak et al., 2001);
- LB agar (Klement et al., 1990).

Cultivation was carried out in flasks containing 150 mL of the appropriate medium at a temperature of 25-27°C on an orbital shaker at a rotation speed of 240 rpm for 5 days (120 hours) until the late stationary phase of growth was reached. The cultures were incubated in the dark to avoid photodegradation of metabolites.

The VOC profile in culture fluids was studied by headspace gas chromatography with a mass spectrometric detector (Headspace-GC-MS) on an Agilent Technologies 6890N chromatographic system equipped with an MSD 5975 mass-selective detector (Agilent Technologies, USA). Chromatographic analysis was performed on a 30 × 0.25 mm HP-5MS capillary column coated with a layer containing 6% cyanopropyl/phenyl and 94% polydimethylsiloxane, 1.4 µm thick. Helium was used as the carrier gas at a flow rate of 1.0 mL/min. The thermostating temperature of the vials was set at 80 °C with a thermostating time of 40 min. The vapour phase injection volume was 1.0 mL. The column temperature program was optimized as follows: initial temperature 40 °C with a holding time of 3 min, increase to 100 °C at a rate of 4 °C/min with a holding time of 3 min, increase to 200 °C at a rate of 8 °C/min with a holding time of 3 min, and a final increase to 240 °C at a rate of 20 °C/min with a 4 min hold. Ionisation was performed by electron impact (EI) with an energy of 70 eV at an ion source temperature of 230 °C. Mass spectra were recorded in full scan mode in the m/z 29-1000 range. Compounds were identified by comparing experimental mass spectra with the NIST MS Search 2.0 library; compounds with a match quality index of at least 80% were used for identification. A semi-quantitative assessment of the VOC content was performed by internal normalisation based on the areas of the chromatographic peaks, and the results were expressed in ppm (parts per million). Sterile culture media served as controls.

Spring wheat (*Triticum aestivum* L.) seeds of the Pecheranka variety were used to study the growth-stimulating activity of the isolated isolate. *Bacillus amyloliquefaciens* E12 was cultivated in a liquid synthetic medium or potato broth for 48 hours at a temperature of 25-27°C. The culture fluid was diluted in ratios of 1:50 and 1:100 for seed treatment. The treatment was carried out by soaking the seeds in the appropriate dilutions of the suspension for 30 minutes, followed by germination of the seeds under high humidity conditions. Laboratory germination, the absence of seed damage by phytopathogenic microfungi (as the main indicator of protective activity) and the dynamics of shoot growth were evaluated.

The results are presented as the mean ± standard deviation (M±SD). Statistical processing of experimental data was performed using the Statistica 6.0 software package (StatSoft Inc., USA) and the built-in statistical functions of Microsoft Excel 2016. The reliability of differences between variants was assessed using Student's t-test for independent samples or one-way ANOVA followed by Tukey's post-hoc test. The difference between variants was considered statistically significant at a significance level of $p < 0.05$.

Results and discussion

Endophytic bacterial isolate E12 was successfully obtained from superficially sterilised seed material without evidence of external contamination. It should be noted that bacterial growth was observed in 15-20% of sterilised seeds (the absence of surface microbiota on which was confirmed by microbiological methods). The isolated bacterial isolate is a typical representative of the endophytic microbiota of Scots pine seeds. On a nutrient medium after 48 hours of incubation at 28°C, it formed white-grey opaque colonies with a diameter of 3-5 mm with cut edges and a characteristic wrinkled surface. The colonies had a dense consistency, which is a typical morphological feature of bacilli that produce exopolysaccharides (Figure 1a) and form biofilms (Figure 1b).

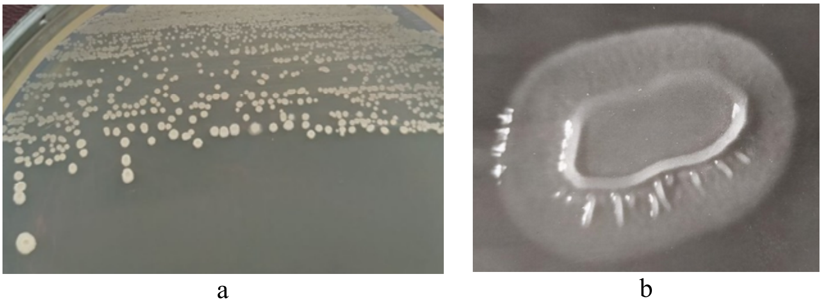
a b

Figure 1. Colonies of isolated endophytic bacteria E12 (a); microphotograph of colonies of endophytic bacteria E12 (b)

Microscopic analysis revealed that the endophytic isolate has rod-shaped cells. After 24-48 hours of cultivation, intense sporulation was observed. The spores were ellipsoidal in shape, centrally and subterminally located, which is characteristic of representatives of the genus *Bacillus*. Spore formation did not lead to significant deformation of the vegetative cell. An important morphological feature of the isolate is its ability to form chains of 2–4 cells when cultivated on a nutrient medium (Figure 2). This characteristic is a key differential feature that distinguishes *Bacillus amyloliquefaciens* from similar *Bacillus subtilis*, which usually form single cells and pairs (De Vos et al., 2009; Priest et al., 1987).

This feature is important for endophytic microorganisms, as the elongated morphology may improve their penetration through intercellular spaces and colonisation of internal plant tissues.

A comparative analysis of the morphological and cultural properties of the endophytic isolate with literature data for *B. subtilis* and *B. amyloliquefaciens* is presented in Table 1.

Analysing the results presented in Table 1, it was determined that the isolated strain E12 corresponds to the phenotypic profile *of B. amyloliquefaciens*, including the key differential feature – the formation of cell chains, which is not characteristic of *B. subtilis* (De Vos et al., 2009 Priest et al., 1987).

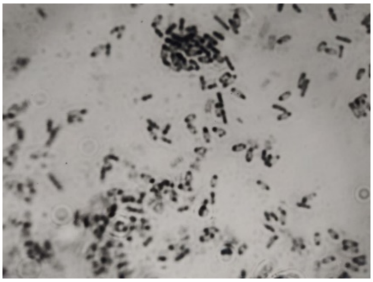

Figure 2. Microphotograph of E12 bacillus cells

Table 1

Morphological and cultural properties of the endophytic isolate

Property	**Isolate E12**	***Bacillus subtilis*** (De Vos et al., 2009)	***Bacillus amyloliquefaciens*** (De Vos et al., 2009; Priest et al., 1987)
Cell shape	Rods forming chains of 2-4 cells	Straight rods 0.7–0.8 × 2.0–3.0 µm, solitary and in pairs	Straight rods 0.7–0.9 × 1.8–3.0 µm, forming chains
Spore formation	+ (ellipsoidal)	+ (ellipsoidal or cylindrical)	+ (ellipsoidal)
Formation of cell chains	+	+/-	+
Gram staining	+	+	+
Mobility	+	+	+
Catalase activity	+	+	+
Growth in 7% NaCl	+	+	+
Growth in 10% NaCl	+	+/-	+/-
Growth in glucose medium aerobically	+	+	+
Growth in glucose medium anaerobically	+	+/-	+/-
Gelatin hydrolysis	+	+	+
Starch hydrolysis	+	+	+
Casein hydrolysis	+	+	+
Esculin hydrolysis	+	+	+
Nitrate reduction	+	+	+
Tyrosine hydrolysis	+	+/-	-
Citrate utilisation	+	+	+
Voges-Proskauer reaction	+	+	+

Identification by MALDI-TOF mass spectrometry

Taxonomic identification of the endophytic isolate was performed using MALDI-TOF mass spectrometry, a modern rapid method for identifying microorganisms based on the analysis of ribosomal protein profiles (Tsuchida et al., 2020). This method provides rapid (less than 30 minutes) and highly accurate identification of bacteria to the species level without the need for long-term cultivation or molecular genetic analysis.

Mass spectrum analysis of endophytic bacteria determined that the isolate belonged to the species *B. amyloliquefaciens* / *B. subtilis* with a confidence criterion of 91%, which corresponds to correct identification at the species level (Figure 3).

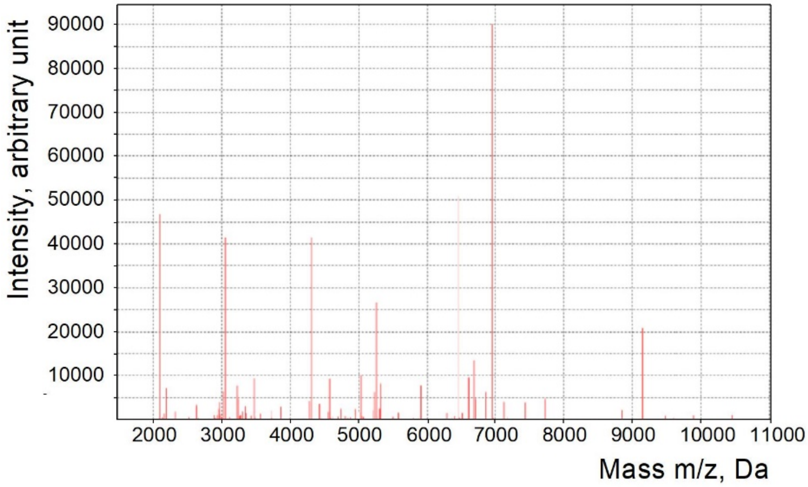

Figure 3. Mass spectrum of endophytic isolate *B. amyloliquefaciens* E12

The 91% confidence criterion confirms the high quality of the obtained mass spectrum and the reliability of identification. The correspondence of the *B. amyloliquefaciens* E12 profile to two species (*B. amyloliquefaciens* and *B. subtilis*) is justified by their high genetic homology (>99% similarity of 16S rRNA) and overlapping ribosomal protein profiles (Dunlap et al., 2016). This required the use of additional identification methods to accurately determine the species affiliation of the endophytic isolate. The MALDI-TOF MS method is widely used for routine identification *of Bacillus* spp. due to its speed, accuracy, and cost-effectiveness (Tsuchida et al., 2020). However, for phylogenetically closely related species of the *B. subtilis* complex (including *B. subtilis*, *B. amyloliquefaciens*, *B. velezensis*, *B. siamensis*, *B. methylotrophicus*, etc.), a combination of MALDI-TOF with additional phenotypic or molecular genetic methods is required (Dunlap et al., 2016).

Physiological and biochemical characteristics of endophytic isolates using VITEK® 2 Compact

The physiological and biochemical characteristics of endophytic bacteria *B. amyloliquefaciens* E12 were determined using the VITEK® 2 Compact automated analyser with a BCL card designed to identify Gram-positive spore-forming microorganisms (Table 2).

Table 2

Biochemical profile of *B. amyloliquefaciens* E12 according to VITEK® 2 BCL

Biochemical test (substrate/enzyme name)	**Mnemonic**	***B. amyloliquefaciens***	
		E12	**According to** (Schober et al., 2025)
Beta-xylosidase	BXYL	+	+
L-lysine arylamidase	LysA	-	-
L-aspartate arylamidase	AspA	+	+
Leucine arylamidase	LeuA	+	+
Phenylalanine arylamidase	PheA	+	+
L-proline arylamidase	ProA	-	-
Beta-galactosidase	BGAL	-	-
L-pyrrolidonyl arylamidase	PyrA	+	+
Alpha-galactosidase	AGAL	+	+/-
Alanine arylamidase	AlaA	+	+
Tyrosine arylamidase	TyrA	+	+
Beta-N-acetyl-glucosaminidase	BNAG	-	-
Ala-phe-pro arylamidase	APPA	+	+
Cyclodextrin	CDEX	-	-
D-galactose	dGAL	-	-
Glycogen	GLYG	+	+
Myo-inositol	INO	-	-
Methyl-Alpha-D-glucopyranoside	MdG	+	+
Ellman	ELLM	-	-
Methyl-D-xyloside	MdX	-	-
Alpha-mannosidase	AMAN	-	-
Maltotriose	MTE	-	-
Glycine arylamidase	GlyA	+	+
D-mannitol	dMAN	-	-
D-mannose	dMNE	+	+/-
D-melezitose	dMLZ	-	-
N-acetyl-D-glucosamine	NAG	-	+/-
Palatinose	PLE	(-)	+/-
L-rhamnose	lRHA	-	-
Beta-glucosidase	BGLU	+	+
Beta-mannosidase	BMAN	+	+
Phosphoryl choline	PHC	-	-
Pyruvate	PVATE	+	+
Alpha-glucosidase	AGLU	+	+
D-tagatose	dTAG	-	-
D-trehalose	dTRE	+	+
Inulin	INU	-	-
D-glucose	dGLU	+	+
D-ribose	dRIB	(+)	+/-
Putrescine assimilation	PSCNa	-	-
Growth in 6.5% NaCl	NaCl	+	+
Kanamycin resistance	KAN	-	-
Oleandomycin resistance	OLD	(-)	-
Esculin hydrolyse	ESC	+	+
Tetrazolium red	TTZ	+	+
Polymixin_B resistance	POLYB_R	+	+

Note: Results in parentheses (-) and (+) indicate weak reactions.

The endophytic isolate *B. amyloliquefaciens* E12 utilises a wide range of carbohydrates: dGLU, dMNE, dTRE, MdG and GLYG. The efficient utilisation of complex polysaccharides, such as glycogen, indicates the presence of glycolytic enzymes and α-/β-glycosidases, which provide a competitive advantage in the rhizosphere and phyllosphere, where polysaccharides are the main sources of carbon.

The lack of INU utilisation is a critically important finding for the differentiation of bacterial species. Inulin (β-2,1-fructose polymer) is utilised by *B. subtilis* bacteria due to the presence of a specific enzyme, inulinase, but *B. amyloliquefaciens* does not produce this enzyme (De Vos et al., 2009; Priest et al., 1987). This specific biochemical test is an important confirmation of the belonging of the isolated endophytic strain to *B. amyloliquefaciens*.

The formation of β-glucosidase (BGLU +) and α-glucosidase (AGLU +) indicates the ability of *B. amyloliquefaciens* E12 to hydrolyse β-1,3- and α-1,4-glucans, components of the cell walls of phytopathogenic fungi. This enzymatic activity is one of the mechanisms of biocontrol that allows bacteria to destroy the integrity of the mycelium of pathogenic fungi (Wang et al., 2021).

An important characteristic *of B. amyloliquefaciens* E12 is its high tolerance to salt stress: the ability to grow in the presence of 6.5% NaCl and variable growth at 10% NaCl. This property indicates the potential for adaptation to osmotic stresses, which is critical for colonisation of the rhizosphere under conditions of soil salinisation.

Differentiation of *B. amyloliquefaciens* from *B. subtilis*

The species *B. amyloliquefaciens* and *B. subtilis* are phylogenetically similar and belong to *the B. subtilis species complex*, which complicates their differentiation based on phenotypic characteristics. During the identification of the isolate by the MALDI-TOF method, 91% similarity to both *Bacillus* species was determined, which is justified by a significant overlap of protein profiles (Dunlap et al., 2016). Such ambiguity in identification required the use of additional differential criteria to accurately determine the species affiliation of endophytic isolate E12.

According to scientific studies (Priest et al., 1987; De Vos et al., 2009), the main phenotypic markers for specifying *B. amyloliquefaciens* and *B. subtilis* are the formation of cell chains and the ability to utilise inulin.

During the analysis of the experimental results obtained, it was determined that the bacterial isolate forms chains of 2-4 cells when cultured on liquid nutrient medium (Figure 3). Also, endophytic bacteria E12 do not utilise inulin according to the results of the VITEK® 2 analysis (Table 2). Both of these specific characteristics indicate that isolate E12 belongs to the species *Bacillus amyloliquefaciens*. Thus, the results of comprehensive identification (phenotypic markers + MALDI-TOF + VITEK® 2 Compact) allow the endophyte to be identified as *B. amyloliquefaciens*.

The species *B. amyloliquefaciens* is known for its biocontrol and growth-stimulating properties due to the production of bioactive metabolites. In particular, these bacteria synthesise lipopeptide antibiotics (bacillomycin D, surfactin, iturin, fengicin), which disrupt the integrity of the cell membranes of phytopathogens (Chen et al., 2009). *B. amyloliquefaciens* also produces polyketides (difucidin, macrolactin, bacillain) that inhibit the synthesis of pathogen nucleic acids, as well as volatile organic compounds (acetoin, 2,3-butanedione, pyrazines) that stimulate plant growth and induce systemic resistance (Borriss, 2011; Chen et al., 2009). The ability to effectively colonise the rhizosphere and internal plant tissues makes *B. amyloliquefaciens* one of the most promising species for the creation of biological products (Borriss, 2011; Compant et al., 2005).

Antagonistic activity of *B. amyloliquefaciens* E12 against phytopathogens

During the evaluation of the antagonistic activity *of B. amyloliquefaciens* E12, a specific biocontrol effect against a wide range of economically important phytopathogenic microorganisms was determined (Table 3, Figure 4).

Table 3

Antibacterial activity *of B. amyloliquefaciens* E12 against phytopathogenic bacteria and microfungi

Test culture	Disease caused	Plant susceptible to infection	Inhibition zone (mm)
Against phytopathogenic bacteria			
P. fluorescens 8573	Seedling blight, root rot	Cereals, legumes, vegetable crops	26 ± 1
P. syringae UCM B-1027	Bacterial blight, bacterial speck	Tomatoes, beans, soybeans, fruit trees (apple, pear, stone fruit)	37 ± 2
X. campestris UCM B-1049	Black rot	Cabbage, rapeseed, radish, mustard, broccoli	42 ± 2
C. michiganensis subsp. *michiganensis* 10_2	Bacterial canker	Tomatoes, peppers, eggplants	22 ± 1
P. carotovorum UCM B-1075	Soft rot, foot rot, blackleg, canker	Potatoes, rice, pears, tomatoes, carrots, cabbage, onions	35 ± 2
A. tumefaciens UCM B-1000	Crown gall	Fruit trees, grapes, roses, tomatoes, sunflowers	0
Against phytopathogenic microfungi			
F. oxysporum GTF1	Fusarium wilt, root rot	Tomatoes, cucumbers, melons, cotton, bananas, cereals, beans, flax	15 ± 1
A. alternata GTA2	Alternaria blight (early blight), stem cancer, leaf spot	Tomatoes, potatoes, wheat, barley, citrus fruits, apple trees, cabbage	18 ± 2

The highest activity *of B. amyloliquefaciens* E12 was detected against *X. campestris* UCM B-1049 (42±2 mm) – the causative agent of black rot in cabbage crops, *P. syringae* UCM B-1027 (37±2 mm) – the causative agent of bacterial blight in a wide range of plants, and *P. carotovorum* UCM B-1075 (35±2 mm) – the causative agent of soft bacterial rot in vegetable crops. Moderate activity *of B. amyloliquefaciens* E12 against *P. fluorescens* 8573 (26±1 mm) and *C. michiganensis* subsp. *michiganensis* 10_2 (22±1 mm) was also determined. The zone of growth inhibition of microfungi was 18±2 mm for *A. alternata* GTA2 and 15±1 mm for *F. oxysporum* GTF1. The lack of activity *of B. amyloliquefaciens* E12 against *A. tumefaciens* UCM B-1000 may be associated with the specific resistance of this pathogen to *Bacillus* antimicrobial metabolites.

The results obtained are consistent with numerous studies of the antagonistic activity of bacilli. Thus, Issazadeh et al. (2012) and Mougou et al. (2018) have determined that *Bacillus* spp. exhibit high antagonistic activity against *Xanthomonas* spp. and *Pectobacterium* spp.

(inhibition zone 14-30 mm), which depends on the production of specific lipopeptides by endophytes, and the ability *of Bacillus* spp. to effectively inhibit *P. syringae* (10-20 mm) through the production of surfactin.

The results of the antagonistic activity *of B. amyloliquefaciens* E12 against phytopathogens (inhibition zones of 15-42 mm for different pathogens) are in the upper range of effectiveness described in the literature, which indicates the high biocontrol potential of the isolated strain.

Differences in the sensitivity of various pathogens to endophytic bacteria *B. amyloliquefaciens* E12 have been determined. Higher activity against Gram-negative bacteria (*X. campestris* UCM B-1049, *P. syringae* UCM B-1027, *P. carotovorum* UCM B-1075) compared to the Gram-positive pathogen *C. michiganensis* UCM B-1075 can be explained by the peculiarities of the structure of cell walls.

Among microfungi, the higher sensitivity *of A. alternata* GTA2 (18 mm) compared to *F. oxysporum* GTF1 (15 mm) may be associated with differences in cell wall structure. *Fusarium* spp. have a thicker cell wall with a high content of β-1,3-glucans and chitin, which provides higher resistance to *Bacillus* lytic enzymes. *Alternaria* spp. have thinner cell walls and higher sensitivity to antifungal lipopeptides, in particular iturins and fungicins (Ongena et al., 2008).

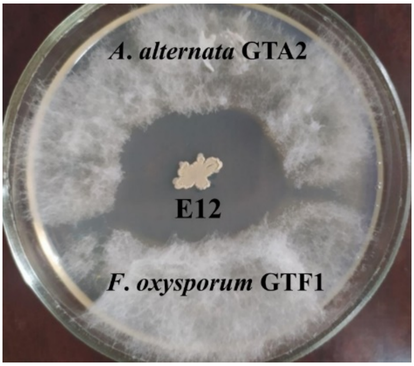

Figure 4. Antagonistic activity of endophytic bacteria *B. amyloliquefaciens* E12 against phytopathogenic microfungi *A. alternata* GTA2 and *F. oxysporum* GTF1

Visual observation revealed not only a delay in the radial growth of pathogens, but also morphological changes in the areas affected by *B. amyloliquefaciens* E12. The mycelium of microfungi became denser, partial fragmentation of hyphae and suppression of sporulation were observed (Figure 4).

The broad spectrum of antagonistic activity *of B. amyloliquefaciens* E12 against phylogenetically different forms of pathogens is due to the ability of this species to produce antimicrobial compounds with different mechanisms of action. *B. amyloliquefaciens* is capable of synthesising more than 20 different antimicrobial metabolites, which can be classified into several groups: lipopeptides, polyketides, ribosomal peptides and lytic enzymes (Chen et al., 2009; Ongena et al., 2008).

Competition for nutrients plays an important role in biocontrol. The high metabolic potential *of B. amyloliquefaciens* E12, confirmed by VITEK® 2 Compact analysis (ability to utilise a wide range of carbohydrates and complex polysaccharides), provides a competitive advantage in the rhizosphere and phyllosphere of plants. Rapid consumption of available nutrients limits the access of pathogens to the resources necessary for growth and colonisation (Compant et al., 2005). *B. amyloliquefaciens* produces siderophores, substances that bind iron ions. Iron deficiency critically limits the growth of most phytopathogens, as iron is necessary for the functioning of many enzymes in the respiratory chain (Chen et al., 2009; Borriss, 2011).

The production of volatile organic compounds also contributes to biocontrol. Some VOCs, such as pyrazines, have a direct antimicrobial effect, inhibiting the growth of phytopathogens and fungal sporulation (Kai et al., 2009). VOCs can also induce systemic resistance in plants by enhancing the defence response (Ryu et al., 2004).

Multifactorial biocontrol mechanisms act synergistically, ensuring stable antagonistic activity *of B. amyloliquefaciens* E12 under various environmental conditions and allowing endophytes to effectively compete with pathogens in complex microbial communities of the rhizosphere and phyllosphere (Borriss, 2011). The combination of direct antimicrobial action, competition for nutrient resources, production of lytic enzymes, and induction of plant resistance characterises *B. amyloliquefaciens* E12 as a promising agent for the development of multifunctional biological products for agriculture.

Protective and growth-stimulating effect of *B. amyloliquefaciens* E12 on wheat seeds in vivo

When assessing the effect of endophytic bacteria *B. amyloliquefaciens* E12 on soft spring wheat seeds (Pecheryanka variety), no phytotoxic effect and a positive effect on the growth parameters *of Triticum aestivum* L. were determined (Table 4).

