UNIVERSIDADE FEDERAL DE VIÇOSA

Technological potential of Lactiplantibacillus plantarum strains focusing on metabolomic analysis and their potential use as bioprotective cultures

Luana Virginia Souza
Doctor Scientiae

VIÇOSA - MINAS GERAIS
2026

LUANA VIRGINIA SOUZA

Technological potential of Lactiplantibacillus plantarum strains focusing on metabolomic analysis and their potential use as bioprotective cultures

Thesis submitted to the Veterinary Medicine Graduate Program of the Universidade Federal de Viçosa in partial fulfillment of the requirements for the degree of *Doctor Scientiae*.

Adviser: Luis Augusto Nero

Co-adviser: Antonio F. de Carvalho