Table 4

Effect of *B. amyloliquefaciens* E12 on the germination (G) and growth of Pecherianka wheat

Treatment option	G (%)	Shoot length				Fungal damage
		4 days		7 days		
		cm	%	cm	%	
Control (water)	90	1.1±0.08	100	6.5±0.34	100	+ (10% seeds)
E12 (synthetic medium, 1:50)	94	1.7±0.12	154	9.5±0.48	146	- (0%)
E12 (synthetic medium, 1:100)	90	1.5±0.10	136	7.5±0.37	115	- (0%)
E12 (Potato Dextrose Broth, 1:50)	91	1.5±0.13	136	7.0±0.35	107	- (0%)
E12 (Potato Dextrose Broth, 1:100)	90	1.3±0.09	118	6.6±0.33	101	- (0%)

Seed germination after treatment with *B. amyloliquefaciens* E12 cell suspension was 90-94%, which did not differ from the control values (90%) and indicates no negative effect of bacteria on seed viability. This is a critically important result, as some *Bacillus* strains can exhibit phytotoxicity at high concentrations due to excessive production of antibiotics or volatile organic compounds. The absence of phytotoxicity in the endophytic isolate *B. amyloliquefaciens* E12 confirms its safety for use in biological products.

The height of one-week-old wheat shoots treated with endophytic bacteria *B. amyloliquefaciens* (synthetic medium, dilution 1:50 and 1:100) was 9.5±0.5 cm and 7.5±0.4 cm, respectively, compared to 6.3±0.3 cm in the control, which is an increase of 46% and 15%, respectively. This result is biologically significant, as the positive effect was observed already 7 days after seed treatment, indicating rapid activation of growth processes. An increase in the height of wheat shoots was observed at both dilutions (1:50 and 1:100), indicating the stability of growth-stimulating activity over a wide range of bacterial suspension concentrations.

A comparison with international studies shows that our results are consistent with the literature data on the growth-stimulating activity of *Bacillus* spp. During the studies (Tahir et al., 2017), it was determined that under laboratory conditions, *B. subtilis* SYST2, producing acetoin, 2,3-butanediol and other phytohormones, increases the length of *Arabidopsis* shoots and roots by 3 times in laboratory conditions, while in field conditions the effect can reach 20-30%. It has also been determined that *Bacillus* spp. usually increase shoot biomass in laboratory conditions less than in field conditions due to rhizosphere colonisation and longer interaction of microorganisms with the plant. Scientists (Maslennikova et al., 2023) found that a mixture *of B. subtilis* and *B. amyloliquefaciens* increases potato yield by about 16% in field conditions. Our results (46% increase in shoot height at a dilution of 1:50 and 15% at a dilution of 1:100 over 7 days) are within the range of effectiveness typical for short-term laboratory experiments. The pronounced effect at the maximum concentration of the bacterial suspension (1:50 dilution) indicates the dose-dependent growth-stimulating activity of *B. amyloliquefaciens* E12. During field trials with long-term monitoring, a more stable growth-stimulating effect can be observed at all stages of plant ontogenesis.

Growth-stimulating activity was also determined when *B. amyloliquefaciens* E12 was cultivated on Potato Dextrose Broth at dilutions of 1:50 and 1:100. The height of one-week-old wheat shoots was 7.0±0.35 cm (1:50 dilution) and 6.6±0.33 cm (1:100 dilution) compared to 6.5±0.34 cm in the control, corresponding to an increase of 7% and 1%, respectively. The lower growth-stimulating activity compared to the results in a synthetic medium may be associated with less efficient production of biologically active metabolites in Potato Dextrose Broth.

The increase in shoot height may be associated with several mechanisms of growth-stimulating action. *B. amyloliquefaciens* is capable of synthesising phytohormones, such as auxins, cytokinins, gibberellin, jasmonic acid and salicylic acid, which stimulate cell elongation and meristem division (Luo et al., 2022).

An important result of the *in vivo* experiment is the discovery of pronounced protective properties of the endophytic isolate *B. amyloliquefaciens* E12. During the germination of wheat seeds treated with a suspension of *B. amyloliquefaciens* E12 cells, no cases of seed infection by microfungi were recorded during the 7 days of the experiment (0% of seeds infected, n=90). The seeds remained visually healthy, with no signs of mycelial growth or necrotic changes. However, in the control variant (seed treatment with sterile water), 10% of seeds were infected with surface saprotrophic microfungi, which is typical for humid chamber conditions. The damage manifested itself in the form of visually noticeable mycelial growth and, in some cases, led to the inhibition of germination or death of seedlings due to the release of mycotoxins and phytotoxic metabolites by fungi.

The complete absence of fungal lesions (100% protection) when wheat seeds were treated with the endophytic isolate *B. amyloliquefaciens* E12 is a significant finding in the study. The antifungal activity of the isolated strain, detected *in vitro*, was confirmed, and it was determined that antimicrobial metabolites are produced in biologically significant concentrations under real conditions of bacteria-plant interaction. The protective effect persisted for 7 days, indicating the stability of antifungal metabolite production and, possibly, the colonisation of the wheat seed surface by *B. amyloliquefaciens* E12 microorganisms with the formation of a protective biofilm that creates a physical barrier to pathogens. Protection was provided against specific saprotrophic fungi (*Aspergillus*, *Penicillium*, *Mucor*), confirming the multi-purpose biocontrol mechanism *of B. amyloliquefaciens* E12 and indicating the production of various antifungal metabolites.

An analysis of international studies has determined that our results correspond to the level of effective *Bacillus* biocontrol strains described in the literature. Scientists (Benhamou et al., 1998) found that *Bacillus* spp. protect tomatoes and plants of the cabbage family from *F. oxysporum* when seeds are treated before sowing. Researchers (Maslennikova et al., 2023) determined that a mixture *of B. subtilis* and *B. amyloliquefaciens* effectively inhibits the growth of *Rhizoctonia solani* microfungi on potatoes, with the disease development index decreasing from 40.9% to 12.0%. Our results (100% protection) confirm the high biocontrol potential of the endophytic isolate *B. amyloliquefaciens* E12 and its competitiveness compared to effective commercial strains.

The mechanisms of protective action *in vivo* include several interrelated processes. *B. amyloliquefaciens*, as endophytic microorganisms, are capable of rapidly colonising the seed surface and forming biofilms – structured microbial communities that create a physical barrier to pathogens and ensure stable production of antimicrobials. Lipopeptide antibiotics (iturins, fungicins) and polyketides are produced directly on the seed surface in high local concentrations, which effectively inhibits fungal spore germination and mycelium growth by disrupting the integrity of cell membranes (Ongena et al., 2008).

The ability of *B. amyloliquefaciens* E12 to provide 100% protection of seeds against fungal infections is critical for practical application. Fungal infections are the main cause of seed germination loss during storage, especially in conditions of high humidity, when saprotrophic fungi are activated, leading to damping-off syndrome and deterioration of sowing qualities (Perez-Garcia et al., 2011). Biological treatment of seeds with endophytic microorganisms *B. amyloliquefaciens* E12 can replace or supplement chemical seed dressings (thiocarbamates, thiabendazoles, triazoles, etc.), reducing the environmental impact, risks to human health and the problem of pathogen resistance to fungicides (Thakur et al., 2020).

Analysing the results of the study of the effect of endophytic isolate on wheat seeds, the dual functionality of *B. amyloliquefaciens* E12 was determined: a pronounced growth-stimulating effect (46% increase in shoot height at a dilution of 1:50 and 15% at a dilution of 1:100 within 7 days), which is a biologically significant effect for a short-term laboratory experiment, and an absolute protective effect (100% protection against fungal infections), which corresponds to the high level of biocontrol of effective commercial *Bacillus* strains. This combination of properties is optimal for the creation of biological products for pre-sowing treatment of grain and vegetable seeds, since a biological product based on endophytic bacteria *B. amyloliquefaciens* E12 can simultaneously protect seeds from pathogens during storage and germination and stimulate early seedling growth, increasing the field germination of plants.

The results obtained are comparable to the characteristics of commercial *Bacillus*-based biological products. The biological product RhizoVital® (ABiTEP GmbH) based on *B. amyloliquefaciens* FZB42 is a biofertiliser with stimulating activity and provides protection against various soil diseases. RhizoPlus® (ABiTEP GmbH) based on *B. subtilis* FZB24 promotes the growth of rhizobacteria and is a biocontrol agent for potatoes, corn, vegetables and fruits. Serenade® (AgraQuest) based on *B. subtilis* QST713 is used for the prevention and control of soil phytopathogens, providing protection for vegetable, fruit and grape crops. SONATA® (AgraQuest), based on *B. pumilus* QST2808, is a biofungicide for the control of powdery mildew (Borriss, 2011).

The endophytic origin of *B. amyloliquefaciens* E12 may provide additional benefits compared to rhizospheric or epiphytic strains. Endophytes are adapted to penetrate plant tissues and establish long-term symbiotic associations through specific mechanisms that overcome the plant's immune response and effectively colonise intercellular spaces. Endophytic colonisation provides protection to the plant throughout the growing season, unlike epiphytic strains, which can be washed away by rain, suppressed by ultraviolet radiation, or eliminated by competitive phyllosphere microbiota. Vertical transmission of endophytes through seed material ensures intergenerational support of symbiosis, which contributes to the stability of protective mechanisms in subsequent generations of plants without the need for re-inoculation (Hardoim et al., 2015; Santoyo et al., 2016). Therefore, given the described properties of endophytic microorganisms, the isolated *B. amyloliquefaciens* E12 is promising for the development of prolonged-action biological products for agriculture and the food industry.

Metabolic profile of *B. amyloliquefaciens* E12: production of volatile organic compounds

During the analysis of volatile organic compounds (VOCs) by vapour-phase gas chromatography with a mass spectrometric detector, a diverse metabolic profile of the endophytic isolate *B. amyloliquefaciens* E12 was revealed, which depends on the composition of the culture medium (Table 5).

Table 5

Profile of volatile organic compounds of the endophytic isolate *B. amyloliquefaciens* E12 on different culture media

Compound	Function	Concentration, ppm	
		Synthetic medium	Potato Dextrose Broth
Acetoin	Growth stimulator	130.3 ± 1.5	45.2 ± 1.5
2,3-Butanedione	Growth stimulator	12.5 ± 0.5	8.1 ± 0.4
Pyrazines	Protective / signalling	0.5 ± 0.1	2.0 ± 0.2
Phenylacetaldehyde	Auxin precursor / growth stimulator	n/d	0.8 ± 0.1
Isovaleric acid	Protective	1.2 ± 0.1	2.5 ± 0.2

Note: n/d - not detected.

In a synthetic medium (limited nitrogen content and high glucose content - 5 g/L), the isolated endophyte *B. amyloliquefaciens* E12 induced high production of acetoin (130.3 ppm), moderate production of 2,3-butanedione (12.5 ppm) and isobutyric acid (1.2 ppm). In potato dextrose broth for *B. amyloliquefaciens* E12, a change in metabolism to the biosynthesis of nitrogen-containing heterocycles – pyrazines (2.0 ppm) and aromatic precursors of auxins – phenylacetaldehyde (0.8 ppm) was detected, and moderate production of acetoin (45.2 ppm), 2,3-butanedione (8.1 ppm) and isobutyric acid (2.5 ppm) was determined.

The results of the variability of the metabolic profile *of B. amyloliquefaciens* E12 confirm the adaptation of the isolated endophyte to different nutrient media through changes in biosynthetic pathways. Under conditions of carbon excess, the butanediol pathway (acetoin/2,3-butanediol pathway) is activated, which is an alternative pathway for pyruvate utilisation during intensive glucose fermentation. This pathway performs a homeostatic function – it maintains intracellular pH during the accumulation of organic acids by removing excess pyruvic acid into neutral products according to the following scheme: Pyruvate → α-Acetolactate → Acetoin → 2,3-Butanediol (Xiao et al., 2007). This biochemical characteristic allows *Bacillus* to maintain a high rate of glycolysis without acidifying the cytoplasm, which is critical for survival in environments with high sugar content and for activating plant growth.

Under conditions of balanced carbon and nitrogen content (potato dextrose broth), metabolism is partially reoriented towards amino acid catabolism, leading to the formation of nitrogen-containing heterocyclic compounds. Pyrazines are synthesised through the condensation of amino acids (leucine, glycine, lysine) with α-dicarbonyl compounds via the Maillard reaction (Wang et al., 2021). Phenylacetaldehyde is formed as an intermediate metabolite of the phenylacetic pathway during the degradation of phenylalanine according to the following scheme: Phenylalanine → Phenylpyruvate → Phenylacetaldehyde →

Phenylacetic acid. This metabolic variability allows *Bacillus* spp. to function effectively in various ecological niches, from sugar-rich root exudates to protein-rich plant residues (Borriss, 2011).

The results obtained are consistent with the literature data. Kai et al. (2009) and Almeida et al. (2023) have determined that the profile of VOCs produced by *B. amyloliquefaciens* depends on the composition of the nutrient medium: the addition of glucose can induce the production of β-phenylethanol, butanedione and acetoin, while the absence of glucose and enrichment of the medium with nitrogen leads to a change in metabolism with the formation of pyrazines and benzaldehyde.

The most important result of metabolomic analysis is the identification of endophytic bacteria *B. amyloliquefaciens* E12 as highly efficient producers of acetoin (3-hydroxy-2-butanone), a compound with growth-stimulating and stress-protective activity. When cultivating the isolated strain on a synthetic medium, a significant content of acetoin (about 130 ppm) was determined, which is consistent with the results of similar studies described in the literature for natural strains of *Bacillus* spp. During a comparative analysis, it was determined that *B. subtilis* GB03 produces 2,3-butanediol and acetoin, which stimulate the growth of *Arabidopsis* and protect the plant from the effects of phytopathogens (Ryu et al., 2004). *B. subtilis* SYST2 produces 1,3-propanediol, acetoin and phytohormones that stimulate the growth of tomato seedlings (Tahir et al., 2017).

Acetoin is a multifunctional metabolite that performs various functions in bacterium-plant interactions. The growth-stimulating effect of acetoin is associated with the induction of systemic plant resistance and stimulation of phytohormone biosynthesis. The mechanism involves the activation of salicylateand jasmonate-dependent signalling cascades, induction of cytokinin and auxin biosynthesis gene expression, and enhancement of photosynthesis through increased chlorophyll content and photosystem efficiency, leading to increased photosynthetic productivity (Tahir et al., 2017).

At the bacterial cell level, acetoin performs a homeostatic function – it maintains the pH of the cytoplasm during intensive glucose fermentation via the butanediol pathway (Xiao et al., 2007).

The high production of acetoin by the isolate *B. amyloliquefaciens* E12 may be related to its endophytic origin. Endophytic bacteria that colonise the internal tissues of plants are found in specific microaerobic conditions with high sugar concentrations and limited access to nitrogen (Hardoim et al., 2015). These conditions promote the activation of the butanediol pathway, and acetoin production may be an adaptive strategy for establishing mutualistic relationships with the plant, providing a growth-stimulating and stress-protective effect (Santoyo et al., 2016).

Changes in the metabolic profile are an important characteristic of plant growth-promoting bacteria for effective functioning in the dynamic conditions of the rhizosphere, where the composition of exudates varies depending on the stage of plant development (young roots secrete a lot of sugars, ageing tissues secrete amino acids and peptides), stress conditions (accumulation of osmolytes during drought or salinisation) and microbial competition for specific substrates (Compant et al., 2005). The ability of *B. amyloliquefaciens* E12 to adapt its metabolism to changing environmental conditions ensures the stability of rhizosphere colonisation by endophytes.

Conclusions

During comprehensive identification (MALDI-TOF mass spectrometry, VITEK® 2 Compact, phenotypic markers), the endophytic isolate E12 was confirmed to belong to the species *Bacillus amyloliquefaciens* (91.0% confidence). Differentiation from the closely related *B. subtilis* was performed based on characteristic features: the ability to form cell chains and the inability to utilise inulin.

It was found that endophytic bacteria *B. amyloliquefaciens* E12 have significant biocontrol potential, exhibiting antagonistic activity against phytopathogenic microfungi (*F. oxysporum* GTF1, *A. alternata* GTA2; growth inhibition zones 15-18 mm) and bacteria (*C. michiganensis* subsp. *michiganensis* 10_2 , *P. fluorescens* 8573, *P. carotovorum* UCM B-1075, *P. syringae* UCM B-1027, *X. campestris* UCM B-1049; inhibition zones 22-42 mm). When assessing the biological activity of endophytic bacteria *B. amyloliquefaciens* E12 on wheat seeds *in vivo*, growth-stimulating potential (46% increase in shoot height) and high protective efficacy were determined, which was expressed in complete (100%) suppression of the development of fungal diseases by the isolated strain.

It has been established that *B. amyloliquefaciens* E12 is an effective producer of acetoin and is characterised by its ability to change metabolic biosynthetic pathways from the production of growth-stimulating metabolites (acetoin, 2,3-butanedione, phenylacetaldehyde) to protective signalling compounds (pyrazines, isobutyric acid) depending on the composition of the culture medium. This metabolic variability characterises the dual biotechnological functionality of the endophytic isolate.

The comprehensive characterisation of the endophytic isolate *B. amyloliquefaciens* E12 confirms its potential for the development of environmentally safe biological products aimed at protecting and stimulating the growth of agricultural crops. High production of growth-stimulating metabolites, effective biocontrol activity against pathogens of major bacterial and fungal plant diseases, and the absence of phytotoxicity create a scientific basis for the further biotechnological application of endophytic bacteria *B. amyloliquefaciens* E12 in biological protection and plant growth stimulation systems in agriculture and the food industry.

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Improved production of selenium nanoparticles using lactic acid bacteria: Role of strain selection and process parameters

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Abstract

Introduction. The aim of this study was to enhance the biotransformation efficiency of inorganic selenium into selenium nanoparticles (SeNPs) through the selection of lactic acid bacteria (LAB) strain composition and determination of biosynthesis parameters.

Materials and methods. To select the LAB composition, strains from the genera *Lactobacillus*, *Streptococcus*, *Lactococcus*, *Leuconostoc,* and *Bifidobacterium* were used. Quantitative determination of SeNPs was carried out spectrophotometrically. Residual sodium selenite was measured spectrophotometrically after reduction to elemental selenium using ascorbic acid. The morphological and elemental characteristics of the synthesized SeNPs were examined using scanning electron microscopy (SEM) and energy-dispersive X-ray spectroscopy (EDX).

Results and discussion. For individual strains and a two-strain composition, cultivation parameters were established, including inoculum concentration (10%), Na_2SeO_3 concentration, agitation speed (110 rpm), cultivation time (72 h), and strain-specific Na_2SeO_3 addition time. Under these conditions, high SeNP concentrations were achieved: *Lactobacillus plantarum* 3201 – 32.21 ± 0.13 µg/mL, *Lactobacillus bulgaricus* 3511 – 31.04 ± 0.19 µg/mL, *Lactococcus cremoris* 1220 – 27.65 ± 0.10 µg/mL, *Streptococcus thermophilus* 2192 – 27.96 ± 0.09 µg/mL, and *Bifidobacterium longum* 4205 – 27.88 ± 0.15 µg/mL. The combination of *L. bulgaricus* and *L. cremoris* (LBLC) produced 38.92 ± 0.23 µg/mL SeNPs, corresponding to 78.27% of the total transformed selenium, with a bacterial biomass survival rate of 78.01%. SEM and EDX analyses confirmed the formation of amorphous, spherical SeNPs with sizes ranging from 76–458 nm (mean 260.95 ± 70.88 nm). The particle size distribution showed two frequency peaks (150–175 nm – 11.05%; 225–250 nm – 17.4%) and one volume-based peak (275–300 nm), with d10, d50, and d90 values of 232, 313, and 400 nm, respectively.

Conclusions. The results obtained under established conditions demonstrate the high potential of the composition LBLC with SeNPs for incorporation into functional food products as a bioavailable source of selenium.

Introduction

The use of lactic acid bacteria (LAB) capable of biotransforming inorganic selenium into selenium nanoparticles (SeNPs) has gained increasing attention as a promising strategy to address selenium deficiency through the development of functional foods enriched with this essential trace element (Kieliszek and Serrano-Sandoval, 2023; Salama et al., 2021; Stabnikov et al., 2025; Stabnikova et al., 2023; Wang et al., 2025). Biosynthesized SeNPs are also considered a promising alternative to conventional dietary supplements containing organic selenium forms, due to their potentially lower toxicity and improved bioavailability. Toxicity decreases as LD_{50} increases. Mice administered Se-methionine and Se-cysteine had LD_{50} values of 8.8 ± 1.37 mg Se/kg and 12.6 mg Se/kg body weight, respectively, whereas SeNPs showed a much higher LD_{50} of 198.1 mg Se/kg, corresponding to approximately 23- and 16-fold lower toxicity (Schrauzer, 2000; Shakibaie et al., 2012; Yang and Jia, 2014).

The ability of LAB to convert inorganic selenium into reduced forms varies widely and depends both on strain-specific characteristics and on the conditions under which the process is performed. Such ability has been reported for *Lactobacillus acidophilus, L. rhamnosus, L. casei, L. delbrueckii* subsp. *bulgaricus, L. plantarum, L. fermentum, L. bulgaricus, L. helveticus, L. reuteri, L. brevis, Streptococcus thermophilus,* and *Bifidobacterium,* among others (Crespo et al., 2021; El-Saadony et al., 2021; Gregirchak et al., 2023; Husen and Siddiqi, 2014; Kurek et al., 2016; Martínez et al., 2020; Pescuma et al., 2017; Pophaly et al., 2014; Spyridopoulou et al., 2021; Stabnikov et al., 2025).

The main group of SeNP-producing bacteria belongs to the *Lactobacillus* genus, which remains the most extensively studied. *Streptococcus thermophilus* exhibits better tolerance to high sodium selenite concentrations compared to *Lactobacillus* species, with growth inhibition observed at concentrations of up to 200 mg/L (Castañeda-Ovando et al., 2019). Representatives of the *Bifidobacterium* genus remain the least studied microorganisms capable of converting inorganic selenium compounds. Nevertheless, several reports demonstrate their ability to transform sodium selenite into both Se-containing organic molecules and elemental selenium. For example, cultivation of *Bifidobacterium* in a medium supplemented with 1 mM sodium selenite yielded 33.3 mg of total transformed selenium per gram of biomass (Stabnikova et al., 2023).

Inoculum based on LAB biomass often combine multiple bacterial strains to broaden functional properties. However, comparative studies of monocultures (*L. casei, L. acidophilus, L. plantarum*) and a three-strain composition revealed higher selenium conversion efficiency in the monocultures (Salama et al., 2021). The key factors influencing selenium biotransformation by LAB are the selenium source and its concentration in the culture medium (Liao and Wang, 2022). Sodium selenite (Na_2SeO_3) is the most commonly used inorganic source (Stabnikova et al., 2023). Reported concentrations vary widely: when the level exceeds 4 mg/L, the metabolic pathway typically shifts from organic selenium compound formation (e.g., selenoamino acids) toward nanoparticle synthesis, which are a less toxic form for microbial cells. Most studies employ sodium selenite at concentrations ranging from 20 to 200 mg/L (Stabnikova et al., 2023), although higher concentrations of up to 1000 mg/L have also been reported (Wang et al., 2025). Most available data address total selenium accumulation without specifying the fraction converted to nanoparticles. The highest reported selenium accumulation (65.00 mg/L) was achieved by *L. paracasei* CH135 (calculated from source data) (Morschbacher et al., 2018), whereas the maximum SeNP yield, 19.76 mg/L, was reported for *L. bulgaricus* (Xia et al., 2007). To further enhance SeNP synthesis, optimization of additional factors, such as pH, cultivation temperature, reaction duration, agitation speed, and inoculum concentration, has been explored (El-Saadony et al., 2021; Yang et al., 2017).

Nevertheless, further studies are needed to determine the optimal cultivation parameters and LAB composition for developing functional supplements in which selenium is predominantly present in nanoparticle form.

Materials and methods

Bacterial strains and culture medium

The study utilized lactic acid bacteria obtained from the Institute of Food Resources of the National Academy of Agrarian Sciences of Ukraine. The strains used were: *Lactobacillus casei* 3321, *Lactobacillus plantarum* 3201, *Lactobacillus acidophilus* 3103, *Lactobacillus bulgaricus* 3511, *Lactobacillus brevis* 3900, *Streptococcus thermophilus* 2192, *Lactococcus cremoris* 1220, *Lactobacillus rhamnosus* 3303, *Leuconostoc lactis* 1404, *Lactococcus lactic* 1107, and *Bifidobacterium longum* 4205. Hydrolyzed milk was used as the growth medium, prepared by reconstituting skimmed milk powder, enzymatic hydrolysis with protosubtilin, and subsequent filtration.

Preparation of inoculum and lactic bacteria cultivation

Collection cultures of lactic acid bacteria were stored in hydrolyzed milk at a temperature of 2–4 °C with periodic subculture. The seed material was prepared by inoculating 50 mL of nutrient medium in 250 mL flasks and incubating for 48 h at 37 °C with shaking in an ES-20/60 orbital shaker-incubator (Biosan SIA, Latvia).

Determination of biomass concentration

Biomass concentration was determined gravimetrically. Aliquots of the culture liquid (10 mL) were transferred to centrifuge tubes and centrifuged at 8,000 rpm for 20 min. The supernatant was decanted, and the pellet was washed three times with 10 mL of PBS. The washed pellet was resuspended in 10 mL of PBS and filtered through pre-weighed filter paper. The filter paper was then dried to constant weight at 105 °C for approximately 60 min and reweighed. Biomass concentration (g/L) was calculated from the difference between the filter mass before and after filtration.

Biotransformation of sodium selenite into selenium nanoparticles

A 25% solution of sodium selenite (Na_2SeO_3) served as the inorganic selenium source. Prior to application, the solution was filtered through a 0.22 µm pore-size filter.

Analysis of the ability to biotransform sodium selenite into selenium nanoparticles (Se-NPs)

The culture liquid (20 mL) was transferred into centrifuge tubes and centrifuged for 15–20 minutes at 8,000 rpm. The supernatant was decanted into a separate container. The presence of Se-NPs was assessed visually based on the color of the pellet: a white pellet indicated pure biomass, whereas a red pellet indicated biomass containing SeNPs.

Determination of Se-NPs

The quantitative determination of Se-NPs in the samples was performed by converting them into a soluble form through the addition of sodium sulfide (Na_2S), followed by spectrophotometric measurement. The obtained values were compared with those of standard solutions using a calibration curve. This method has been employed in several studies (Biswas et al., 2011; Wang et al., 2025) and was modified to suit the conditions of the present study.

Preparation of standard solutions. Standard solutions (10–250 µg/mL) were prepared by adding calculated amounts of 0.25% sodium selenite to test tubes and bringing the volume to 1 mL with distilled water. A 0.1 M ascorbic acid solution was then added, and the mixture was incubated for 30 min to allow the reduction reaction and color development. The total volume of each sample was adjusted to 6 mL with distilled water. Subsequently, 0.1 mL of 1 M sodium sulfide was added, followed by a 10 min incubation to stabilize the color. Freshly prepared samples were immediately analyzed using a spectrophotometer (Thermo Spectronic UV300, Spectronic Unicam, England) at 500 nm.

Preparation of experimental samples. The culture liquid (20 mL) were transferred to centrifuge tubes and centrifuged at 8,000 rpm for 15–20 min. The supernatant was decanted, and the pellet was washed three times to remove residual medium and unmetabolized sodium selenite by resuspending it in 10 mL of 1 M NaCl, followed by centrifugation under the same conditions.

The washed pellet was resuspended in 1 mL of distilled water, after which 1.5 mL of 0.1 M ascorbic acid solution was added. The total volume was adjusted to 6 mL with distilled water. Subsequently, 0.1 mL of 1 M sodium sulfide was added, and the mixture was incubated for 10 min to stabilize the color. The samples were then centrifuged at 15,000 rpm for 3 min to separate the biomass pellet.

Freshly prepared samples were immediately analyzed using a spectrophotometer at 500 nm, and absorbance values were compared with the calibration curve obtained from the standards.

Determination of residual sodium selenite

The quantitative determination of residual sodium selenite in the solution was carried out based on its reduction to elemental selenium (Se^0) in the presence of ascorbic acid (Shahabadi et al., 2021), followed by spectrophotometric measurement. The obtained values were compared with those of standard solutions using a calibration curve.

The calibration curve for SeNP determination was constructed based on measurements of standard solutions of sodium selenite with concentrations ranging from 10 to 200 µg/mL. Calculated amounts of 0.25% sodium selenite were added to test tubes, followed by 1 mL of distilled water. Then, 0.1 M ascorbic acid solution was added, and the mixture was incubated for 30 minutes to allow the reduction reaction and stabilize the color change. The total volume of each sample was adjusted to 6 mL by adding distilled water. Freshly prepared samples were immediately measured using a spectrophotometer at a wavelength of 300 nm.

For the determination of experimental samples, 1 mL of the supernatant was mixed with 1.5 mL of 0.1 M ascorbic acid solution and incubated for 30 minutes to stabilize the color change. The solution changed its color from light yellow to red. After incubation, the total sample volume was adjusted to 6 mL by adding distilled water. The samples were then centrifuged at 15,000 rpm for 3 minutes to remove possible residual biomass particles. The freshly prepared samples were immediately analyzed using a UV-300 spectrophotometer at a wavelength of 300 nm.

Determination of SeNP biosynthesis conditions

The influence of key parameters, namely inoculum concentration and sodium selenite concentration in the medium, was evaluated by conducting experiments to assess their direct effects within defined ranges for each strain. Measurements included accumulated biomass concentration, residual sodium selenite in the medium, and synthesized selenium nanoparticle (SeNPs) concentration. Based on these values, the degree of sodium selenite utilization, the fraction of selenium present as nanoparticles, and the biomass survival rate were calculated.

Additional parameters, such as agitation speed and the timing of sodium selenite addition, were examined by assessing their parallel effects at different settings.

To design and evaluate the multifactorial experiment for optimizing SeNP synthesis, a mathematical experimental design approach was applied, including response surface construction using the least squares method. A full-factorial experimental design was implemented, with all measurements performed in parallel for each strain. SeNP concentration served as the criterion of optimality.

The lactic acid bacterial strains were cultivated under conditions in which one or more parameters were varied according to the experimental matrix, and all experiments were conducted in randomized order.

Morphology and size characterization of selenium nanoparticles (SeNPs)

Samples for SEM and EDX analyses were prepared using a modified protocol previously described for lactic acid bacteria associated with selenium nanoparticles (Martínez, 2020).

Culture suspensions were centrifuged at 8,000 rpm for 20 min to pellet the cells. The resulting biomass was washed three times with phosphate-buffered saline (PBS).

A 4% glutaraldehyde solution was added to the sediment as a fixative to stabilize the cellular structure. Samples were incubated for 2 h at room temperature. After fixation, the samples were washed three times with PBS to remove residual fixative.

Dehydration was performed by sequential incubation in an ethanol gradient (30%, 50%, 70%, 80%, 90%, and 100%), holding the samples for 10 min at each concentration. After centrifugation with 100% ethanol, the residual solvent was partially removed, and the samples were air-dried for 4 h until complete evaporation of ethanol. The dried samples were placed in a vacuum chamber, where a gold/palladium alloy layer (~20 nm thick) was sputter-coated onto the surface in the presence of argon.

Microscopic analysis was carried out using a TESCAN MIRA3 scanning electron microscope equipped with BSE and SE detectors.

Morphology and size of SeNPs were examined using a Tescan MIRA3 scanning electron microscope (SEM) (Tescan Orsay Holding, Czech Republic) equipped with both backscattered (BSE) and secondary (SE) electron detectors. The dimensional characteristics of the nanoparticles and bacterial cells were evaluated by analyzing SEM images using the integrated image analysis software.

The particle size distribution was determined by direct measurement of individual nanoparticles in several random fields of view (field size 5–15 µm; total n = 500 particles). The following parameters were calculated: mean particle size, standard deviation (SD), d10, d50, d90, and span. Particle distribution was assessed by constructing relative frequency, volume density, and cumulative volume density plots.

The relative fraction of particles was calculated for each size range (step = 25 nm, within 50–500 nm) according to the equation (1):

$$\text{Relative fraction, \%}= \frac{N_i}{\sum N_i}, \tag{1}$$

where N_i is the number of particles within a given size range, and $\sum N_i$ is the total number of particles analyzed (n = 500).

The volume fraction for each size range was calculated by formula (2):

$$\text{Volume fraction, \%}= \frac{V_i}{\sum V_i}, \tag{2}$$

where V_i is the total volume of particles within a given range, and $\sum V_i$ is the sum of the total particle volumes.

The volume of each particle was calculated using the equation (3):

$$V = \frac{4}{3}\pi\left(\frac{d_i}{2}\right)^3 \tag{3}$$

where d_i is the measured diameter of the particle.

Cumulative volume densities were determined by summing the individual particle volumes in ascending order of size. These data were used to construct an integral cumulative volume curve.

The particle sizes corresponding to 10%, 50%, and 90% cumulative volume densities were designated as D10, D50, and D90, respectively.

The span, indicating the width of the particle size distribution, was calculated by formula:

$$Span = \frac{D90-D10}{D50} \tag{4}$$

Elemental composition analysis

Elemental composition was analyzed using an EDX (Energy-Dispersive X-ray) detector integrated with a Tescan VEGA3 SBU scanning electron microscope (Tescan Orsay Holding, Czech Republic). After visual differentiation of nanoparticles and bacterial cells, two EDX measurements were performed: s1 — in a region containing potential SeNP aggregates to determine selenium content in the nanoparticles, and s2 — in a SeNP-free region to determine the elemental composition of the bacterial biomass alone.The obtained spectra were compared, and the elemental peaks were identified to determine the percentage composition of the major components in each measurement. The gold and palladium peaks originating from the sputter-coating process were excluded from the quantitative analysis.

Statistical analysis

All experimental assays were performed in three independent replicates for each sample to ensure data reliability and enable statistical comparison between experimental groups. Statistical analysis was carried out using Statistica software (StatSoft Inc., version 12) and Microsoft Excel.

Within-sample statistical evaluation of measurement results for each parameter was performed by calculating the arithmetic mean (Mean) and standard deviation (SD). The relative standard deviation (RSD) was used to assess data consistency, with an acceptability criterion of RSD ≤ 15%.

Between-sample statistical evalua`tion included testing the homogeneity of variances =sing Levene’s test, followed by one-`way analysis of variance (ANOVA) to assess the statistical significance of differences among groups. When significant effects were identified, Tukey's honest significant difference (HSD) test was performed for post-hoc pairwise comparisons.

Results and discussion

Confirmation of selenium nanoparticle formation

The ability of the studied bacterial strains to synthesize selenium nanoparticles was confirmed by the appearance of a characteristic red coloration in the medium, indicating the formation of amorphous elemental selenium in the sediment after cultivation with 50 µg/mL sodium selenite. This concentration was selected as a universal working level, since some strains (*Lactobacillus rhamnosus* 3303, *Leuconostoc lactis* 1404) did not produce detectable nanoparticles at lower selenite concentrations (10–20 µg/mL), whereas others showed only slight color changes at these levels.

These observations are consistent with previous reports describing red precipitate formation by various lactic acid bacteria exposed to selenite, including *Lactobacillus bulgaricus* (1–64 µg/mL) (Xia et al., 2007), *Lactococcus lactis* NZ9000 (0.6 mM ≈ 100 µg/mL) (Xu et al., 2019), *Lactobacillus delbrueckii* subsp. *bulgaricus* CICC 20247 (1000 mg/mL), among others.

Moreover, the change in the medium color to red during nanoparticle formation is a universal indicator, which has also been confirmed in studies on other microorganisms, such as the yeast *Saccharomyces cerevisiae* (Skrotska et al., 2025).

Quantitative assessment of SeNP synthesis by lactic acid bacteria

The ability of the studied strains to synthesize selenium nanoparticles was evaluated under standardized conditions (1 % inoculum, 50 µg/mL sodium selenite, 37 °C). After 24 h (Table 1), distinct color changes indicative of SeNP formation were recorded for *L. casei* 3321, *L. cremoris* 1220, *S. thermophilus* 2192, and *B. longum* 4205, which produced 3.25±0.06, 2.42±0.05, 1.25±0.01, and 2.42±0.04 µg/mL of SeNPs, respectively. The remaining strains produced lower SeNP concentrations, all below 0.68 µg/mL.

After 48 h, *L. casei* 3321 exhibited the highest SeNP level (4.21±0.04 µg/mL), while *L. bulgaricus* 3511 demonstrated the greatest overall increase (2.18 µg/mL). *L. cremoris* 1220 and *B. longum* 4205 reached 3–4 µg/mL, whereas *L. acidophilus* 3103, *L. brevis* 3900, and *S. thermophilus* 2192 accumulated 2–3 µg/mL. In contrast, *L. plantarum* 3201, *L. rhamnosus* 3303, *L. lactis* 1404, and *L. lactic* 1107 remained below 1 µg/mL, confirming their low reducing activity toward sodium selenite.

At 72 h, *L. plantarum* 3201 showed a delayed SeNP accumulation (3.26 ± 0.07 µg/mL; increase 2.83 µg/mL), while previously high producers reached only minor increases.

Cultivation beyond 72 h did not significantly change SeNP yields ($p > 0.05$). Given these results, a universal cultivation time of 72 hours was selected, since most cultures reached their maximum concentration of synthesized selenium nanoparticles within this period, with no significant changes observed thereafter.

Considering the low productivity of certain strains, subsequent optimization studies focused on *L. casei* 3321, *L. cremoris* 1220, *L. plantarum* 3201, *B. longum* 4205, *L. bulgaricus* 3511, *L. acidophilus* 3103, *S. thermophilus* 2192, and *L. brevis* 3900.

Table 1

Production of SeNPs, µg/mL, during LAB cultivation

Strains	Cultivation time, h			
	24	48	72	96
Lactobacillus casei SP14	3.25±0.06^{a}	4.21±0.04^{b}	4.25±0.01^{b}	4.26±0.08^{b}
Lactococcus cremoris SP03	2.42±0.05^{a}	3.73±0.06^{b}	3.82±0.08^{b}	3.81±0.04^{b}
Lactobacillus plantarum SP13	0.23±0.03^{a}	0.43±0.02^{a}	3.26±0.07^{b}	3.28±0.01^{b}
Bifidobacterium longum SP12	2.42±0.04^{a}	3.15±0.07^{b}	3.26±0.09^{b}	3.24±0.02^{b}
Lactobacillus bulgaricus SP21	0.25±0.02^{a}	2.43±0.01^{b}	3.01±0.04^{c}	2.99±0.07^{c}
Lactobacillus acidophilus SP09	0.42±0.01^{a}	2.32±0.04^{b}	2.92±0.06^{c}	2.92±0.05^{c}
Streptococcus thermophilus SP10	1.25±0.01^{a}	1.87±0.01^{b}	2.19±0.04^{c}	2.18±0.03^{c}
Lactobacillus brevis SP04	0.57±0.01^{a}	1.68±0.05^{b}	2.14±0.02^{c}	2.17±0.04^{c}
Lactobacillus ramnosus SP05	0.68±0.03^{a}	0.73±0.02^{a}	0.84±0.05^{a}	0.84±0.02^{a}
Leuconostoc lactis SP11	0.35±0.04^{a}	0.37±0.05^{a}	0.56±0.02^{b}	0.55±0.03^{b}
Lactococcus lactic SP01	0.25±0.01^{a}	0.52±0.01^{b}	0.59±0.01^{b}	0.60±0.02^{b}

$^{a-d}$ The mean values in the same row with different superscripts differ significantly when $p<0.05$

Determination of SeNP biosynthesis conditions for monocultures of LAB

The first step in enhancement of SeNP synthesis was to evaluate the effect of inoculum dose. The concentrations tested were 1%, 5%, 10%, and 15%, while other conditions were standardized for all samples: sodium selenite at 50 µg/mL and incubation temperature of 37°C. Analysis showed that for most strains, maximum SeNP concentrations were achieved at a 10% inoculum (Table 2).

Table 2

Impact of inoculum dose on SeNP production, µg/mL, by the studied LAB strains

Strains	Inoculum dose, %			
	1	5	10	15
L. casei 3321	4.25±0.10^{a}	5.57±0.05^{b}	6.21±0.08^{c}	5.21±0.03^{d}
L. plantarum 3201	3.26±0.07^{a}	4.23±0.03^{b}	7.57±0.05^{c}	3.52±0.10^{a}
L. acidophilus 3103	2.92±0.01^{a}	3.31±0.05^{b}	5.25±0.03^{c}	1.23±0.02^{d}
L. bulgaricus 3511	3.01±0.02^{a}	5.81±0.01^{b}	7.52±0.04^{c}	2.54±0.04^{d}
L. brevis 3900	2.14±0.02^{a}	2.39±0.02^{a}	4.02±0.05^{b}	2.04±0.04^{a}
S. thermophilus 2192	2.19±0.04^{a}	4.21±0.02^{b}	5.29±0.06^{c}	2.18±0.06^{a}
L. cremoris 1220	3.82±0.08^{a}	5.12±0.04^{b}	6.54±0.03^{c}	3.45±0.01^{a}
B. longum 4205	3.26±0.09^{a}	3.45±0.07^{a}	4.23±0.02^{b}	4.27±0.03^{b}

$^{a-d}$ The mean values in the same row with different superscripts differ significantly when $p<0.05$.

Specifically, *L. plantarum* 3201 and *L. bulgaricus* 3511 exhibited the highest levels of SeNP production, namely, 7.57±0.05 µg/mL and 7.52±0.04 µg/mL, respectively, which were more than twice the values obtained at a 1% inoculum. High SeNP concentrations were also recorded for *L. casei* 3321, reaching 6.21±0.08 µg/mL at a 10% inoculum. Increasing the inoculum to 15% generally reduced SeNP synthesis in these strains, likely due to elevated metabolic activity diminishing the stress conditions required for selenium nanoparticle formation. Other strains, including *L. acidophilus* 3103, *L. brevis* 3900, *S. thermophilus* 2192, and *L. cremoris* 1220, exhibited gradual increases in SeNP production up to 10% inoculum, followed by stabilization or slight decreases. For instance, SeNP concentrations for *L. acidophilus* 3103 elevated from 2.92±0.06 µg/mL (1%) to 5.25±0.03 µg/mL (10%), then decreased to 1.23±0.02 µg/mL (15%). *B. longum* 4205 showed a more uniform increase from 3.26±0.09 µg/mL (1%) to 4.27±0.03 µg/mL (15%). Based on these results, an inoculum of 5–10% was determined to be optimal for most strains, as further increases did not enhance SeNP production and, in some cases, led to reductions.

The results regarding the residual sodium selenite content (Table 3) indicate that inoculum levels of 1% and 5% are insufficient for complete utilization of the inorganic selenium source, whereas the addition of 10% inoculum increases the conversion rate to approximately 89–99%. An inoculum concentration of 15% ensures nearly complete conversion by all strains, reaching 98–99%.

Table 3

Impact of inoculum dose on residual Na_2SeO_3 concentration, µg/mL

Strains	**Inoculum dose, %**			
	1	**5**	**10**	**15**
L. casei 3321	37.84±0.47^{a}	19.95±0.45^{b}	0.30±0.04^{c}	0.21±0.02^{c}
L. plantarum 3201	22.07±0.52^{a}	17.31±0.42^{b}	6.40±0.15^{c}	0.27±0.02^{d}
L. acidophilus 3103	30.75±0.54^{a}	14.49±0.39^{b}	0.56±0.05^{c}	0.78±0.06^{c}
L. bulgaricus 3511	25.17±0.46^{a}	11.74±0.35^{b}	5.15±0.14^{c}	0.73±0.09^{d}
L. brevis 3900	44.49±0.72^{a}	12.58±0.32^{b}	4.44±0.54^{c}	0.28±0.04^{d}
S. thermophilus 2192	30.35±0.64^{a}	15.53±0.42^{b}	1.02±0.07^{c}	0.79±0.07^{c}
L. cremoris 1220	27.96±0.53^{a}	21.60±0.32^{b}	1.12±0.11^{c}	0.48±0.09^{c}
B. longum 4205	40.54±0.86^{a}	27.02±0.56^{b}	1.51±0.19^{c}	0.89±0.03^{c}

$^{a\text{-}d}$ The mean values in the same row with different superscripts differ significantly when $p<0.05$.

The degree of sodium selenite (Na_2SeO_3) conversion into SeNP also depended on the inoculum dose (Table 4).

Maximum selenium conversion was generally observed at a 10% inoculum, with *L. plantarum* 3201 and *L. bulgaricus* 3511 achieving the highest rates (33.16% and 32.94%, respectively). *L. casei* 3321 and *L. cremoris* 1220 reached 27.20% and 28.65%, while *B. longum* 4205 showed a gradual increase, peaking at 18.70% at a 15% inoculum. Increasing the inoculum to 15% generally reduced conversion for most strains, likely due to substrate limitation and decreased metabolic activity. Notably, *L. acidophilus* 3103, *L. brevis* 3900, and *S. thermophilus* 2192 exhibited pronounced decreases at the highest inoculum, reflecting strain-specific differences in selenium reduction capacity and stress tolerance.

Table 4

Impact of inoculum dose on Na_2SeO_3 conversion into SeNP, %

Strains	Inoculum dose, %			
	1	5	10	15
L. casei 3321	18.62^{a}	24.40^{b}	27.20^{c}	22.82^{b}
L. plantarum 3201	14.28^{a}	18.53^{b}	33.16^{c}	15.42^{a}
L. acidophilus 3103	12.79^{a}	14.50^{b}	23.00^{c}	5.39^{d}
L. bulgaricus 3511	13.19^{a}	25.45^{b}	32.94^{c}	11.13^{a}
L. brevis 3900	9.37^{a}	10.47^{a}	17.61^{b}	8.94^{a}
S. thermophilus 2192	9.59^{a}	18.44^{b}	23.17^{c}	9.55^{a}
L. cremoris 1220	16.73^{a}	22.43^{b}	28.65^{c}	15.11^{a}
B. longum 4205	14.28^{a}	15.11^{a}	18.53^{b}	18.70^{b}

$^{a\text{-}d}$ The mean values in the same row with different superscripts differ significantly when $p<0.05$.

Analysis of biomass combined with SeNP revealed dose-dependent increases across most strains (Table 5).

Table 5

Impact of inoculum dose on biomass + SeNP concentration, g/L

Strains	C	Inoculum dose, %			
		1	5	10	15
L. casei 3321	7.30 ± 0.40^{a}	1.34 ± 0.05^{b}	3.47 ± 0.08^{c}	4.54 ± 0.28^{d}	4.78 ± 0.32^{d}
L. plantarum 3201	1.70 ± 0.10^{a}	0.43 ± 0.02^{b}	0.79 ± 0.01^{c}	1.02 ± 0.06^{d}	1.32 ± 0.09^{e}
L. acidophilus 3103	6.10 ± 0.32^{a}	1.56 ± 0.05^{b}	2.25 ± 0.21^{c}	3.21 ± 0.23^{d}	4.42 ± 0.35^{e}
L. bulgaricus 3511	9.10 ± 0.53^{a}	2.32 ± 0.02^{b}	2.71 ± 0.16^{c}	4.25 ± 0.25^{d}	4.78 ± 0.31^{d}
L. brevis 3900	5.95 ± 0.30^{a}	0.98 ± 0.06^{b}	1.25 ± 0.04^{c}	1.43 ± 0.14^{c}	1.45 ± 0.04^{c}
S. thermophilus 2192	$10.10\ \pm0.42^{a}$	2.67 ± 0.12^{b}	3.89 ± 0.24^{c}	5.02 ± 0.35^{d}	5.12 ± 0.36^{d}
L. cremoris 1220	8.85 ± 0.43^{a}	2.15 ± 0.11^{b}	2.73 ± 0.20^{c}	3.47 ± 0.16^{d}	4.50 ± 0.24^{e}
B. longum 4205	5.98 ± 0.31^{a}	1.01 ± 0.03^{b}	1.83 ± 0.19^{c}	2.52 ± 0.21^{d}	2.69 ± 0.15^{d}

C – control (without adding Na_2SeO_3); $^{a\text{-}e}$ The mean values in the same row with different superscripts differ significantly when $p<0.05$.

Maximum biomass was generally observed at 15% inoculum, e.g., *L. casei* 3321 (4.78±0.32 g/L), *L. acidophilus* 3103 (4.42±0.35 g/L), *L. bulgaricus* 3511 (4.78±0.31 g/L), *L. cremoris* 1220 (4.50±0.24 g/L), and *B. longum* 4205 (2.69±0.15 g/L). Some strains, such as *S. thermophilus* 2192, stabilized at 5.02–5.12 g/L, whereas *L. plantarum* 3201 and *L. brevis* 3900 showed gradual or minimal changes. Even at 15% inoculum, all biomass values remained below the control, indicating the persistent influence of sodium selenite.

Overall, increasing the inoculum dose to 10–15% reduced growth inhibition and enhanced SeNP production for most strains. However, complete restoration to control biomass levels was not achieved. Considering SeNP yield, sodium selenite conversion, and biomass accumulation, an inoculum dose of 10% was selected as optimal for subsequent experiments. The second stage of optimization focused on evaluating the effect of sodium selenite concentration on SeNP synthesis, using a range of 50–250 µg/mL. After 72 hours of cultivation with a 10% inoculum, SeNP concentration exhibited a clear dose-dependent profile, with an optimum in the range of 150–200 µg/mL Na_2SeO_3 (Table 6).

Table 6

Impact of Na_2SeO_3 concentration on SeNP accumulation, μg/mL

Strains	**Na_2SeO_3 concentration, μg/mL**				
	50	**100**	**150**	**200**	**250**
L. casei 3321	6.23 ± 0.03^{a}	7.38 ± 0.04^{b}	18.73 ± 0.06^{c}	13.52 ± 0.05^{d}	11.11 ± 0.02^{e}
L. plantarum 3201	7.56 ± 0.04^{a}	14.76 ± 0.11^{b}	28.75 ± 0.05^{c}	25.84 ± 0.12^{d}	17.42 ± 0.04^{e}
L. acidophilus 3103	5.23 ± 0.04^{a}	5.82 ± 0.06^{a}	22.45 ± 0.07^{b}	16.14 ± 0.10^{c}	16.04 ± 0.05^{c}
L. bulgaricus 3511	7.48 ± 0.02^{a}	13.22 ± 0.10^{b}	27.04 ± 0.07^{c}	26.77 ± 0.16^{c}	21.52 ± 0.09^{d}
L. brevis 3900	3.39 ± 0.06^{a}	7.62 ± 0.04^{b}	15.21 ± 0.10^{c}	14.39 ± 0.07^{c}	10.52 ± 0.01^{d}
S. thermophilus 2192	5.24 ± 0.03^{a}	8.29 ± 0.07^{b}	26.39 ± 0.09^{c}	27.91 ± 0.12^{c}	32.29 ± 0.06^{d}
L. cremoris 1220	6.60 ± 0.05^{a}	11.54 ± 0.09^{b}	24.34 ± 0.13^{c}	25.61 ± 0.11^{c}	24.20 ± 0.05^{c}
B. longum 4205	4.26 ± 0.02^{a}	9.23 ± 0.05^{b}	24.23 ± 0.11^{c}	25.12 ± 0.16^{c}	24.97 ± 0.06^{c}

$^{a\text{-}d}$ The mean values in the same row with different superscripts differ significantly when $p<0.05$.

Maximum selenium conversion was generally observed at a 10% inoculum, with *L. plantarum* 3201 and *L. bulgaricus* 3511 achieving the highest rates (33.16% and 32.94%, respectively). *L. casei* 3321 and *L. cremoris* 1220 reached 27.20% and 28.65%, while *B. longum* 4205 showed a gradual increase, peaking at 18.70% at a 15% inoculum. Increasing the inoculum to 15% generally reduced conversion for most strains, likely due to substrate limitation and decreased metabolic activity. Notably, *L. acidophilus* 3103, *L. brevis* 3900, and *S. thermophilus* 2192 exhibited pronounced decreases at the highest inoculum, reflecting strain-specific differences in selenium reduction capacity and stress tolerance.

In contrast, *L. casei* 3321 and *L. brevis* 3900 showed sharp peaks at 150 μg/mL (18.73±0.06 μg/mL and 15.21±0.10 μg/mL, respectively), followed by marked decreases at higher concentrations. *L. acidophilus* 3103 also demonstrated an optimal yield at 150 μg/mL (22.45±0.07 μg/mL) but maintained relatively stable values up to 250 μg/mL. Overall, an increase in Na_2SeO_3 concentration from 50 μg/mL to 150–200 μg/mL led to 3–5-fold enhancement in SeNP formation, confirming that the optimal selenite range for most strains lies within this interval, beyond which inhibitory effects likely emerge.

Residual Na_2SeO_3 concentrations increased with higher initial doses across all strains (Table 7).

Table 7

Impact of Na_2SeO_3 concentration on residual Na_2SeO_3 concentration, μg/mL

Strains	**Na_2SeO_3 concentration, μg/mL**				
	50	**100**	**150**	**200**	**250**
L. casei 3321	0.30 ± 0.06^{a}	42.62 ± 0.21^{b}	49.59 ± 0.24^{c}	146.56 ± 0.54^{d}	211.11 ± 0.46^{e}
L. plantarum 3201	6.40 ± 0.13^{a}	21.53 ± 0.25^{b}	46.05 ± 0.27^{c}	133.26 ± 0.33^{d}	198.12 ± 0.31^{e}
L. acidophilus 3103	0.56 ± 0.05^{a}	45.42 ± 0.19^{b}	57.85 ± 0.32^{c}	147.31 ± 0.29^{d}	202.31 ± 0.35^{e}
L. bulgaricus 3511	5.15 ± 0.09^{a}	23.22 ± 0.30^{b}	58.44 ± 0.21^{c}	122.73 ± 0.54^{d}	197.98 ± 0.63^{e}
L. brevis 3900	4.44 ± 0.13^{a}	45.22 ± 0.51^{b}	55.09 ± 0.49^{c}	148.24 ± 0.42^{d}	222.21 ± 0.67^{e}
S. thermophilus 2192	1.02 ± 0.08^{a}	25.16 ± 0.26^{b}	46.80 ± 0.43^{c}	125.56 ± 0.39^{d}	168.04 ± 0.43^{e}
L. cremoris 1220	1.12 ± 0.03^{a}	20.45 ± 0.16^{b}	45.23 ± 0.10^{c}	134.89 ± 0.24^{d}	191.10 ± 0.42^{e}
B. longum 4205	1.51 ± 0.04^{a}	21.57 ± 0.34^{b}	32.40 ± 0.41^{c}	136.52 ± 0.35^{d}	186.92 ± 0.56^{e}

$^{a\text{-}d}$ The mean values in the same row with different superscripts differ significantly when $p<0.05$.

At 50 µg/mL, most cultures left ≤1.5 µg/mL unutilized, although *L. plantarum* 3201, *L. bulgaricus* 3511, and *L. brevis* 3900 retained 4–6 µg/mL. Sodium selenite utilization ranged from 87.2–99.4%, reflecting efficient biotransformation. At 100–150 µg/mL, strains diverged into two groups: those efficiently reducing selenite (residuals ~20–25 µg/mL; conversion 74.8–79.6%), such as *L. cremoris* 1220, *L. plantarum* 3201, *B. longum* 4205, *L. bulgaricus* 3511, and *S. thermophilus* 2192, and those reducing it more slowly (residuals ~42–45 µg/mL; conversion 54.6–57.4%), such as *L. casei* 3321, *L. brevis* 3900, and *L. acidophilus* 3103. At 150 µg/mL, overall sodium selenite utilization ranged from 61–78%. Increasing the dose to 200–250 µg/mL led to massive accumulation of unutilized Na_2SeO_3 and conversion efficiencies below 40%.

The proportion of SeNP among total selenium forms generally increased with Na_2SeO_3 concentration (Table 8).

Table 8

Impact of Na_2SeO_3 concentration on SeNP fraction, %

Strains	**Na_2SeO_3 concentration, µg/mL**				
	50	**100**	**150**	**200**	**250**
L. casei 3321	27.45^a	28.17^a	40.86^b	55.41^c	62.57^d
L. plantarum 3201	37.98^a	41.20^a	60.58^b	84.80^c	73.54^c
L. acidophilus 3103	23.17^a	23.35^a	53.36^b	67.09^c	73.67^d
L. bulgaricus 3511	36.53^a	37.71^a	64.68^b	75.88^c	90.61^d
L. brevis 3900	16.30^a	30.47^b	35.10^c	60.89^d	82.91^e
S. thermophilus 2192	23.43^a	24.26^a	47.30^b	82.12^c	86.29^d
L. cremoris 1220	29.57^a	31.77^b	50.88^c	86.15^d	89.99^e
B. longum 4205	19.24^a	25.78^b	45.13^c	86.67^d	86.70^d

$^{a\text{-}d}$ The mean values in the same row with different superscripts differ significantly when $p<0.05$.

For *L. plantarum* 3201, the highest SeNP yield (84.7%) was observed at 200 µg/mL. However, doses of 200–250 µg/mL were suboptimal for SeNP formation, as overall selenite utilization remained low (<40%). The most favorable concentration for both yield and utilization was 150 µg/mL, providing SeNP proportions of ~35–64% and selenite utilization of ~41–78% across most strains. Sodium selenite also influenced biomass formation (Table 9).

Higher concentrations increased growth inhibition for all strains. At 50–100 µg/mL, minimal inhibition was observed for *L. plantarum* 3201 and *L. casei* 3321 (≈33–40%), moderate for *L. acidophilus* 3103 and *S. thermophilus* 2192 (≈47–50%), and higher for *L. bulgaricus* 3511, *B. longum* 4205, and *L. cremoris* 1220 (≈54–60%). *L. brevis* 3900 exhibited the highest sensitivity (≈75–77%). In the 150–250 µg/mL range, inhibition increased for all strains, with L. brevis 3900 remaining the most affected.

Overall, 150 µg/mL Na_2SeO_3 was identified as the optimal concentration for SeNP synthesis, maximizing nanoparticle yield while maintaining acceptable selenite utilization, particularly for *L. plantarum* 3201, *L. bulgaricus* 3511, *S. thermophiles* 2192, *L. cremoris* 1220, and *B. longum* 4205.The third stage of optimization focused on the parallel evaluation of critical process parameters, specifically the medium agitation rate and the time of Na_2SeO_3 addition. These factors were investigated in combination using a mathematically designed experimental framework, with response surfaces constructed using the least squares method for each strain. Optimization criteria included SeNP concentration (C(SeNPs), µg/mL). A full-factorial design was employed, and all measurements were performed in parallel to ensure robustness and reproducibility across strains.

Table 9

Impact of Na_2SeO_3 concentration on biomass + SeNP sediment content, g/L

Strains	Na_2SeO_3 concentration, µg/mL					
	C	50	100	150	200	250
L. casei 3321	7.30 ± 0.40^{a}	4.57 ± 0.12^{b}	4.36 ± 0.09^{b}	4.21 ± 0.04^{b}	4.05 ± 0.06^{b}	3.84 ± 0.09^{b}
L. plantarum 3201	1.70 ± 0.10^{a}	1.14 ± 0.02^{b}	1.03 ± 0.02^{b}	0.93 ± 0.01^{c}	0.90 ± 0.01^{c}	0.87 ± 0.03^{c}
L. acidophilus 3103	6.10 ± 0.32^{a}	3.26 ± 0.11^{b}	3.10 ± 0.07^{b}	3.03 ± 0.05^{b}	2.70 ± 0.10^{c}	2.56 ± 0.08^{c}
L. bulgaricus 3511	9.10 ± 0.53^{a}	4.19 ± 0.09^{b}	4.04 ± 0.12^{b}	3.89 ± 0.13^{b}	3.81 ± 0.15^{b}	3.75 ± 0.08^{b}
L. brevis 3900	5.95 ± 0.30^{a}	1.48 ± 0.05^{b}	1.34 ± 0.01^{b}	1.25 ± 0.07^{c}	1.19 ± 0.04^{c}	1.06 ± 0.01^{c}
S. thermophilus 2192	10.10 ± 0.42^{a}	5.29 ± 0.14^{b}	5.04 ± 0.14^{b}	4.89 ± 0.17^{b}	4.63 ± 0.09^{b}	4.21 ± 0.16^{c}
L. cremoris 1220	8.85 ± 0.43^{a}	3.51 ± 0.06^{b}	3.48 ± 0.11^{b}	3.41 ± 0.03^{b}	3.25 ± 0.14^{b}	3.03 ± 0.12^{b}
B. longum 4205	5.98 ± 0.31^{a}	2.66 ± 0.03^{b}	2.52 ± 0.08^{b}	2.47 ± 0.06^{b}	2.41 ± 0.05^{b}	2.23 ± 0.08^{b}

$^{a\text{-}d}$ The mean values in the same row with different superscripts differ significantly when $p<0.05$.

Based on the experimental data (Figure 1), an agitation rate of 110 rpm was established as optimal for subsequent experiments. The optimal sodium selenite addition times were determined for each strain as follows: *Lactobacillus plantarum* 3201, 6 h; *Lactobacillus bulgaricus* 3511, 2 h; *Streptococcus thermophilus* 2192, 0 h; *Lactococcus cremoris* 1220, 2 h, and *Bifidobacterium longum* 4205, 3 h.

Control measurements were performed under the optimized conditions, yielding the following results (Table 10).

Table 10

Outcomes obtained under optimized conditions

Strains	C(SeNPs), µg/mL	F(SeNPs), %	SUE (Na_2SeO_3), %	BSR, %
L. plantarum 3201	32.21 ± 0.13^{a}	63.37^{a}	74.22^{a}	51.36^{a}
L. bulgaricus 3511	31.04 ± 0.19^{a}	62.64^{a}	72.35^{b}	53.74^{a}
L. cremoris 1220	27.65 ± 0.11^{b}	54.93^{b}	74.33^{a}	66.71^{b}
S. thermophilus 2192	27.96 ± 0.09^{b}	59.15^{b}	68.25^{c}	51.90^{a}
B. longum 4205	27.88 ± 0.15^{b}	45.85^{c}	85.61^{d}	64.57^{b}

$^{a\text{-}d}$ The mean values in the same column with different superscripts differ significantly when $p<0.05$. F (SeNP) - fraction of transformed selenium present as nanoparticles, %; SUE (Na_2SeO_3) -substrate utilization efficiency, %; BSR – bacterial survival rate, %.

The obtained values significantly exceed the highest reported literature value of 19.76 mg/L (calculated from the data provided in the source) for *L. bulgaricus* (Xia et al., 2007), which can be explained by the optimization of cultivation conditions and the specific characteristics of the strains used in this study.

The subsequent stage focused on the formulation of two-strain consortia and the assessment of their synergistic impact on SeNP biosynthesis. Ten distinct compositions were evaluated (Table 11). The time of Na_2SeO_3 addition to the composition was determined as the average of the optimal times for the individual strains.

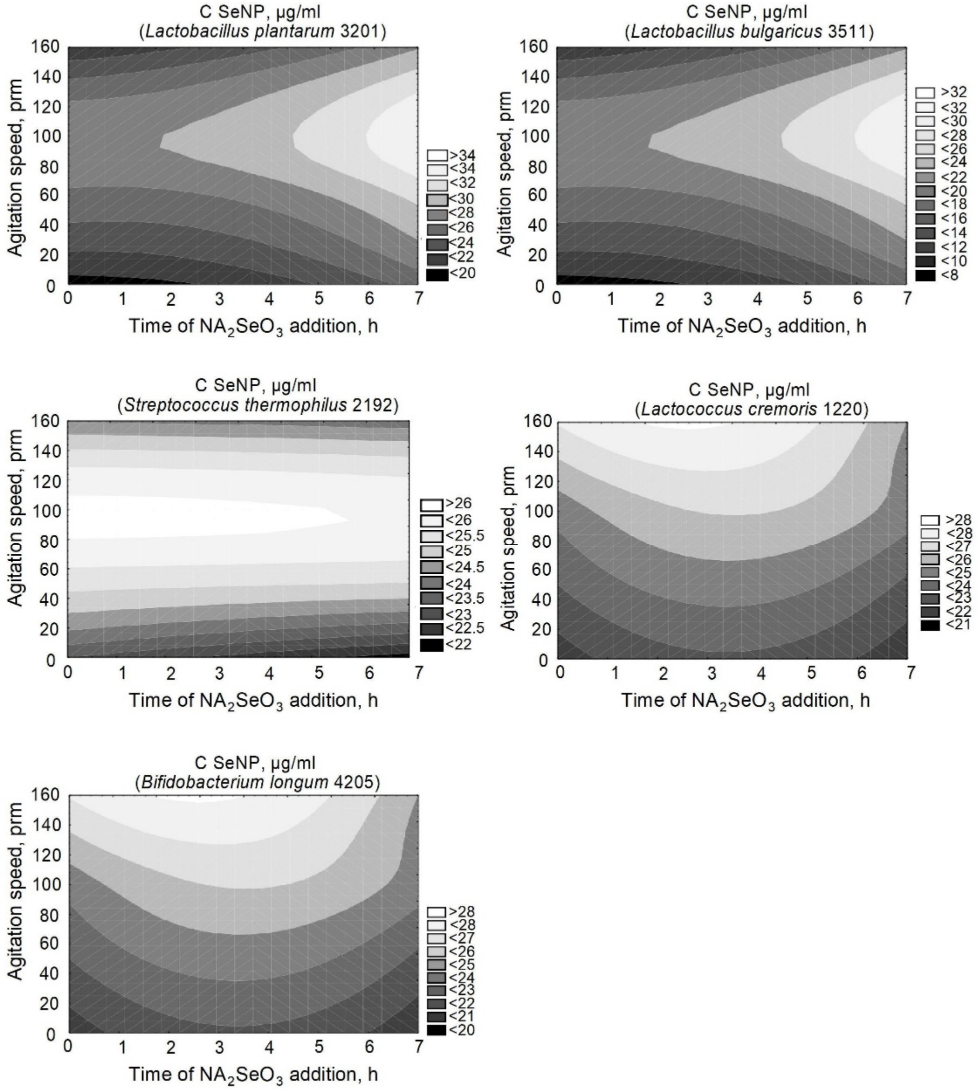

Figure 1. Response surfaces of selenium nanoparticle concentration as function of stirring speed and Na_2SeO_3 addition time

The LBLC dual-strain composition exhibited a pronounced difference compared to all other combinations (Table 12). Notably, it was the only formulation achieving both a high selenium content (78.27%) and an elevated total concentration (38.92 ± 0.23 µg/mL). The LBST and LCST combinations displayed slightly lower values than LBLC but still surpassed the performance of the individual strains. All remaining dual-strain formulations yielded results comparable to or below those of the single-strain cultures.

Table 11

Compositions of the two-strains

Name	Composition
LPLB	*Lactobacillus plantarum* 3201; *Lactobacillus bulgaricus* 3511
LPLC	*Lactobacillus plantarum* 3201; *Lactococcus cremoris* 1220
LPST	*Lactobacillus plantarum* 3201; *Streptococcus thermophilus* 2192
LPBL	*Lactobacillus plantarum* 3201; *Bifidobacterium longum* 4205
LBLC	*Lactobacillus bulgaricus* 3511; *Lactococcus cremoris* 1220
LBST	*Lactobacillus bulgaricus* 3511; *Streptococcus thermophilus* 2192
LBBL	*Lactobacillus bulgaricus* 3511; *Bifidobacterium longum* 4205
LCST	*Lactococcus cremoris* 1220; *Streptococcus thermophilus* 2192
LCBL	*Lactococcus cremoris* 1220; *Bifidobacterium longum* 4205
STBL	*Streptococcus thermophilus* 2192; *Bifidobacterium longum* 4205

Table 12

Outcomes obtained for two-strain compositions

Composition	C(SeNPs), µg/mL	F(SeNPs), %	SUE (Na_2SeO_3), %	BSR, %
LPLB	31.26±0.09^{a}	55.51^{a}	82.22^{a}	51.36^{a}
LPLC	26.12±0.19^{b}	40.00^{b}	95.35^{b}	59.74^{b}
LPST	27.16±0.16^{b}	47.03^{c}	84.33^{a}	56.71^{a}
LPBL	30.04±0.11^{a}	55.42^{a}	79.14^{c}	51.90^{a}
LBLC	38.92±0.23^{c}	78.27^{d}	72.61^{d}	78.01^{c}
LBST	34.16±0.17^{d}	65.17^{e}	76.54^{e}	72.53^{b}
LBBL	29.60±0.09^{a}	62.66^{f}	68.98^{f}	72.15^{b}
LCST	32.41±0.21^{e}	67.93^{g}	69.67^{g}	75.10^{c}
LCBL	29.52±0.15^{a}	49.74^{h}	86.65^{h}	81.05^{d}
STBL	24.16±0.09^{f}	42.97^{i}	82.10^{a}	82.56^{d}

$^{a-i}$ The mean values in the same column with different superscripts differ significantly when $p<0.05$. F (SeNP) - fraction of transformed selenium present as nanoparticles, %; SUE (Na_2SeO_3) -substrate utilization efficiency, %; BSR – bacterial survival rate, %.

These results demonstrate that the use of multi-strain compositions can enhance the yield of selenium nanoparticles (SeNPs). In particular, the obtained data contrast with another study, in which monocultures (*L. casei*, *L. acidophilus*, *L. plantarum*) showed higher SeNP synthesis compared to a three-component composition based on the same strains (Salama et al., 2021).

Characterization of synthesized SeNPs

The morphology of both individual strains (LB and LC) and the dual-strain composition (LBLC) was examined using a TESCAN MIRA3 scanning electron microscope (SEM). As illustrated in Figures 2 and 3, *Lactococcus cremoris* 1220 and *Lactobacillus bulgaricus* 3511 produced spherical nanoparticles, characteristic of biogenic SeNP synthesis by lactic acid bacteria. Notably, *L. cremoris* 1220 cells exhibited small surface pores, suggesting intracellular nanoparticle formation and subsequent release into the medium—an observation not previously reported in the literature. Cells were predominantly arranged as diplococci or short chains, typical for these species. In *L. bulgaricus* 3511, nanoparticles were primarily associated with the cell periphery, with rare instances of membrane-bound SeNPs (Figure 2) and localized lighter structures indicative of nanoparticle accumulation (Figure 2).

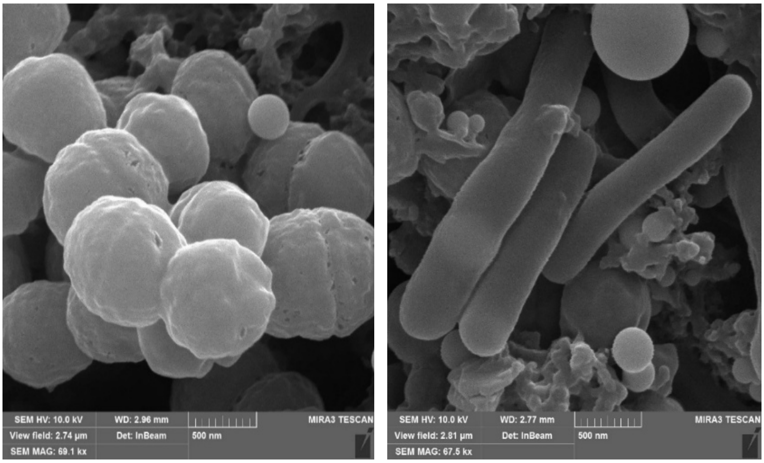

Figure 2. SEM micrographs of the cells with SeNPs:
Lactococcus cremoris **1220 (left),** ***Lactobacillus bulgaricus*** **3511 (right)**

Exposure to 150 µg/mL Na_2SeO_3 altered cell morphology: cell lengths decreased from ~6 µm to 2–4 µm, likely as a stress adaptation, consistent with previous reports (Shao, 2014). Shorter cells are known to be more resilient under adverse conditions.

SEM analysis of the LBLC dual-strain composition confirmed the presence of both *L. cremoris* 1220 and *L. bulgaricus* 3511 (Figure 3).

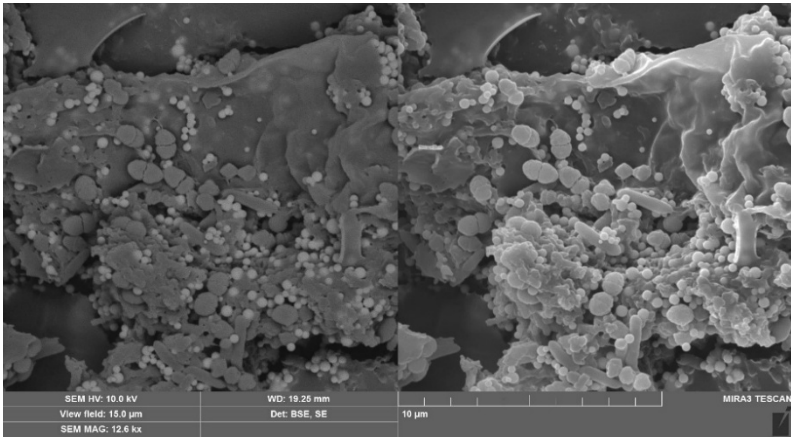

Figure 3. SEM micrographs of the composition LBLC with SeNPs

Nanoparticles were densely distributed, with approximately 125±18 particles per 10 μm².

Analysis of the fractional particle size distribution (Figure 4) revealed two distinct peaks: 150–175 nm (11.05%) and 225–250 nm (17.4%), indicating heterogeneity resulting from dual-strain synthesis.

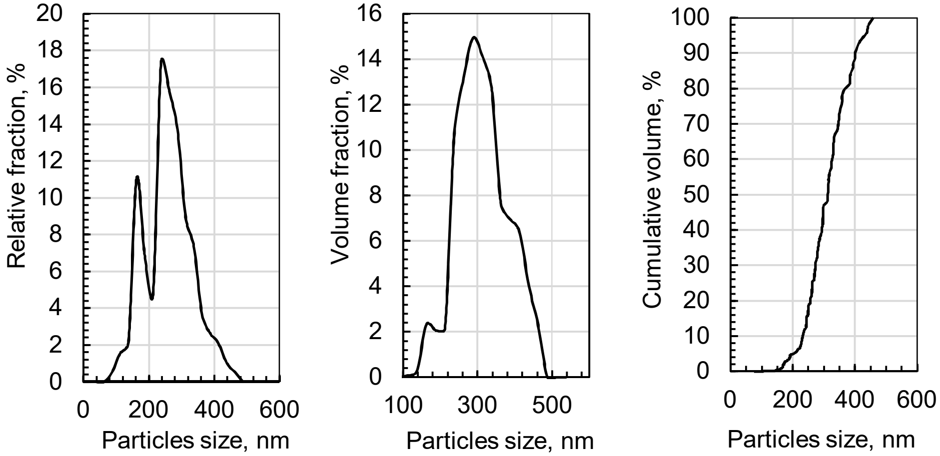

Figure 4. Graphs of particle size distribution:
Fractional (left), Volume-based (central), Cumulative volume-based (right)

When examined separately, *L. bulgaricus* 3511 predominantly produced particles within the 225–250 nm range, whereas *L. cremoris* 1220 mainly generated particles sized 150–175 nm. Volume-based particle size distribution indicated a median particle size (d50) of 313 nm, with 10% below 232 nm and 90% below 400 nm (Figure 4). The calculated span (0.54) indicates a moderately narrow distribution, with the main volumetric peak at 275–300 nm. Compared with literature, reported SeNP sizes from related strains range from 38–550 nm, aligning with our results, though differences arise from cultivation conditions and dual-strain interactions.

EDX analysis confirmed the SeNP composition. Regions with nanoparticles contained 48.34% mass fraction Se and 13.20% atomic fraction Se, compared with 8.39% mass fraction Se and 1.76% atomic fraction Se in nanoparticle-free areas (Figure 5; Table 13).

The Se energy peak (~1.4 keV) corresponds with literature values (El Saadony et al., 2021; Muthu et al., 2023; Spyridopoulou et al., 2021; Xia et al., 2007), confirming the presence of pure selenium. Additional peaks for Au and Pd reflect SEM coating, while O, C, N, P, Na and Ca arise from media components or extracellular polymeric substances surrounding the nanoparticles. Taken together, the spherical morphology, characteristic EDX peaks, and red coloration indicate that the synthesized SeNPs are amorphous and biogenic, consistent with nanoparticles formed by lactic acid bacteria through Se oxyanion reduction (Spyridopoulou et al., 2021). These findings confirm that the dual-strain LBLC composition produces SeNPs comparable to those reported for other lactic acid bacteria.

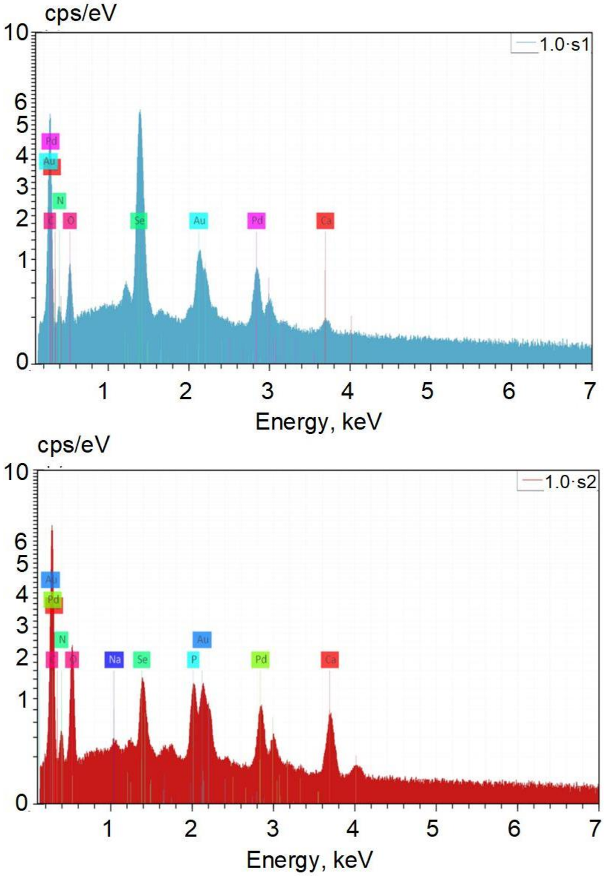

Figure 5. Elemental composition of the sample determined by EDX:
Field s1 (with SeNPs) (left), Field s2 (without SeNPs) (right)

Table 13

Elemental composition of the samples determined by EDX

Element	Field s1 (with SeNPs)		Field s2 (without SeNPs)	
	Mass fraction, %	Atomic fraction, %	Mass fraction, %	Atomic fraction, %
Carbon (C)	41.32	74.20	42.55	58.76
Nitrogen (N)	2.39	3.68	6.25	7.40
Oxygen (O)	5.72	7.72	21.47	22.26
Calcium (Ca)	2.22	1.20	13.87	5.74
Selenium (Se)	48.34	13.20	8.39	1.76
Sodium (Na)	b.d.l.	b.d.l.	0.44	0.32
Phosphorus (P)	b.d.l.	b.d.l.	7.02	3.76
Total	**100.00**	**100.00**	**100.00**	**100.00**

b.d.l. – below detection limit.

Conclusions

In this study, the cultivation parameters were optimized, including a 10% inoculum concentration, strain-specific Na_2SeO_3 dosing, an agitation speed of 110 rpm, a 72-hour cultivation period, and defined timing for Na_2SeO_3 addition: 6 h for *Lactobacillus plantarum* 3201, 2 h for *Lactobacillus bulgaricus* 3511, 0 h for *Streptococcus thermophilus* 2192, 2 h for *Lactococcus cremoris* 1220, and 3 h for *Bifidobacterium longum* 4205. Under these optimized conditions, all selected strains produced high concentrations of SeNPs, reaching 32.21±0.13 µg/mL for *L. plantarum* 3201, 31.04±0.19 µg/mL for *L. bulgaricus* 3511, 27.65±0.10 µg/mL for *L. cremoris* 1220, 27.96±0.09 µg/mL for *S. thermophilus* 2192, and 27.88±0.15 µg/mL for *B. longum* 4205. The two-strain LBLC composition demonstrated the highest performance, synthesizing 38.92±0.23 µg/mL SeNP (78.27% of the total transformed selenium) while ensuring a biomass survival rate of 78.01%. SEM and EDX analyses confirmed that LBLC generated amorphous, spherical SeNPs ranging from 76 to 458 nm, with an average diameter of 260.95±70.88 nm. The fractional distribution revealed two prominent particle-size peaks (150–175 nm and 225–250 nm) and a volumetric peak at 275–300 nm, with distribution metrics of d_{10} = 232 nm, d_{50} = 313 nm, and d_{90} = 400 nm. Overall, the LBLC composition producing selenium nanoparticles shows strong potential for application as a dietary supplement.

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Effect of surfactants produced by *Rhodococcus erythropolis* IMV Ac-5017 on single- and dual-species biofilms of phytopathogenic bacteria

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Abstract

Introduction. The aim of this study was to investigate the disruption of biofilms formed by phytopathogenic bacteria using surfactants produced by *Rhodococcus erythropolis* IMV Ac-5017 in the presence of a yeast-derived inducer and precursors of phytohormone biosynthesis.

Materials and methods. The surfactant producer was cultivated in a liquid mineral medium supplemented with tryptophan and/or erythritol. Inactivated cells of *Saccharomyces cerevisiae* BTM-1 or the corresponding supernatant were used as an inducer. Surfactants were extracted from the culture liquid using modified Folch mixture. The degree of biofilm destruction was determined spectrophotometrically.

Results and discussion. Surfactants synthesized under different cultivation conditions of *R. erythropolis* IMV Ac-5017, at low concentrations (0.94–120 μg/mL), effectively disrupted pre-formed mono- and dual-species biofilms of phytopathogenic bacteria, with destruction ranging from 7–84% and 4–69%, respectively. Notably, surfactants produced in the presence of a eukaryotic inducer, even without phytohormone biosynthesis precursors, were effective against dual-species phytopathogenic biofilms at concentrations of 0.94–3.75 μg/mL, achieving 51–69% disruption. The addition of inactivated *S. cerevisiae* BTM-1 cells or the corresponding supernatant to the cultivation medium further enhanced the synthesis of surfactants, resulting in increased biofilm disruption by 6–42% for single-species biofilms and 3–57% for dual-species biofilms compared with preparations obtained in medium containing erythritol and/or tryptophan without an inducer. These results suggest that the presence of a eukaryotic inducer can modulate the composition and activity of surfactants, potentially by influencing the relative proportions of glyco-, amino-, and neutral lipids in the preparation. Consequently, these findings represent one of the first reports demonstrating the destruction of both single- and dual-species biofilms of phytopathogenic bacteria from the genera *Xanthomonas*, *Pseudomonas*, *Agrobacterium*, *Pectobacterium*, and *Clavibacter* by microbial surfactants, highlighting their potential as a sustainable strategy for controlling plant pathogens in agricultural settings.

Conclusions. The present data demonstrate the potential of surfactants in crop production to combat phytopathogenic bacterial biofilms, ensuring the safety and quality of raw materials for the food and processing industries.

Introduction

Plant diseases caused by phytopathogens (fungi, bacteria, nematodes) pose a growing threat to global agriculture, leading to significant economic losses and affecting food security (Fontana et al., 2025; Pierre et al., 2025).

Bacterial diseases of agricultural crops have traditionally received less attention than diseases caused by other phytopathogens. A review (Mulungu, 2024) notes that despite the relatively lower level of damage caused by phytopathogenic bacterial diseases, the associated costs remain significant and require attention. Phytopathogenic bacteria disrupt plant growth and metabolism, reducing crop productivity and, in turn, may lead to crop losses of up to 100%, annual economic losses of up to US$1 billion and deterioration in the quality of agricultural products, depending on the type of phytopathogen, crop and geographical location (Mulungu, 2024). Despite their lower prevalence compared with fungal diseases, bacterial infections are often difficult to control because of their predominantly polycyclic nature and the absence of systemic antibacterial substances (Oerke, 2006).

A study (Mansfield et al., 2012) notes that, according to a survey of bacteriologists collaborating with the journal *Molecular Plant Pathology*, the most significant phytopathogenic bacteria, which pose serious threats to crops, belong to eight genera (*Pseudomonas, Ralstonia, Agrobacterium, Xanthomonas, Erwinia, Xylella, Pectobacterium* and *Dickeya*), in particular *Pseudomonas syringae*, *Ralstonia solanacearum*, *Agrobacterium tumefaciens*, *Xanthomonas oryzae* pv. *oryzae*, *Xanthomonas campestris*, *Xanthomonas axonopodis*, *Erwinia amylovora*, *Xylella fastidiosa*, *Pectobacterium carotovorum.*

One of the factors contributing to the virulence of phytopathogenic bacteria is their ability to form biofilms (Li et al., 2023; Mina et al., 2019). Biofilms protect bacterial cells from abiotic and biotic stresses (UV radiation, pH fluctuations, osmotic stress, dehydration), as well as from antimicrobial substances and toxic compounds produced by plants (Bogino et al., 2013). Biofilms of phytopathogenic bacteria, formed on leaves, in the rhizosphere, and/or in vascular bundles, reduce crop yield and quality and affect the safety of agricultural products intended for human consumption and animal feeding. In addition, biofilm structures of phytopathogenic bacteria interfere with the proper functioning of plant tissues and organs (Carezzano et al., 2023).

Current strategies to combat biofilms involve the use of pesticides and antibiotics, but their effectiveness is limited and accompanied by risks. Once treatment is discontinued, the infection typically recurs, and excessive use of chemical products leads to water and soil pollution (Agboola et al., 2022; Carezzano et al., 2023). Surfactants of microbial origin are considered a promising alternative to chemical pesticides due to their antimicrobial activity, low toxicity, and high biodegradability (Ashby et al., 2020; Pierre et al., 2025; Pirog et al., 2019).

In recent years, there has been growing scientific interest in so-called integrated technologies, in which associated metabolites of practical importance are synthesised alongside the target product (Katagi et al., 2024; de Siqueira et al., 2024). It has been previously established that the strains *Rhodococcus erythropolis* IMV Ac-5017, *Acinetobacter calcoaceticus* IMV B-7241, and *Nocardia vaccinii* IMV B-7405 simultaneously synthesise surface-active substances with biological activity against phytopathogenic bacteria, as well as stimulatory phytohormones: auxins, cytokinins, gibberellins (Leonova et al., 2020).

Later studies have shown that auxin synthesis can be enhanced in combination with surface-active substances when tryptophan (a precursor of auxin phytohormone biosynthesis) is present in the culture medium (Pirog et al., 2020a). The addition of erythritol (a precursor of gibberellic phytohormone biosynthesis) to the cultivation medium of *N. vaccinii* IMV B-7405 was accompanied by an increase in the concentration of biologically active gibberellins (Pirog et al., 2025). The results indicate the high potential of using complex preparations of surface-active substances and phytohormones in crop production to stimulate plant growth and to control phytopathogenic bacterial numbers. This is important for ensuring the safety and quality of food and food-processing industry products, as food safety begins at the field level.

It should be noted that the surfactants synthesised by *R. erythropolis* IMV Ac-5017 exhibited lower antimicrobial activity compared to other known surface-active amino-, rhamno-, and sophorolipids. However, the antimicrobial and antibiofilm activities of these surfactants against opportunistic pathogens were significantly enhanced when the IMV Ac-5017 strain was cultivated in the presence of *Saccharomyces cerevisiae* BTM-1 yeast in various physiological states (Okhmakevych et al., 2025).

Based on the above, it was hypothesized that the biological activity of *R. erythropolis* IMV Ac-5017 surfactants against phytopathogenic bacteria, as well as the synthesis of auxins and gibberellins, could be simultaneously enhanced by adding tryptophan, erythritol, and a yeast inducer to the culture medium.

The aim of this study was to investigate the disruption of single- and dual-species biofilms formed by phytopathogenic bacteria under the action of surfactants produced by *Rhodococcus erythropolis* IMV Ac-5017 in the presence of *Saccharomyces cerevisiae* BTM-1 and phytohormone biosynthesis precursors.

Materials and methods

Research objects

The main object of the study was the strain of oil-degrading bacteria *Rhodococcus erythropolis* EK-1, registered as *Rhodococcus erythropolis* IMV Ac-5017 in the Microorganism Depository of the D.K. Zabolotny Institute of Microbiology and Virology of the National Academy of Sciences of Ukraine. *Saccharomyces cerevisiae* BTM-1 yeast from the collection of live cultures of the Department of Biotechnology and Microbiology of the National University of Food Technologies was used as a biological inducer.

Phytopathogenic bacteria from the Ukrainian Collection of Microorganisms (UCM) were used: *Pseudomonas syringae* UCM B-1027^T, *Agrobacterium tumefaciens* UCM B-1000, *Xanthomonas vesicatoria* UCM B-1106, and *Pectobacterium carotovorum* UCM B-1075^T. Also, strains from the Collection of the Department of Phytopathogenic Bacteria of the D.K. Zabolotny Institute of Microbiology and Virology of the National Academy of Sciences of Ukraine were used: *Clavibacter michiganensis* subsp. *michiganensis* IMV B-10_2 and *Pseudomonas syringae* pv. *tomato* IMV B-9167.

All phytopathogenic bacterial strains were kindly provided by the D.K. Zabolotny Institute of Microbiology and Virology of the National Academy of Sciences of Ukraine.

Cultivation conditions for the producer of surface-active substances

R. erythropolis IMV Ac-5017 was cultivated in flasks on a shaker at 30°C and 320 rpm for 5 days in a liquid mineral medium of the following composition, g/L: $NaNO_3$, 1.3; $MgSO_4{\cdot}7H_2O$, 0.1; NaCl, 1.0; Na_2HPO_4, 0.6; KH_2PO_4, 0.14; $FeSO_4{\cdot}7H_2O$, 0.001; pH 6.8–7.0. Ethanol 2% (*v/v*) was used as a carbon source. Precursors of phytohormone biosynthesis, tryptophan (300 mg/L) and/or erythritol (400 mg/L), were also added.

The seed material was grown in a medium of identical composition with ethanol, 0.5% (*v/v*), without precursors of phytohormone biosynthesis, in flasks on a shaker at 30°C and 320 rpm for 2 days and was added to the medium in an amount of 10% (*v/v*) to obtain the surfactants.

Experimental variants: control (without inducer and precursors of phytohormone biosynthesis); inducer; tryptophan; erythritol; tryptophan and erythritol; inducer and tryptophan; inducer and erythritol; inducer, tryptophan and erythritol.

Preparation of yeast inducer

S. cerevisiae BTM-1 was grown in a liquid mineral medium of the above composition with glucose (0.5%, *w/v*) in flasks on a shaker at 30°C and 320 rpm for 1 day. The resulting culture fluid was poured into sterile Eppendorf tubes (1.5 mL each) and centrifuged in an ultracentrifuge (10.000 g, 15 min). After centrifugation, the supernatant was poured into sterile tubes and added at a rate of 2.5 mL per 100 mL of medium for culturing the surfactant producer. The biomass (sediment) that remained in the Eppendorf tubes was resuspended in sterile tap water to a final volume corresponding to the volume of culture fluid taken for centrifugation and sterilised in an autoclave at 131°C for 1 hour to obtain inactivated inducer cells (added at a rate of 10 mL of suspension per 100 mL of nutrient medium).

Living inducer cells were not used, as there is a possibility that yeast will consume tryptophan and erythritol as carbon sources.

Isolation and preparation of surfactants

25 mL of the obtained culture fluid was centrifuged (5000 g, 20 min) to obtain the supernatant. Surfactants were isolated from the supernatant using the Bligh and Dyer method, using a modified Folch mixture (chloroform, methanol, hydrochloric acid, 4:3:2) as described in the article (Pirog et al., 2020b). The obtained extracts were evaporated on an IP-1M2 rotary evaporator at 50-60 ºC and an absolute pressure of 0.4 atm to a constant weight. The concentration of surfactants was determined gravimetrically.

The dry residue of surfactants was dissolved in pre-heated distilled water (25 mL). Aqueous solutions of surfactants were sterilised in an autoclave at 112 °C for 30 min.

Determination of single-species phytopathogenic bacterial biofilm destruction by surfactants

For the preliminary formation of single-species biofilms, under sterile conditions, 180 µL of meat-peptone broth (MPB) and 20 µL of a suspension of test cultures of phytopathogenic bacteria were added to the wells of polystyrene immunological plates and incubated for 24 hours at 30°C. After that, the culture fluid was removed and the medium and microorganism suspensions were reintroduced, incubated for another 24 hours at the same temperature.

Next, the culture fluid was removed, the microplate wells (with a pre-formed biofilm of the test culture) were washed twice with sterile tap water, and 200 µL of surfactant preparations at various concentrations (0.94-120 µg/mL) were added. For control wells, 200 µL of sterile tap water was added instead of surfactants. The plates were incubated for 1 day at the same temperature.

After that, the plates were gently rinsed three times with tap water, the biofilms were fixed with methanol for 15 minutes, stained with a 0.1% (*w/v*) solution of gentian violet, and washed off into immunological tubes with a 33% (*v/v*) solution of acetic acid. The degree of biofilm destruction (%) was determined spectrophotometrically as the difference in cell adhesion between untreated and surfactant-treated wells (Gomes et al., 2012).

Determination of the destruction of dual-species phytopathogenic bacterial biofilms by surfactants

180 μL of MPB and 10 μL an aqueous suspension of each of the two phytopathogenic test cultures were added to the wells of immunological plates and incubated for 24 hours at 30°C. After that, the culture fluid was removed and the medium and microorganism suspensions were reintroduced, incubated for another 24 hours.

The culture fluid was removed, the wells were washed twice with sterile tap water, and 200 μL of surfactant preparations at various concentrations (0.94-120 μg/mL) were added. In the control wells, 200 μL of sterile tap water was added instead of surfactants. The plates were incubated for 1, 2 or 4 days.

After that, the plates were gently rinsed three times with tap water, the biofilms were fixed with methanol for 15 minutes, stained with a 0.1% (*w/v*) solution of gentian violet and washed off into immunological tubes with a 33% (*v/v*) solution of acetic acid. The degree of biofilm destruction (%) was determined spectrophotometrically as the difference in cell adhesion between untreated and surfactant-treated wells.

Results and discussion

Destruction of single-species biofilms of phytopathogenic bacteria by surfactants produced by *R. erythropolis* IMV Ac-5017 under different cultivation conditions

Studies have shown that regardless of the cultivation conditions of *R. erythropolis* IMV Ac-5017 (the presence of erythritol and/or tryptophan in the medium, yeast inducer in different physiological states), the synthesised surfactants in a wide range of concentrations (0.94-120 μg/mL) disrupted the previously formed single-species biofilms of phytopathogenic bacteria. However, the degree of destruction depended on the cultivation conditions, the type of test culture and the concentration of surfactants.

Tables 1 and 2 show the maximum biofilm destruction under the action of surfactants synthesised by the strain IMV Ac-5017 in the presence of inactivated cells and supernatant of *S. cerevisiae* BTM-1, with phytohormone biosynthesis precursors in the culture medium. These data indicate that the introduction of a yeast inducer into the medium was accompanied by the synthesis of surfactants, which destroyed phytopathogen biofilms 6-42% more effectively than preparations formed in a medium without an inducer.

The highest degree of destruction (73-84%) was determined for the biofilm of *A. tumefaciens* UCM B-1000 after treatment with surfactants synthesised in the presence of inactivated yeast cells (Table 1). Notably, this level of destruction was achieved at an extremely low concentration of surfactant solutions, only 0.94–3.75 μg/mL. At the same concentration of surfactants formed in the medium with inactivated cells of *S. cerevisiae* BTM-1, maximum destruction (37-65%) of biofilms of *P. syringae* UCM B-1027^T and *P. syringae* pv. *tomato* IMV B-9167 was determined (Table 1). For other strains of phytopathogenic bacteria (*P. carotovorum* UCM B-1075^T, *C. michiganensis* subsp. *michiganensis* IMV B-10$_2$, *X. vesicatoria* UCM B-1106), the maximum destruction of their biofilms (25-48%) was achieved at higher concentrations of surfactants (1.88-120 μg/mL) synthesised in the presence of inactivated yeast cells.

Table 1

Destruction of phytopathogenic bacterial biofilms by surfactants synthesised by *R. erythropolis* IMV Ac-5017 in a medium with phytohormone biosynthesis precursors and inactivated *S. cerevisiae* BTM-1 cells as an inducer

Biofilm destruction, %	Phytohormone biosynthesis precursor					
	Erythritol		Tryptophan		Erythritol + tryptophan	
	Presence of an inducer in the medium					
	-	+	-	+	-	+
P. syringae pv. *tomato* IMV B-9167	10	40	30	37	23^{*}	57^{*}
A.tumefaciens UCM B-1000	42	84	47	78	36^{**}	73^{**}
P. syringae UCM B-1027^{T}	35	65	53^{**}	63^{**}	n.d.	n.d.
P. carotovorum UCM B-1075^{T}	26^{*}	38^{*}	25^{***}	38^{***}	19^{****}	25^{****}
C. michiganensis subsp. *michiganensis* IMV B-10_2	18^{**}	47^{**}	18^{***}	36^{***}	n.d.	n.d.
X. vesicatoria UCM B-1106	27^{*****}	48^{*****}	n.d.	n.d.	27^{******}	48^{******}

Notes: The error in biofilm destruction determination did not exceed 5%. The surfactant concentration was 0.94 µg/mL; * – 1.88 µg/mL; ** – 3.75 µg/mL; *** – 30 µg/mL; **** – 120 µg/mL; ***** – 15 µg/mL; ****** – 60 µg/mL; n.d. – not determined.

Table 2

Effect of phytohormone biosynthesis precursors and *S. cerevisiae* BTM-1 supernatant as an inducer in the culture medium of *R. erythropolis* IMV Ac-5017 on the ability of the synthesised surfactants to destroy phytopathogenic bacterial biofilms

Biofilm destruction, %	Phytohormone biosynthesis precursor					
	Erythritol		Tryptophan		Erythritol + tryptophan	
	Presence of an inducer in the medium					
	-	+	-	+	-	+
A. tumefaciens UCM B-1000	30^{*}	42^{*}	47	59	36^{**}	52^{**}
P. syringae UCM B-1027^{T}	41^{**}	58^{**}	n.d.	n.d.	19	33
P. carotovorum UCM B-1075^{T}	26^{**}	60^{**}	33^{***}	47^{***}	27^{**}	33^{**}
C. michiganensis subsp. *michiganensis* IMV B-10_2	7^{****}	43^{****}	n.d.	n.d.	22^{*****}	43^{*****}
P. syringae pv. *tomato* IMV B-9167	22^{******}	64^{******}	n.d.	n.d.	23	30

Notes: The error in biofilm destruction determination did not exceed 5%. The surfactant concentration was 0.94 µg/mL; * – 1.88 µg/mL; ** – 3.75 µg/mL; *** – 15 µg/mL; **** – 60 µg/mL; ***** – 120 µg/mL; ****** – 7.5 µg/mL; n.d. – not determined.

When the supernatant of *S. cerevisiae* BTM-1 was used as an inducer, the destruction of biofilms of *A. tumefaciens* UCM B-1000, *P. syringae* UCM B-1027^T, *P. carotovorum* UCM B-1075^T, and *P. syringae* pv. *tomato* IMV B-9167 was also highest (30–64%) at low concentrations (0.94–15 µg/mL) of surfactants synthesised under these conditions (Table 2). To achieve maximum destruction (43%) of the biofilm of *C. michiganensis* subsp. *michiganensis* IMV B-10$_2$, a higher concentration (60–120 µg/mL) of surfactants obtained in the presence of phytohormone synthesis precursors in the yeast supernatant was required (Table 2).

Two methods are commonly used to evaluate the disruption of biofilms formed by phytopathogenic bacteria. In the first method, the test culture and the antibiofilm agent at various concentrations are added simultaneously to the wells of a microtiter plate (Dey et al., 2022; Long et al., 2023). This approach primarily assesses the ability of the agent to inhibit biofilm formation. In the second method, the biofilm is first allowed to form, after which the antibiofilm agent is applied (Abdallah et al., 2018; Papaianni et al., 2020). This approach evaluates the ability of the agent to disrupt preformed biofilms. Due to the use of different methodologies, it is often difficult to directly compare the effectiveness of antibiofilm agents across studies.In most studies, the first method was used to determine the destruction of phytopathogenic biofilms (Chen et al., 2016; Xia et al., 2023).

The second method for determining biofilm disruption is considered more appropriate for assessing the antibiofilm activity of a given compound. This is supported by studies in which the degree of biofilm disruption was evaluated using both methods (Fontana et al., 2025; Ni et al., 2020). Thus, Ni et al. (2020) demonstrated that carvacrol at 2 mg/mL inhibited the formation of *Pseudomonas syringae* pv. *actinidiae* biofilms over 48 h, but did not affect preformed biofilms. Fontana et al. (2025) reported that methanol and ethanol extracts of hops at concentrations of 1 and 10 mg/mL suppressed biofilm formation by *Xanthomonas campestris* pv. *campestris* and *Erwinia amylovora*, while disruption of preformed biofilms of both phytopathogens was greater at 10 mg/mL.

In this study, the antibiofilm activity of surfactants produced by strain IMV Ac-5017 was evaluated based on their ability to disrupt preformed biofilms of phytopathogenic bacteria.

The disruption of biofilms by microbial surfactants involves several mechanisms (Paraszkiewicz et al., 2021; Zhao et al., 2017). First, they reduce the surface and interfacial tension within the biofilm matrix. Second, their antimicrobial activity disrupts the integrity of microbial cytoplasmic membranes, increases membrane permeability, and leads to loss of cell viability. Third, their anti-adhesive effects, associated with changes in the surface charge of cells and substrates, decrease microbial attachment to surfaces.

This complex mechanism of action allows microbial surfactants to efficiently disrupt biofilms. Moreover, compared to conventional biofilm-disrupting agents, microbial surfactants have several additional advantages (Banat et al., 2014; Zhao et al., 2017): (1) no environmental pollution, as microbial surfactants are biodegradable; (2) possibility of their use in various environmental conditions due to their stable physicochemical properties; (3) their specific mode of action significantly reduces the likelihood of developing resistant microbial forms.

Although numerous studies have reported the use of microbial surfactants for the biocontrol of phytopathogens, they primarily focus on antimicrobial activity and provide limited information on their effects on phytopathogenic biofilms (Pierre et al., 2025; Sani et al., 2024). Information on the disruption of phytopathogenic bacterial biofilms remains scarce, with only a few reports describing the ability of microbial surfactants to affect such structures. For example, lipopeptide-containing supernatants of *Bacillus amyloliquefaciens*

A3 and *Bacillus velezensis* A2 exhibited similar levels of biofilm inhibition and disruption of the sweet potato rot pathogen *Dickeya dadanti* (60–61% for strain A3 and 44–48% for strain A2) (Hossain et al., 2020). Lipopeptides from *B. amyloliquefaciens* 32a at concentrations of 40 and 80 μg/mL caused 55–67% disruption of biofilms formed by *Agrobacterium tumefaciens* C58 and B6 (Abdallah et al., 2018). Moreover, the lipopeptide-containing supernatant of *Paenibacillus polymyxa* ShX301 inhibited *D. dadanti* biofilm formation by 39% after 24 h of exposure (Hossain et al., 2023).).

Previous studies have demonstrated that surfactants produced by *N. vaccinii* IMV B-7405 effectively disrupt biofilms formed by phytopathogenic bacteria (*A. tumefaciens* UCM B-1000, *P. syringae* UCM B-1027T, *X. vesicatoria* UCM B-1106, *P. carotovorum* UCM B-1075T, *C. michiganensis* subsp. *michiganensis* IMV B-102, and *P. syringae* pv. *tomato* IMV B-9167). Biofilm disruption was observed over a wide range of surfactant concentrations (0.78–100 μg/mL). However, the extent of disruption was concentration-dependent. At higher concentrations (50–100 μg/mL), biofilm disruption reached 51–88%, whereas lower concentrations (0.78–1.5 μg/mL) resulted in 10–47% disruption (Pirog et al., 2025).

The results of this study indicate that, in contrast to the surfactants produced by *N. vaccinii* IMV B-7405 (Pirog et al., 2025) and lipopeptides of *B. amyloliquefaciens* 32a (Abdallah et al., 2018), the maximum destruction of most of the investigated phytopathogenic bacterial biofilms was achieved at lower concentrations (0.94-15 μg/mL) of surfactants synthesised by *R. erythropolis* IMV Ac-5017 in a medium with phytohormone biosynthesis precursors and a yeast inducer (Tables 1 and 2).

There are reports in the literature on the disruption of phytopathogenic bacterial biofilms by various antibiofilm agents: natural plant-derived compounds (Fontana et al., 2025; Han et al., 2021), microbial exometabolites other than surfactants (Dey et al., 2022; Hao et al., 2024), metal nanoparticles (Mishra et al., 2020), phages (Umrao et al., 2021), and combinations of phages with natural biocides (Ni et al., 2020; Papaianni et al., 2020).

It should be noted that, while there is abundant literature on the antimicrobial activity of metal nanoparticles against phytopathogens (Orfei et al., 2023; Wasule et al., 2024) and of bacteriophages (Nawaz et al., 2023; Stefani et al., 2021), few studies have addressed their ability to disrupt biofilms of phytopathogenic bacteria. Thus, in a study (Mishra et al., 2020), it was reported that biogenic silver nanoparticles at concentrations of 5-50 μg/mL inhibited the formation of *Xanthomonas oryzae* pv. *oryzae* biofilm. Phage ϕsp1 inhibited the formation of *Ralstonia solanacearum* biofilm by 73.68% and destroyed the pre-formed 24-hour biofilm by 94.73% (Umrao et al., 2021).

Papaianni et al. (2020) showed that 6-pentyl-α-pyron (a secondary metabolite of *Trichoderma atroviride* P1) at a concentration of 0.001 μg/mL destroyed the pre-formed biofilm of *X. campestris* pv. *campestris* by 70%. When 6-pentyl-α-pyron was used in combination with bacteriophage Xccφ1 and hydroxyapatite, the degree of biofilm disruption increased slightly (by 5-7%).

Lipid-rich, unpurified endometabolites of *Penicillium* sp. PM031 (a concentrate of ethyl acetate mycelium extract dissolved in dimethyl sulfoxide) inhibited the formation of *R. solanacearum* biofilm by 32.24, 34.34 and 33.88% after 24 h at concentrations of 625, 1250 and 2500 μg/mL, respectively (Dey et al., 2022).

Lactonase MzmL from *Mesoflavibacter zeaxanthinifaciens* XY-85 at a concentration of 5 μg/mL destroyed almost 50% of the biofilm of the phytopathogen *P. carotovorum* subsp. *carotovorum* within 4 days (Hao et al., 2024).

Most literature focuses on the effect of plant-derived compounds on the biofilms of phytopathogenic bacteria. Long et al. (2023) synthesised a series of derivatives of the 7-aliphatic amine tryptanthrin (an indolequinazoline plant alkaloid) and investigated their efficacy against the biofilm of *X. oryzae* pv. *oryzae*. One of the compounds (6e) at a concentration of 25.5 µg/mL inhibited biofilm formation by 80%.

The formation of *R. solanacearum* biofilm was inhibited by approximately 53% in the presence of 8 µg/mL resveratrol (phytoalexin) after 24 hours of incubation, in contrast to coumarin, which in a wide range of concentrations (8-64 µg/mL) had almost no inhibitory effect on the biofilm formation of this phytopathogen over 24 and 48 hours (Chen et al., 2016).

Harmin, also known as telepatin (a plant alkaloid), at concentrations of 20-60 mg/L inhibited *R. solanacearum* biofilm formation by 60-70% (Xia et al., 2023).

It should be noted that in the above articles, the authors studied the effect of natural biocides on the inhibition or destruction of the biofilm of one, and in rare cases two, phytopathogenic bacteria (*X. oryzae* pv. *oryzae, X. campestris* pv. *campestris*, *P. syringae* pv. *actinidiae*, *R. solanacearum, E. amylovora, D. dadanti*).

A key difference in our study is the use of six strains of phytopathogens belonging to different genera of phytopathogenic bacteria (*Xanthomonas, Pseudomonas, Agrobacterium, Pectobacterium, Clavibacter*) as test cultures. Importantly, the surfactants synthesised by *R. erythropolis* IMV Ac-5017 in medium with different compositions effectively destroyed the pre-formed single-species biofilms of all studied phytopathogens, especially at low concentrations.

Effect of surfactants produced by *R. erythropolis* IMV Ac-5017 under different cultivation conditions on dual-species phytopathogenic bacterial biofilms

At the next stage, we investigated the ability of surfactants produced by *R. erythropolis* IMV Ac-5017 to disrupt dual-species biofilms of phytopathogenic bacteria, depending on the cultivation conditions (Tables 3-5).

It should be noted that the efficiency of dual-species biofilms disruption depended on the incubation time of pre-formed biofilms with surfactants. At exposure times of 24 hours (similar to the study of the destruction of single-species biofilms, see Tables 1 and 2) and 48 hours, the disruption of all studied dual-species biofilms did not exceed 3-5%. Therefore, in further studies, the duration of treatment with surfactant solutions was increased to 96 hours.

The data presented in Table 3 show that the destruction of dual-species biofilms ranged from 11 to 69% following treatment with low concentrations (0.94-3.75 µg/mL) of surfactants synthesised in the presence of yeast inducer in various physiological states only, without phytohormone synthesis precursors. The highest degree of destruction (59-69%) was observed for *C. michiganensis* subsp. *michiganensis* IMV B-10$_2$ + *P. syringae* pv. *tomato* IMV B-9167 biofilm after treatment with surfactants synthesised in the presence of yeast *S. cerevisiae* BTM-1 supernatant as an inducer. When surfactants produced in a medium with inactivated yeast cells were used to treat this biofilm, the degree of its destruction was 10-48% lower and amounted to 21-49% (Table 3).

It should be noted that the destruction of dual-species biofilms under the action of surfactants synthesised in a medium containing only phytohormone biosynthesis precursors (without a yeast inducer) was 4-45% depending on the surfactant concentration (0.94-60 µg/mL) (Tables 4 and 5).

At the same time, the addition of inactivated yeast cells to a medium containing erythritol and/or tryptophan was in most cases accompanied by the synthesis of surfactants, under the action of which the degree of destruction of dual-species biofilms increased by 3-31 % compared with the destruction rates after treatment with preparations obtained without an inducer (Table 4). The exceptions were biofilms of *X. vesicatoria* UCM B-1106 + *P.*

syringae pv. *tomato* IMV B-9167 and *C. michiganensis* subsp. *michiganensis* IMV B-10$_2$ + *P. syringae* pv. *tomato* IMV B-9167. Their destruction was the same (39 and 44-45%, respectively) after treatment with surfactants synthesised in a medium containing only phytohormone synthesis precursors, as well as with precursors and inactivated yeast cells (Table 4).

Table 3

Destruction of dual-species biofilms of phytopathogenic bacteria under the action of surfactants synthesised by *R. erythropolis* IMV Ac-5017 in a medium with a yeast biological inducer

Biofilm destruction, %	**Inducer**					
	Inactivated cells			**Supernatant**		
	Surfactant concentration, µg/mL					
	0.94	**1.88**	**3.75**	**0.94**	**1.88**	**3.75**
C. michiganensis subsp. *michiganensis* IMV B-10$_2$ + *X. vesicatoria* UCM B-1106	41	51	38	44	44	n.d.
X. vesicatoria UCM B-1106 + *P. syringae* pv. *tomato* IMV B-9167	36	36	41	53	35	11
C. michiganensis subsp. *michiganensis* IMV B-10$_2$ + *P. syringae* pv. *tomato* IMV B-9167	21	21	49	67	69	59

Notes: The error in biofilm destruction determination did not exceed 5%; N.d. – not determined.

Table 4

Destruction of dual-species biofilms of phytopathogenic bacteria under the action of surfactants synthesised by *R. erythropolis* IMV Ac-5017 in a medium containing precursors of phytohormone biosynthesis and inactivated cells of *S. cerevisiae* BTM- 1 as a biological inducer

Biofilm destruction, %	**Phytohormone biosynthesis precursor**					
	Erythritol	**Tryptophan**			**Erythritol + tryptophan**	
	Presence of an inducer in the medium					
	-	+	-	+	-	+
C. michiganensis subsp. *michiganensis* IMV B-10$_2$ + *X. vesicatoria* UCM B-1106	9	40	15*	21*	4	7
X. vesicatoria UCM B-1106 + *P. syringae* pv. *tomato* IMV B-9167	7**	32**	39	39	n.d.	n.d.
C. michiganensis subsp. *michiganensis* IMV B-10$_2$ + *P. syringae* pv. *tomato* IMV B-9167	24	49	39*	51*	44***	45***

Notes: The error in biofilm destruction determination did not exceed 5%. The surfactant concentration was 15 µg/mL; * – 60 µg/mL; ** – 7.5 µg/mL; *** – 0.94 µg/mL; n.d. – not determined.

Table 5

Effect of precursors of phytohormone biosynthesis and *S. cerevisiae* BTM-1 supernatant as an inducer in the *R. erythropolis* IMB Ac-5017 culture medium on the ability of synthesised surfactants to destroy dual-species biofilms of phytopathogenic bacteria

Biofilm destruction, %	**Phytohormone biosynthesis precursor**					
	Erythritol		**Tryptophan**		**Erythritol + tryptophan**	
	Presence of an inducer in the medium					
	-	+	-	+	-	+
C. michiganensis subsp. *michiganensis* IMV B-10$_2$ + *X. vesicatoria* UCM B-1106	10	54	17	44	4*	61*
X. vesicatoria UCM B-1106 + *P. syringae* pv. *tomato* IMV B-9167	30**	49**	28***	42***	n.d.	n.d.
C. michiganensis subsp. *michiganensis* IMV B-10$_2$ + *P. syringae* pv. *tomato* IMV B-9167	38	42	37	37	45	49

Notes: The error in biofilm destruction determination did not exceed 5%. The surfactant concentration was 0.94 µg/mL; * – 15 µg/mL; ** – 3.75 µg/mL; *** – 1.88 µg/mL; n.d. – not determined.

Destruction of dual-species biofilms *C. michiganensis* subsp. *michiganensis* IMV B-10$_2$ + *X. vesicatoria* UCM B-1106 and *X. vesicatoria* UCM B-1106 + *P. syringae* pv. *tomato* IMV B-9167 under the action of surfactants produced in a medium containing erythritol and/or tryptophan and the supernatant of *S. cerevisiae* BTM-1 was 14-57% higher than that observed after treatment with preparations synthesised in the presence of only phytohormone synthesis precursors without an inducer (Table 5). At the same time, the degree of destruction of biofilm *C. michiganensis* subsp. *michiganensis* IMV B-10$_2$ + *P. syringae* pv. *tomato* IMV B-9167 was virtually the same after treatment with surfactant solutions synthesised either with only phytohormone biosynthesis precursors or with precursors combined with yeast supernatant.

Dual-species and polymicrobial biofilms represent a serious challenge, as, compared with single-species biofilms, they exhibit greater structural stability, altered metabolic interactions, and enhanced resistance to antimicrobial agents (Nithyanand et al., 2025; Yuan et al., 2020).

Several studies have reported the role of various biocides in disrupting polymicrobial biofilms of human pathogens (Fanaei Pirlar et al., 2020; Yuan et al., 2025), including microbial surfactants (da Silva et al., 2025; Srikanth et al., 2021). Surfactants produced by *R. erythropolis* IMV Ac-5017 in the presence of a biological yeast inducer have also been shown to effectively disrupt dual-species bacterial and bacterial–yeast biofilms (Okhmakevych et al., 2025).

Despite the well-documented ability of phytopathogenic bacteria, particularly *P. syringae*, *X. campestris*, and *R. solanacearum,* to form biofilms (Carezzano et al., 2023), and the limited information available on the disruption of their single-species biofilms, no publications were found reporting the disruption of dual-species biofilms of phytopathogens.

The study of dual-species biofilm formation by phytopathogenic bacteria was guided by data on the prevalence of these pathogens in Ukraine (Kolomiiets et al., 2019) and worldwide (Hubab et al., 2025), as well as by reports of the simultaneous detection of *P. syringae* pv. *tomato*, *X. vesicatoria*, and *C. michiganensis* subsp. *michiganensis* on tomato seeds (Xhemali et al., 2024), and the documented synergistic interactions between *Pseudomonas* and

Xanthomonas species during co-infection development (Aiello et al., 2017). Thus, Aiello et al. (2017) examined in detail the role of biofilms in these synergistic interactions between *Pseudomonas* spp. and *Xanthomonas perforans* in tomato core necrosis. It was shown that strains with high biofilm-forming activity (*P. fluorescens* 3P2S1, *P. putida* P9, *P. protegens* Pf5) simultaneously enhanced disease symptoms and the density of *X. perforans* populations in tomato tissues most strongly.

It should be noted that surfactants synthesised by *R. erythropolis* IMV Ac-5017 in medium of different compositions destroyed the pre-formed dual-species biofilms of all studied phytopathogens. However, the degree of destruction depended on the type of test cultures, the concentration of surfactants, the presence of phytohormone biosynthesis precursors in the medium, and the physiological state of the inducer (inactivated yeast cells or supernatant) (Tables 1–5).

Previous studies (Pirog et al., 2021) demonstrated that the antibiofilm activity of surfactants produced by *R. erythropolis* IMV Ac-5017 against human pathogens depended on the cultivation conditions of the producer, particularly the nature and concentration of the carbon source in the medium. The strain synthesizes a complex mixture of glyco-, amino-, and neutral lipids. According to the literature, aminolipids are more potent antimicrobial agents than glycolipids, while neutral lipids exhibit very weak antimicrobial activity (Cochrane and Vederas, 2016). An increase in the content of calcium cations—activators of NADP$^+$-dependent glutamate dehydrogenase, a key enzyme in the biosynthesis of surface-active aminolipids—led to the production of surfactants with enhanced biofilm-disrupting activity compared with those formed in the basic medium (Pirog et al., 2021).

It is likely that during the cultivation of *R. erythropolis* IMV Ac-5017 in a medium containing phytohormone biosynthesis precursors and a biological inducer, the content of aminolipids in the synthesised surfactant complex changes, which accompanied by a corresponding change in its antibiofilm activity. Clarifying these points will be the focus of our further research.

There are few reports in the literature on the destruction of bacterial or yeast biofilms in the presence of surfactants from the genus *Rhodococcus.* Thus, surfactants from *R. fascians* BD8 at a concentration of 250 µg/mL, were shown to destroy biofilms of *Enterococcus hirae* ATCC 10542, *E. faecalis* ATCC 29212, and *Candida albicans* SC5314 on silicone, polystyrene, and glass (Janek et al., 2018). The surfactants we studied, synthesised by *R. erythropolis* IMV Ac-5017, are effective destructors of biofilms of both human pathogens (Okhmakevych et al., 2025; Pirog et al., 2021), and phytopathogenic bacteria (this article) at concentrations one to two orders of magnitude lower than those of *R. fascians* BD8. Furthermore, the results presented in this study represent one of the first reports on the disruption of both single-species and dual-species biofilms formed by phytopathogenic bacteria from the genera *Xanthomonas*, *Pseudomonas*, *Agrobacterium*, *Pectobacterium*, and *Clavibacter* in the presence of microbial surfactants.

The addition of a yeast inducer in different physiological states (inactivated cells or supernatant of *S. cerevisiae* BTM-1) to the culture medium of *R. erythropolis* IMV Ac-5017 containing precursors of phytohormone biosynthesis resulted in the production of surfactants that were more effective at disrupting both single-species and dual-species biofilms of phytopathogenic bacteria compared with preparations formed in medium without an inducer (Tables 1, 2, 4, 5). It is anticipated that the presence of the yeast inducer, together with erythritol and/or tryptophan, will not interfere with the synthesis of auxin- and gibberellin-type phytohormones by the surfactant-producing strain. Further studies will be conducted to investigate this possibility.

Conclusions

This study demonstrated that surfactants produced by *R. erythropolis* IMV Ac-5017 in a medium containing precursors of phytohormone biosynthesis and a yeast inducer effectively disrupted both single- and dual-species biofilms of phytopathogenic bacteria. The ability to disrupt dual-species biofilms of phytopathogens was reported here for the first time. In the absence of the inducer, the surfactants disrupted single-species biofilms by 7–53% and dual-species biofilms by 4–45%, depending on cultivation conditions. The presence of inactivated *S. cerevisiae* BTM-1 cells or its supernatant enhanced antibiofilm activity, resulting in increases of 6–42% for single-species biofilms and 3–57% for dual-species biofilms compared with preparations obtained without the inducer.

These findings highlight the potential application of microbial surfactants for managing phytopathogenic biofilms, offering a promising tool for sustainable plant protection and for ensuring the safety and quality of raw materials in the food and processing industries. Further studies are warranted to optimize production conditions and to evaluate practical applications in agricultural settings.

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Smart approaches to two-stage dough processing and quality control

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Abstract

Introduction. The aim of this study is to develop an intelligent system for optimizing the two-stage bread dough production process based on predictive machine learning models, enabling adaptive control of product quality parameters while minimizing production costs.

Materials and methods. The two-stage process of wheat dough preparation (pre-dough and dough) is described using a systems analysis approach. To assess and predict the quality of pre-dough and dough, mathematical models were developed using binary regression tree algorithms. Optimization, taking into account the influence of the pre-dough preparation stage on the dough production stage, was carried out using the hierarchical game method (Stackelberg Game).

Results and discussion. Through systems analysis using prograph modeling, quantitative relationships were established between input disturbances (flour gluten properties and yeast milk activity) and dough quality indicators. It was determined that the developed binary regression tree models demonstrate high predictive performance, with an accuracy of $R^2 > 0.93$ for key indicators such as titratable acidity, leavening capacity, final temperature, and moisture content. The study also revealed that adaptive optimization of water temperature and fermentation time, based on the identified raw material characteristics, makes it possible to reduce the variability of final dough quality indicators by 12–15% compared to industrial standards. The implementation of five modeling scenarios demonstrated that minimizing the generalized quality criterion ensures the stability of the biotechnological environment even under raw material variability of up to 10%. Comparative analysis confirmed that the application of the Stackelberg game-based approach provides effective synchronization of the pre-dough and dough stages, enabling the achievement of target dough quality indicators. The use of the proposed optimization scenarios makes it possible to stabilize dough temperature, acidity, and leavening capacity at the output, mitigating the impact of variability in raw material quality (gluten properties and yeast milk activity). This ensures the production of dough with optimal technological characteristics for each individual batch, which is critical for automated bakery production lines.

Conclusion. An integrated control strategy based on machine learning models and game-theoretic optimization of the dough production process makes it possible to optimize the specified process variables within recipe constraints and reduce the generalized dough quality loss index by more than 30%.

Introduction

Smart Manufacturing integrates advanced digital technologies with traditional production processes, creating a flexible, efficient, and adaptive manufacturing system. The main goal of this approach is to increase productivity, improve product quality, and reduce costs by using the Internet of Things, big data, and artificial intelligence to make informed real-time decisions (Miranda et al., 2019; Wetzel and Damsgard, 2020). Automation of technological processes plays a key role in this approach, enabling the reduction of human errors, increasing production speed, and ensuring continuous monitoring and optimization of operations (Konur et al., 2023; Wang et al., 2021). Despite significant progress in the development of automated control systems (ACS), there remains a need for further optimization of technological processes to achieve even higher product quality and production efficiency. Traditional control methods, based on empirical data and the experience of process operators, do not always ensure optimal results, as they fail to account for the variability of input raw material quality indicators from batch to batch. This variability can lead to instability in the quality of finished products, which is a serious challenge for manufacturers.

The dough preparation process is one of the key stages in the baking industry, determining the quality of the final product. The pre-dough method of dough preparation has a long history and is widely used in bakery production due to its ability to ensure high quality of finished products (Amr et al., 2022; Brandt, 2019). This method involves the preliminary preparation of pre-dough, which is then added to the main dough mixing process. Pre-dough consists of yeast, flour, and water, which are left to ferment for a certain period of time, allowing yeast cells to develop and forming aromatic compounds that give bread its characteristic taste and aroma (Islam et al., 2024; Yang et al., 2022).

Traditionally, the dough preparation process is controlled based on the experience of bakers within the framework of established technological regulations (Trystram, 2022). The main process parameters, such as the consumption of flour and yeast milk, fermentation time of pre-dough and dough, water temperature, and others, are determined on the basis of the recipe, empirical data, and operator experience. However, these methods are often insufficiently accurate and may lead to variability in the quality of the final product, especially when the quality indicators of raw materials are subject to change.

In the scientific community, numerous innovations have emerged in the bakery sector, related both to changes in technological processes and to the improvement of existing ones. The review by (Cappelli et al., 2021) analyzes technological innovations and improvement strategies aimed at increasing sustainability, productivity, and quality in the production of flour, pasta, bread, and bakery products. The main focus is placed on the importance of the stages of wheat cultivation, milling, dough processing, and baking, using life cycle assessment (LCA) to develop environmentally friendly strategies at the early stages of production. The study by (Mitelut et al., 2021) presents current scientific literature data on bread and bakery products, trends, and innovations in these fields, which are aimed at developing new functional bakery products through the use of various functional ingredients and extending the shelf life of these products by means of both conventional and innovative processing and preservation technologies. The authors of the review by (Mesta-Corral et al., 2024) also analyze the physicochemical and rheological changes in dough, as well as the sensory properties of bread, through the inclusion of alternative flours such as beans, lentils, and soy into the dough.

Part of the innovations are also aimed at the use of AI and ML, which in the food industry are applied to optimize production processes, improve product quality, and reduce losses through data analysis and prediction of potential problems (Abinaya et al., 2024; Lutska et al., 2023). For example, computer vision models are used to determine the quality of raw and final products (da Silva Cotrim et al., 2020; Medina-García et al., 2024; Saha and Manickavasagan, 2021), detecting product defects at processing and packaging stages.

Overall, the literature demonstrates the significant potential of machine learning for improving technological processes in the food industry. The implementation of these technologies can reduce quality variability and the percentage of defective products, lower production costs, and increase the competitiveness of enterprises. Thus, the literature review confirms the relevance and prospects of research in the field of applying machine learning to optimize dough preparation processes using the pre-dough method.

The primary aim of this study is to develop a method for optimizing process variables in two consecutive technological stages, namely pre-dough and dough preparation, based on machine learning models, in order to improve product quality and reduce production costs. To achieve this aim, a comprehensive analysis of existing machine learning approaches applied in the food industry is conducted, followed by a systems-level investigation of the pre-dough and dough preparation processes to identify key quality parameters and influencing variables. Based on these findings, predictive machine learning models are developed and validated to describe and forecast the final characteristics of both pre-dough and dough. In addition, optimization criteria and scenario-based strategies are established, taking into account the two-stage nature of the process, thereby enabling the development of a method for optimizing process variables at the level of individual production batches.

Materials and methods

The process of two-stage dough preparation (based on traditional thick pre-dough) was investigated using a batch method in order to improve product quality stability and reduce production costs. The technological processes were described using a systems analysis approach, in which resources, operations, and system objectives were identified through prograph modeling.

For monitoring, data acquisition, and analysis of technological variables, automated instrumentation of the process control system was used, along with quality indicators obtained from technological logs, representing the results of measurements and calculations performed by process engineers.

Mathematical models were developed using machine learning methods, in particular binary regression tree algorithms, which made it possible to predict key quality indicators (temperature, moisture, acidity, and leavening capacity) with an accuracy exceeding 0.93.

Adjustment of technological regimes was carried out through a two-stage optimization procedure based on the Stackelberg hierarchical game, enabling adaptation of the control strategy for each individual production batch.

Dough preparation using pre-dough method

The study was carried out on the technological process of dough preparation using thick wheat pre-dough with a moisture content of 48–52%, corresponding to typical industrial wheat bread production conditions. In this study, the pre-dough refers to a thick sponge (stiff pre-ferment) consisting of flour, water, and yeast milk. For consistency, the term “pre-dough” is used throughout the manuscript. The technological process of dough preparation using the pre-dough method is multifactorial and includes several critical stages, each of which affects the quality of the final product. A key component of success is the control and optimization of process variables that ensure stability and high bread quality. In this study, yeast milk (a suspension of yeast in water) was selected as the yeast source due to its suitability for automated dosing in large-scale bakeries, which are the subject of digitalization within this work. The use of a liquid phase allows for more precise control of input variables in machine learning models. Traditionally, the consumption of yeast milk is determined according to standards depending on the recipe and type of bread, ranging from 0.5–1.5% of the flour weight. The water temperature for pre-dough preparation should be 35–40 °C, while for dough kneading it should be 20–30 °C. The kneading time of the pre-dough ranges from 10 to 20 minutes, and fermentation lasts from 3 to 6 hours. The water consumption for dough kneading is 50–60% of the flour weight, and the consumption of salt solution is 1.5–2% of the flour weight. The kneading time of the dough ranges from 10 to 30 minutes, and fermentation lasts from 1 to 3 hours, depending on bakery conditions and the desired result.

System analysis of the dough preparation process based on prograph modelling

To formalize and comprehensively analyze the technological process of dough preparation under conditions of input variability, a scenario-based approach was applied, specifically prograph modeling. The constructed prograph makes it possible to represent the hierarchical structure of relationships between resources, operations, and process events.

A prograph reflects the sequence of operations oriented toward the final result and includes scheduling, responsible performers, and management actions. Operations transform objects using resources, which may be material or financial, and show the transfer of objects between operations through events and inter-operational links.

The basic prograph of the system is defined by the following set:

$$B = \langle F, O, R, C \rangle,$$

where $F = \{f_1, \ldots, f_6\}$ – the set of operations;

$O = \{o_1, \ldots, o_6\}$ – the set of objects;

$R = \{ r_1, \ldots, r_8\}$ – the set of resources;

$C = \{c_1, \ldots, c_{41}\}$ – the set of goals.

Methods and conditions for mathematical model development

Mathematical modeling of the pre-dough and dough preparation stages was carried out using nonlinear machine learning algorithms based on collected production data. The models were developed as predictive tools linking variable input resources (flour characteristics and yeast milk activity) and controllable process variables (kneading time, fermentation duration, and water temperature) with output quality indicators, including temperature, moisture, acidity, and leavening capacity of semi-finished products (Table 1). This approach enables automatic adaptation of technological regimes to the specific characteristics of each batch of raw materials, ensuring the stability of target product quality indicators under conditions of input uncertainty.

The variable “quantity and quality of gluten” (z_2) is represented in the model as an integral indicator, where gluten quantity is determined by the percentage content of wet gluten, and gluten quality is assessed using the IDK-1 device (instrument units). This combination into a single vector input is обусловлено спецификой алгоритма decision tree, which allows the relationship between these characteristics and the elasticity and extensibility of the dough to be identified without losing the significance of each individual parameter.

Table 1

Technological variables

Symbol	Variable decryption	Pre-dough model	Dough model
Flour characteristics			
z_1	Flour strength	–	+ (independent inputs)
z_2	Quantity and quality of gluten	+ (independent inputs)	+ (independent inputs)
z_3	Gas-forming capacity	+ (independent inputs)	+ (independent inputs)
z_4	Water absorption capacity	+ (independent inputs)	+ (independent inputs)
z_5	Autolytic activity	+ (independent inputs)	+ (independent inputs)
z_6	Flour acidity	+ (independent inputs)	+ (independent inputs)
Yeast milk characteristics			
z_7	Acidity of yeast milk	+ (independent inputs)	–
z_8	Yeast milk fermentation activity	+ (independent inputs)	–
Process variables of pre-dough preparation			
u_1	Yeast milk consumption	+ (changes within the recipe)	–
u_2	Water temperature	+ (changes within the recipe)	–
u_3	Pre-dough kneading time	+ (changes within the recipe)	–
u_4	Pre-dough fermentation time	+ (changes within the recipe)	–
Process variables of dough preparation			
u_5	Salt solution consumption	–	+ (changes within the recipe)
u_6	Water consumption for dough	–	+ (changes within the recipe)
u_7	Dough kneading time	–	+ (changes within the recipe)
u_8	Water temperature	–	+ (changes within the recipe)
u_9	Dough fermentation time	–	+ (changes within the recipe)
Technological variables of the ready pre-dough			
y_1 (z_9)	Pre-dough temperature	+ (outputs)	+ (dependent inputs)
y_2 (z_{10})	Pre-dough moisture	+ (outputs)	+ (dependent inputs)
y_3 (z_{11})	Pre-dough leavening capacity	+ (outputs)	+ (dependent inputs)
y_4 (z_{12})	Pre-dough titratable acidity	+ (outputs)	+ (dependent inputs)
Technological variables of the ready dough			
y_5	Dough temperature	–	+ (outputs)
y_6	Dough moisture	–	+ (outputs)
y_7	Dough leavening capacity	–	+ (outputs)
y_8	Dough titratable acidity	–	+ (outputs)

Regression decision trees were selected to model complex nonlinear relationships in the dough preparation process. Although ensemble methods (such as Random Forest and Gradient Boosting) provide higher accuracy, they are computationally complex and exhibit low interpretability ("black-box" behavior), which limits their practical applicability for process engineers. The use of individual decision trees ensures the required transparency of the decision-making logic while maintaining sufficient predictive accuracy. To improve prediction reliability and minimize the risk of overfitting on limited datasets, separate models were developed for each output parameter (temperature, moisture, acidity, and leavening capacity). Such a decomposition-based approach allows for the optimization of model training parameters for each specific physicochemical relationship, thereby enhancing the generalization ability of the algorithm.

The mathematical models were developed based on a dataset of experimental data obtained under batch dough preparation conditions, where each sample corresponds to a complete technological cycle of the two-stage "pre-dough–dough" system. Model training was carried out with consideration of constraints on the ranges of variable variation in accordance with current regulatory documentation (recipes), ensuring the adequacy of predictions within real production tolerances.

Optimization of the dough preparation process

Considering the two-stage nature of the dough preparation process, a game-theoretic approach – specifically, the hierarchical (Stackelberg) game – is employed to integrate these stages into a unified optimization framework. Since pre-dough preparation precedes dough preparation, the leader should be the dough preparation stage, as its variables have the greatest impact on the final product. This approach allows the process to be optimized step by step, taking into account the influence of the first stage on the second.

Stackelberg game algorithm

Input data (Table 1):

- Flour and yeast milk characteristics: $z_1, z_2, \dots, z_8$;
- Initial control variables for the dough preparation process (leader): $u_5, \dots, u_9$;
- Initial control variables for the pre-dough preparation process (follower): u_1, u_2, u_3, u_4;
- Target outputs of the dough: y_5, y_6, y_7, y_8;
- Target intermediate variables (pre-dough results influencing the dough): y_1, y_2, y_3, y_4.
- Weighting factors w_i for the dough and pre-dough processes, respectively, and scaling coefficients k_i, $i = 1, 2, \dots, 8$.

Step 1. The leader (dough) determines the optimal control variables:

- Define the loss function for the dough (minimization criterion):

$$J_2 = \sum_{i=5}^{8} \frac{w_i}{k_{i,}} (y_i - y_i^*)^2 \,;$$

- The variables $u_5, \dots, u_9$ are optimized under the assumption that the pre-dough outputs $z_9, z_{10}, z_{11}, z_{12}$ can also be adjusted.

Step 2. Information transfer to the follower (pre-dough):

- The optimal values $z_9, z_{10}, z_{11}, z_{12}$ are transmitted to the first stage (pre-dough) as the specified output values $y_i^*, i = 1, 2, 3, 4$.

Step 3._ The follower (pre-dough) searches for the optimal control variables:

- Define the loss function for the pre-dough (minimization criterion):

$$J_1 = \sum_{i=1}^{4} \frac{w_i}{k_i,} (y_i - y_i^*)^2\,;$$

- The variables u_1, u_2, u_3, u_4 are optimized to achieve the outcomes specified by the leader.

Step 4. Result verification:

- The final dough outputs y_5, y_6, y_7, y_8 are checked for compliance with the desired values;
- The iteration is repeated if necessary.

Results and discussion

Results of process system modelling

Variability in raw materials represents a critical challenge for bakery production. Fluctuations in flour properties and yeast milk activity can directly affect dough consistency and rheological behavior, complicating the maintenance of stable production processes. Such variability may also result in undesirable changes in the texture, taste, aroma, and appearance of bread, thereby reducing its consumer acceptability. Furthermore, instability in raw material quality often necessitates additional adjustments to technological processes and increases the costs associated with quality control. To establish the relationship between process variables and quality characteristics, the dough preparation process should be analyzed taking into account resources, criteria, goals, objects, operations, and events. For this purpose, the process is represented in the form of a prograph.

The prograph for the dough preparation process is shown in Figure 1.

To construct the prograph, tables were prepared: goals (Table 2), operations and objects (Table 3) and resources (Table 4). Achievement of these goals takes place through the execution of a certain sequence of actions, called processes or operations f_i, each of which is directed toward achieving the specified goals c_j with the use of resources r_k. The result of each operation is represented by objects o_i.

The use of prograph modeling made it possible to clearly identify the points of application of control actions and input disturbances (such as variability in flour quality parameters and yeast milk activity) at each stage—from pre-dough preparation to dough kneading (Prokopenko and Ladanyuk, 2015). Such a system-based description provides a logical foundation for the further development of mathematical models and optimization algorithms, as it allows for an unambiguous determination of the composition of each phase and the sequence of ingredient dosing, thereby minimizing the risk of deviations from the technological regime under changing raw material characteristics.

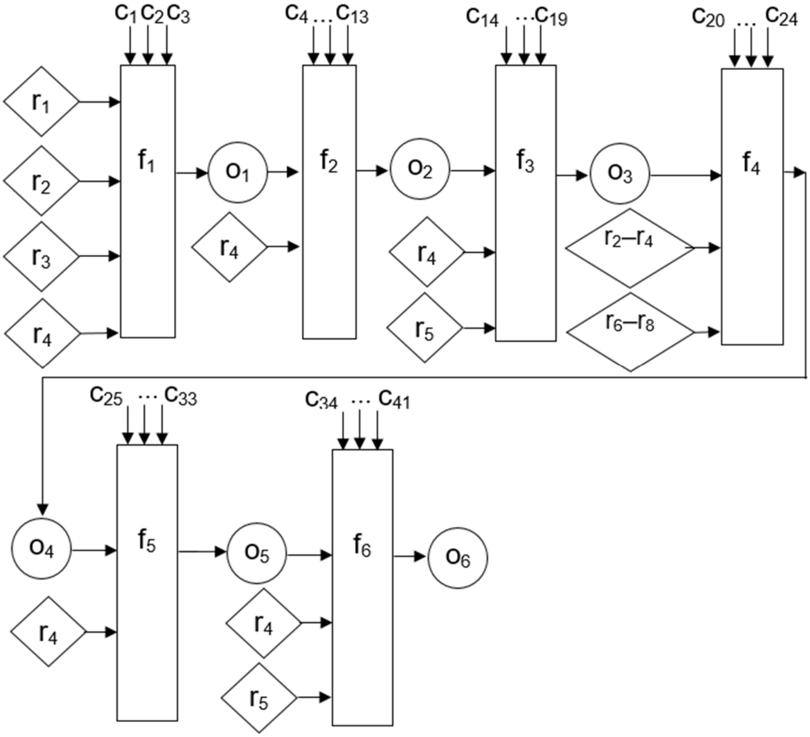

Figure 1. Prograph of the dough preparation process

Optimization of the two-stage dough preparation process through the control of critical parameters, such as temperature and moisture, forms the basis for ensuring stable product quality and minimizing production risks associated with raw material variability (Konur et al., 2023). The implementation of analytical models and machine learning algorithms makes it possible not only to predict the impact of variable ingredients on the final product but also to automatically adapt kneading and fermentation cycles, enabling the achievement of optimal organoleptic properties of bread while simultaneously reducing resource consumption (Wang et al., 2021). Such a system-based approach to automation and standardization, through continuous monitoring and real-time adjustment of process parameters, opens up new opportunities for improving the efficiency of food production and enhancing enterprise competitiveness.

Thus, the optimization method for an individual product batch is implemented as follows (Figure 2): first, the initial conditions for raw materials and control actions are initialized, and the target variables for each production stage (pre-dough and dough) are defined; then, using process models, the outputs for pre-dough and dough are predicted and possible deviations from the desired values are calculated; next, the optimization algorithm (in our case, the hierarchical game) is applied to identify the optimal control actions; after prediction and optimization, adjustments are made to the production parameters before processing the next batch; finally, after the batch is produced, the actual output parameters are compared with the predicted ones, and, if necessary, the model or approach is adjusted.

This approach ensures that the optimization strategy is adapted for each batch individually, thereby increasing flexibility and stability of product quality.

Table 2

Goals of the dough preparation process

Symbol	Resource content
c_1	Distribute ingredients evenly
c_2	Initiate fermentation processes
c_3	Reduce the time required for mixing without compromising the quality of the mixture
c_4	Ensure homogeneity of the pre-dough for proper fermentation
c_5	Ensure that pre-dough kneading is carried out according to technological regulations
c_6	Prevent overheating of the pre-dough
c_7	Reduce the time needed to achieve the desired consistency without loss of quality
c_8	Reduce the amount of residues and waste during pre-dough kneading
c_9	Reduce electricity consumption for kneading without decreasing productivity
c_{10}	Obtain the required pre-dough consistency
c_{11}	Ensure moisture control
c_{13}	Monitor key technological parameters of the kneading process and adjust them in time in case of deviations
c_{14}	Ensure proper fermentation time of the pre-dough
c_{15}	Regulate pre-dough acidity
c_{16}	Maintain temperature affecting fermentation rate and pre-dough quality
c_{17}	Ensure uniform fermentation of the pre-dough throughout the entire volume
c_{18}	Achieve stable pre-dough quality from batch to batch
c_{19}	Reduce energy consumption during the control of technological parameters
c_{20}	Ensure even incorporation of pre-dough with new ingredients
c_{21}	Control moisture and texture of pre-dough with new ingredients
c_{22}	Ensure proper ratio between pre-dough and other ingredients for stable dough quality
c_{23}	Reduce energy consumption during the mixing process
c_{24}	Prepare the pre-dough for further kneading
c_{25}	Improve dough texture
c_{26}	Avoid dough overheating during kneading
c_{27}	Reduce the time to reach the required consistency while maintaining dough quality
c_{28}	Ensure uniform dough rising
c_{29}	Obtain proper dough texture for further processing stages
c_{30}	Ensure stable dough quality according to the recipe
c_{31}	Create conditions for final fermentation
c_{32}	Reduce energy consumption during kneading without reducing process efficiency
c_{33}	Control kneading technological parameters to achieve the specified result
c_{34}	Ensure dough rising
c_{35}	Ensure control of dough temperature, moisture, and consistency
c_{36}	Regulate dough pH
c_{37}	Ensure achievement of the required level of acidity, structure, and dough rising in the shortest possible time
c_{38}	Control gas formation
c_{39}	Minimize energy consumption for heating, humidifying, and ventilating fermentation chambers
c_{40}	Ensure the possibility of controlling and regulating dough fermentation parameters
c_{41}	Prepare the dough for subsequent stages (dividing, shaping, final fermentation, baking, etc.)

Table 3

Operations and objects

Symbol	Content of operations	Symbol	Content of objects
f_1	Mixing of pre-dough ingredients	o_1	Mixture after kneading
f_2	Kneading of pre-dough	o_2	Pre-dough after kneading
f_3	Fermentation of pre-dough	o_3	Ready pre-dough
f_4	Mixing of pre-dough with other ingredients	o_4	Pre-kneaded dough
f_5	Kneading of dough	o_5	Dough
f_6	Fermentation of dough	o_6	Fermented dough

Table 4

Resources of the dough preparation process

Symbol	Content of resources
r_1	Yeast milk
r_2	Flour
r_3	Water
r_4	Electricity
r_5	Hot water
r_6	Salt
r_7	Sugar
r_8	Additional ingredients according to the recipe

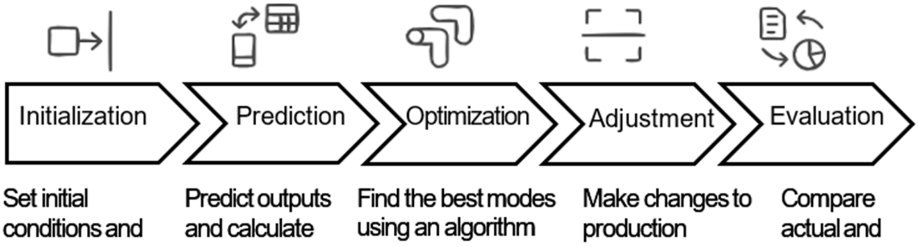

Figure 2. Main stages of the optimization method for process variables

Results of model development

The concept is based on the construction of predictive models to determine optimal control variables that ensure target quality of semi-finished products under varying raw material characteristics. The pre-dough model links yeast milk consumption, flour properties, and kneading conditions with output indicators such as temperature, moisture, leavening capacity, and acidity (Table 1). The dough model additionally incorporates the characteristics of the resulting pre-dough and the dosage of salt solution to predict final quality indicators. The developed models serve as a basis for minimizing deviations from technological targets under conditions of input variability (Hassoun et al., 2023; Lamrini et al., 2012; Lee et al., 2023; Sharma et al., 2021; Toska et al., 2021). For further optimization, several machine learning models were considered (Addanki et al., 2022; Khan et al., 2022), including regression decision trees and ensemble methods based on them.

Interpretability is also enhanced (Zgodavova et al., 2020): with four separate trees, the model becomes more transparent and understandable, as it is possible to clearly see how each factor affects each output. This is valuable for process operators (Al-Anqoudi et al., 2021), enabling them to better understand the data, make more informed decisions (Lutoslawski et al., 2021; Takahashi et al., 2022), and effectively control product quality (Drozd et al., 2021; Isleroglu et al., 2020; Santos et al., 2021). Finally, the approach with four trees provides greater flexibility in choosing algorithms and hyperparameters for each output and can significantly reduce training time through the use of parallel learning.

The mathematical model for evaluating and predicting the quality indicators (Drozd et al., 2021) of pre-dough preparation is described by the following dependence:

$$\overline{y^{pr}} = \left[Tree_{pr1}\left(\overline{x^{pr}}\right), Tree_{pr2}\left(\overline{x^{pr}}\right), Tree_{pr3}\left(\overline{x^{pr}}\right), Tree_{pr4}\left(\overline{x^{pr}}\right)\right]$$

$$\overline{y^{pre}} = [y_1, y_2, y_3, y_4], \qquad \overline{x^{pr}} = [z_2, z_3, \ldots, z_8, u_1, u_2, u_3, u_4]$$

$Tree_{pri}\left(\overline{x^{pr}}\right)$ – a set of 4 decision trees, with each tree describing the dependence of one output variable on the input data; $\overline{y^{pr}}$, $\overline{x^{pr}}$ – accordingly, the output and input vectors of the pre-dough preparation model (Table 5).

The mathematical model of dough preparation can be expressed by the following relation:

$$\overline{y^{d}} = \left[Tree_{d1}\left(\overline{x^{d}}\right), Tree_{d2}\left(\overline{x^{d}}\right), Tree_{d3}\left(\overline{x^{d}}\right), Tree_{d4}\left(\overline{x^{d}}\right)\right]$$

$$\overline{y^{d}} = [y_5, y_6, y_7, y_8], \qquad \overline{x^{d}} = [z_1, z_2, \ldots, z_6, z_9, z_{10}, z_{11}, z_{12}, u_5, u_6, \ldots, u_9]$$

$Tree_{di}\left(\overline{x^{d}}\right)$ – a set of 4 decision trees, each modeling the relationship between input data and one output variable; $\overline{y^{d}}$, $\overline{x^{d}}$ – accordingly, the output and input vectors of the dough preparation model (Table 1).

Table 5 presents the results of building the models of the pre-dough and dough preparation processes. These models are represented by binary regression trees, which show varying adequacy depending on the importance of predictors and metrics. For the pre-dough model, all four trees demonstrate low MSE values and high R^2 both on the training and testing data (Lutska et al., 2022; Zaiets et al., 2024). For the dough model, the first and fourth trees exhibit relatively low MSE values on the training data, indicating good accuracy, although the fourth shows a somewhat lower R^2 on the test data. Tree 2 and Tree 3 have very low MSE values as well as high R^2 on the test set, which indicates their excellent generalization ability. In particular, Tree 2 and Tree 3 provide the best results, with strong ability to explain data variation, making them the most adequate models among those presented.

Table 5

Machine learning models

Model	Predictor importance	General settings	Metrics
$Tree_{pr1}\left(\overline{x^{pr}}\right)$	[0.23, 0, 0.09, 0.04, 0, 0.02, 0.18, 0.04, 0.40, 0, 0]	MinLeafSize=5, MaxDepth=7, Prune="on"	MSE TRAIN: ~ 0 MSE TEST: ~ 0 R^2 TRAIN: 0.998 R^2 TEST: 0.995
$Tree_{pr2}\left(\overline{x^{pr}}\right)$	[0, 0, 0.16 0, 0.54, 0.01, 0, 0.27, 0.02, 0, 0]	MinLeafSize=5, MaxDepth=5, Prune="on"	MSE Train: ~ 0 MSE Test: ~ 0 R^2 Train: 0.995 R^2 Test: 0.992
$Tree_{pr3}\left(\overline{x^{pr}}\right)$	[0, 0.16, 0.13, 0, 0, 0.05, 0, 0, 0.06, 0.06, 0.54]	MinLeafSize=5, MaxDepth=5, Prune="on"	MSE Train: ~ 0 MSE Test: ~ 0 R^2 Train: 0.998 R^2 Test: 0.998
$Tree_{pr4}\left(\overline{x^{pr}}\right)$	[0.39, 0, 0.04, 0, 0, 0, 0.42, 0.08, 0, 0, 0.08]	MinLeafSize=5, MaxDepth=7, Prune="on"	MSE Train: ~ 0 MSE Test: ~ 0 R^2 Train: 0.998 R^2 Test: 0.998
$Tree_{d1}\left(\overline{x^{d}}\right)$	[0, 0, 0.32, 0, 0, 0, 0.10, 0.10, 0, 0.21, 0, 0, 0, 0.11, 0.16]	MinLeafSize=5, MaxDepth=7, Prune="on"	MSE Train: 0.057 MSE Test: 0.003 R^2 Train: 0.962 R^2 Test: 0.997
$Tree_{d2}\left(\overline{x^{d}}\right)$	[0, 0, 0.31, 0.40, 0, 0, 0.05, 0.05, 0, 0, 0, 0, 0.06, 0.05, 0.08]	MinLeafSize=5, MaxDepth=7, Prune="on"	MSE Train: ~ 0 MSE Test: ~ 0 R^2 Train: 0.999 R^2 Test: 0.998
$Tree_{d3}\left(\overline{x^{d}}\right)$	[0.04, 0.27, 0.05, 0, 0.02, 0.03, 0, 0, 0, 0.15, 0, 0.10, 0, 0, 0.34]	MinLeafSize=5, MaxDepth=5, Prune="on"	MSE Train: 0.007 MSE Test: ~ 0 R^2 Train: 0.997 R^2 Test: 0.995
$Tree_{d4}\left(\overline{x^{d}}\right)$	[0, 0, 0.050, 0.01, 0, 0.05, 0, 0, 0.20, 0.18, 0.01, 0, 0, 0, 0.50]	MinLeafSize=5, MaxDepth=5, Prune="on"	MSE Train: 0.007 MSE Test: 0.038 R^2 Train: 0.988 R^2 Test: 0.931

Among all control variables in the pre-dough preparation model, the most significant is the variable pre-dough fermentation time u_4, which appears in all derived models and has a strong impact on the final pre-dough indicators. Among all control variables in the dough preparation model, the most significant is the variable dough fermentation time u_9, which is present in all obtained models and strongly influences the quality of the final product. Dough fermentation time determines structure, leavening capacity, and acidity, which are key indicators for forming bread texture and flavor (Nnyigide et al., 2023; Raj et al., 2024). Its consistent influence across all models confirms the importance of controlling this variable to achieve stable quality and consistency of the final product. Figure 3 shows the change in dough characteristics relative to the most influential factors: flour gas-forming capacity and dough fermentation time (Raj et al., 2024). These characteristics are also influenced by the properties of the ready pre-dough, particularly $y_4(z_{12})$ (Figure 4).

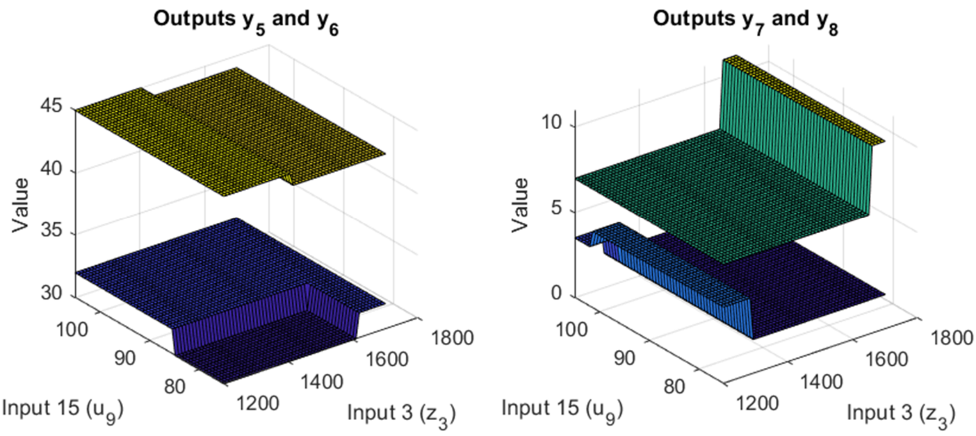

Figure 3. Dependence of dough characteristics on the most influential input features (flour gas-forming capacity and dough fermentation time)

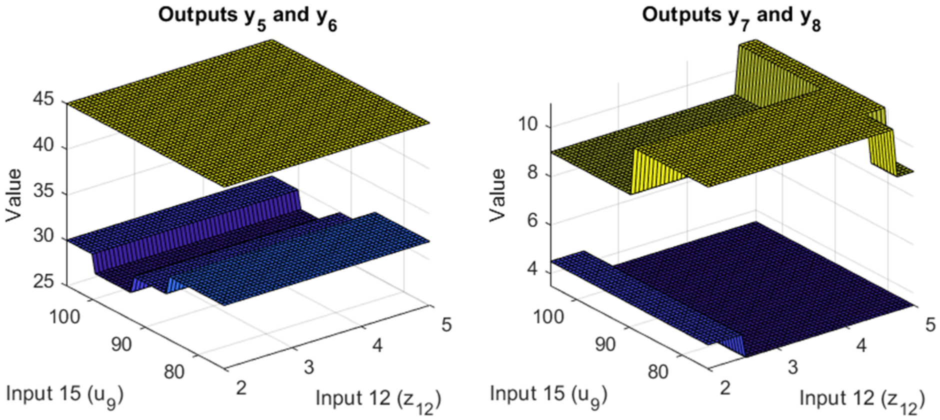

Figure 4. Dependence of dough characteristics on pre-dough titratable acidity and dough fermentation time

Results of optimization procedure

To account for technological fluctuations in raw material quality and to ensure model adaptability to changing conditions, five scenarios were formulated for simulation, reflecting typical situations in bakery production. All scenarios differ in the values of independent input variables – flour characteristics ($z_1 - z_6$) and yeast milk characteristics ($z_7 - z_8$), which are not subject to optimization but are determined by supply. For each scenario, optimization of process variables is carried out to ensure high dough quality (Table 6). All other variables (u_1 – u_9) are optimized within the permissible recipe constraints for each scenario.

Table 6

Defined modeling scenarios

Scenario no.	**Raw material description**	**Z_1**	**Z_2**	**Z_3**	**Z_4**	**Z_5**	**Z_6**	**Z_7**	**Z_8**
1	Reference quality of raw materials	M	M	M	M	M	M	M	M
2	Weak flour (low strength and gluten content)	L	L	M	H	M	H	M	M
3	Low-quality yeast milk	L	M	M	M	M	M	L	L
4	Flour with high autolytic activity	L	M	M	M	H	H	M	M
5	High-quality raw materials	H	H	H	M	M	M	M	H

* L – low level, M – medium level, H – high level.

The simulation results are presented in Table 7. During the simulation, five scenarios were implemented to optimize the technological parameters of the dough preparation process, taking into account the target values of temperature, moisture, leavening capacity, and acidity. For each scenario, the degree of reduction of the integral dough quality criterion J_2 was assessed in comparison with the initial value without changing the operating mode, as well as the optimization criterion for the pre-dough J_1, which plays a key role in shaping the microbiological environment at the early stages of fermentation.

Table 7

Modeling results

Scenario	**Temp (°C)**	**Moisture (%)**	**Gas-forming capacity**	**Acidity**	**J_2**	**J_1**
Target Values	30.00	44.50	9.50	3.00	–	–
1	29.90 **(–0.10)**	44.00 **(–0.50)**	8.88 **(–0.62)**	3.22 **(+0.22)**	0.015 **(–80.8%)**	0.06
2	29.90 **(–0.10)**	43.00 **(–1.50)**	11.00 **(–0.62)**	3.50 **(+0.50)**	0.078 **(–45.0%)**	4.06
3	28.00 **(–2.00)**	44.00 **(–0.50)**	8.88 **(–0.62)**	3.22 **(+0.22)**	0.078 **(–32.8%)**	0.25
4	28.00 (–**2.00)**	44.00 **(–0.50)**	8.88 **(–0.62)**	3.22 **(+0.22)**	0.078 **(–32.2%)**	4.25
5	29.90 (–**0.10)**	45.00 **(+0.50)**	9.00 **(–0.50)**	3.22 **(+0.22)**	0.013 **(–84.3%)**	1.00

The most effective scenarios were 1 and 5 (Figure 5). In Scenario 5, the minimum value of the criterion J_2=0.013 was achieved, corresponding to a reduction of 84.3% compared to the initial level. The deviations of the technological variables in this case were minimal, which indicates the effectiveness of the chosen optimization approach. Scenario 1 also demonstrated high efficiency, reducing J_2 to 0.015 (−80.8%), while the pre-dough was characterized by the lowest value of the criterion J_1 =0.06, confirming the stability of the microbiological process at the initial stage (Brandt, 2019).

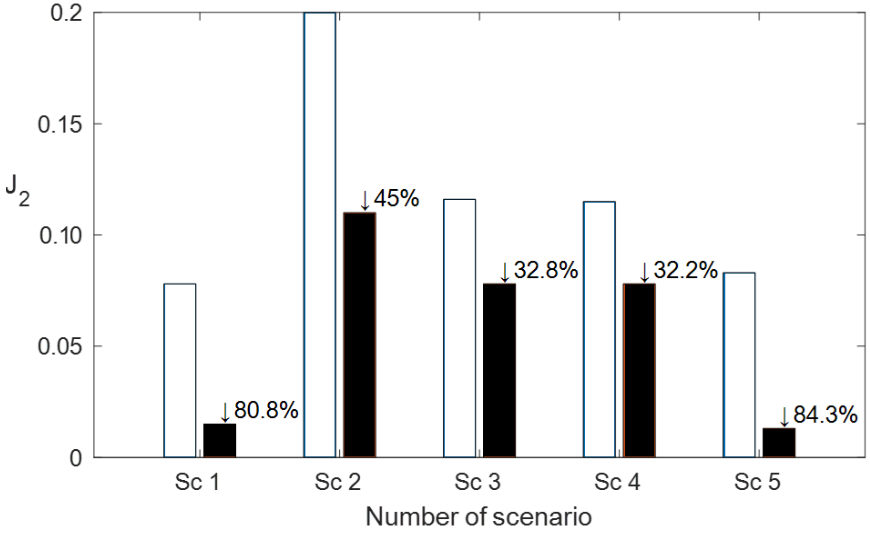

Figure 5. Values of criterion J_2 before (white bar) and after (black bar) optimization

The results showed that temperature and acidity have the greatest impact on the integral quality of the dough (Islam et al., 2024). A decrease in temperature by 2 °C in Scenarios 3 and 4 caused a significant reduction in leavening capacity, as well as a smaller decrease in J_2 (-32.2 та 32.8 %), despite the proximity of other variables to the target values. It was also noted that in Scenario 2 a significant increase in J_1 =4.06 led to a deviation in acidity by +0.5 units, which overall negatively affected the final result.

Analysis of J_1 values indicates that insufficiently precise regulation of pre-dough parameters (Scenarios 2 and 4) significantly complicates the achievement of the desired dough quality (Amr et al., 2022). For example, when J_1 >4.0, even minor deviations in technological parameters do not lead to the expected decrease in the final indicator J_2. This highlights the need for a comprehensive approach to optimizing both fermentation stages – pre-dough and dough.

In the present study, simulations were also carried out taking into account changes in nine control actions ($u_1 - u_9$), which cover the technological parameters of pre-dough preparation and dough kneading processes. The aim was to identify such combinations of control actions that provide the lowest values of the integral dough quality criterion J_2. This made it possible to draw the following conclusions:

- An increase in yeast milk consumption u_1 had a positive effect on the reduction of J_2 in Scenarios 1 and 5. Clearly, the additional amount of active yeast milk stimulates fermentation, improving the acidity and leavening capacity of the dough. However, in Scenario 2, where consumption was reduced, an increase in acidity was observed along with a sharp deterioration of the indicator J_1=4.06, indicating a disruption of microbiota activity. Thus, increasing u_1 (within the recipe limits) provides a positive effect on dough quality and the stability of the fermentation process.
- Temperature actions u_2 and u_8 have a cumulative effect. A decrease in water temperature in Scenarios 3 and 4 led to the worst results in terms of leavening capacity and insufficient fermentation activity, despite adherence to other parameters. In

Scenario 5, the temperature was increased (within the recipe limits), which ensured the lowest J_2=0.013 (−84.3 %). Thus, an increase in water temperature within the recipe limits is a key positive influence that accelerates fermentation and contributes to dough quality.

- In the scenarios with prolonged pre-dough kneading u_3 (Scenarios 1 and 5), an improvement in dough quality indicators was observed. This is due to better yeast milk distribution and more uniform flour hydration. Thus, increasing u_3 within the recipe limits contributes to greater homogeneity and stability of the pre-dough
- Extended fermentation time u_4 (in Scenarios 1, 4, and 5) makes it possible to achieve the target acidity values. Conversely, its reduction (Scenario 2) resulted in unstable dough structure and increased J_1. Therefore, a longer fermentation time is advisable for establishing stable fermentative activity.
- A reduction in salt solution consumption u_5 in Scenarios 1 and 5 allowed a decrease in acidity and improved the overall fermentation balance. In Scenario 2, by contrast, an increase in consumption led to fermentation inhibition. Thus, reducing u_5 within permissible limits helps to avoid excessive osmotic pressure while maintaining yeast milk activity.
- An increase in water consumption for dough u_6 (Scenarios 3–5) showed a positive effect on dough hydration and consistency. The effect was particularly pronounced in Scenario 5, where this action was accompanied by the lowest value of J_2. Thus, increasing water consumption for dough is an effective control action that optimizes dough plasticity.
- Extended kneading time u_7 (Scenarios 4 and 5) positively influenced dough homogeneity and improved leavening capacity. A reduction in time (Scenario 2) had the opposite effect – reduced volume and poorer structure. Therefore, optimizing u_7 towards longer kneading ensures better mixing of components and the development of the gluten network.

An increase in dough fermentation time u_9 (Scenarios 1, 4, and 5) reduced the value of J_2 to minimal levels, particularly to 0.013 in Scenario 5. In Scenario 2, with a shortened fermentation time, the worst balance between pre-dough and dough was recorded. Thus, extending u_9 ensures the completion of the fermentation cycle and dough maturation (Trystram, 2022).

The simulation results confirm that optimizing control actions within the recipe limits can significantly reduce the integral dough quality index, in some cases by 80–84%. The most effective was Scenario 5, in which:

- Increased yeast milk consumption, water usage, kneading and fermentation time;
- Increased water temperature;
- Reduced salt solution consumption.

Such a combination of control actions makes it possible to synchronize the processes of fermentation and acidity formation in the pre-dough with the enzymatic transformations in the dough. In addition, the presented simulation results confirm the traditional technological understanding of specialists in the bakery industry and allow for a quantitative assessment of the contribution of each control action to the overall quality indicator. This provides additional value for decision-making when adapting recipes or automating production.

The proposed optimization algorithm is not tied to a specific recipe and operates within predefined technological constraints. When the recipe, pre-dough parameters, or dough preparation method change, the models must be retrained using new production data, after which the algorithm retains its functionality without requiring structural modifications.

The obtained results demonstrate the effectiveness of the hierarchical game approach for optimizing bakery production processes using mathematical models. The developed approach makes it possible to significantly improve dough quality with minimal changes to the basic technological parameters. This opens up prospects for the implementation of intelligent decision support systems in production environments.

Conclusions

The use of machine learning methods for optimizing technological processes demonstrates high efficiency due to their ability to analyze complex nonlinear dependencies, adapt to changing production conditions, and make optimal decisions in real time. Machine learning algorithms improve the accuracy of predicting the quality characteristics of the final product, enabling more effective control of production parameters. They also contribute to automated process adjustments, minimizing the influence of the human factor and ensuring the stability of the technological regime.

Optimization of parameters using machine learning makes it possible to reduce raw material and energy costs, thereby increasing production efficiency. In addition, automated systems help reduce technological deviations, which positively affects the quality of the final product. One of the key advantages of such methods is their adaptability to variable conditions, allowing technological parameters to be adjusted according to changes in raw material characteristics or external factors.

Machine learning methods also provide the possibility of multifactor analysis, taking into account numerous variables and their interrelationships, which is not accessible through traditional optimization methods. Thus, machine learning is a powerful tool for improving product quality, reducing costs, and ensuring the stability of the production process. Further research may focus on the development of hybrid models that combine machine learning with optimal control methods and game theory to achieve even greater efficiency in complex technological processes.

This article addresses the problem of two-stage optimization of the bread production process using the Stackelberg hierarchical game. The first stage of the process – pre-dough preparation – is represented as the follower, while the second stage – dough preparation – acts as the leader. The proposed optimization method takes into account the interrelationship between the two stages: the outputs of the first stage become the input variables for the second. The leader (dough preparation stage) first determines the optimal values of its control variables and sets the desired values of intermediate indicators (temperature, acidity, leavening capacity, and pre-dough moisture). The follower (pre-dough stage) then adjusts its control variables in such a way as to achieve these desired parameters.

The developed approach makes it possible to:

- Ensure high quality of the final product through targeted control of key dough characteristics;
- Flexibly adjust the pre-dough preparation process according to the requirements of the subsequent stage;
- Apply game theory methods to optimize technological processes with a sequential structure.

The simulation results show that the hierarchical control strategy reduces deviations of dough technological variables from the desired values, thereby improving the stability of the production process. The proposed approach can be applied not only in bakery production but also in other industries involving multistage technological processes with interdependent variables.

Within the framework of this study and the proposed optimization method, further work is planned on developing new and improving existing static and dynamic models based on machine learning for predicting baking characteristics and enhancing the quality of the final product.

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Minimal size of the article (without the Abstract and References) is 10 pages. For review article is 25 pages (without the Abstract and References).

Manuscript should include:

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Abstract. The **abstract** should contain the following mandatory parts:

Introduction provides a rationale for the study (2–3 lines).

Materials and methods briefly describe the materials and methods used in the study (3–5 lines).

Results and discussion describe the main findings (20–26 lines).

Conclusions provides the main conclusions (2–3 lines).

The abstract should not contain any undefined abbreviations or references to the articles.

Keywords. Immediately after the abstract provide 4 to 6 keywords.

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Manuscripts should be divided into the following sections:

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Редакційна колегія наукового періодичного видання «**Ukrainian Food Journal**» запрошує Вас до публікації результатів наукових досліджень.

Вимоги до оформлення статей

Мова статей – англійська.

Мінімальний обсяг статті – **10 сторінок** формату А4 (без врахування анотацій і списку літератури**).**

Для всіх елементів статті шрифт – **Times New Roman**, кегль – **14**, інтервал – 1.

Всі поля сторінки – по 2 см.

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1. **Назва статті.**
2. Автори статті (ім’я та прізвище повністю, приклад: Денис Озерянко).
3. *Установа, в якій виконана робота.*
4. Анотація. **Обов’язкова** структура анотації:
   - Вступ (2–3 рядки).
   - Матеріали та методи (до 5 рядків)
   - Результати та обговорення (пів сторінки).
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Пункти 2–6 виконати англійською і українською мовами.

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За необхідності можна додавати інші розділи та розбивати їх на підрозділи.

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У списку літератури повинні переважати англомовні статті та монографії, які опубліковані після 2010 року.

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Кількість авторів статті	**Приклад цитування у тексті**
1 автор	(Arych, 2019)
2 автора	(Kuievda and Bront, 2020)
3 і більше авторів	(Bazopol et al., 2022)

Приклад тексту із цитуванням: It is known (Arych, 2019; Bazopol et al., 2022), the product yield depends on temperature, but, there are some exceptions (Kuievda and Bront, 2020).
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В Ukrainian Food Journal взято за основу загальноприйняте в світі спрощене оформлення списку літератури згідно стандарту Garvard. Всі елементи посилання розділяються **лише комами**.

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Kochubei-Lytvynenko O., Kuzmyk U., Yushchenko N. (2022), Spices for dairy products, In: O. Paredes-López, O. Shevchenko, V. Stabnikov, V. Ivanov (Eds.), *Bioenhancement and Fortification of Foods for a Healthy Diet*, pp. 157–178, CRC Press, Boca Raton, https://doi.org/10.1201/9781003225287-11

4. Тези доповідей конференції:

Arych M. (2018), Insurance's impact on food safety and food security, *Resource and Energy Saving Technologies of Production and Packing of Food Products as the Main Fundamentals of Their Competitiveness: Proceedings of the 7th International Specialized Scientific and Practical Conference, September 13, 2018,* NUFT, Kyiv, pp. 52–57, https://doi.org/5533.935-3.

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Тематика публікацій в Ukrainian Food Journal:

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Результати досліджень, представлені в журналі, повинні бути новими, мати чіткий зв'язок з харчовою наукою і представляти інтерес для міжнародного наукового співтовариства.

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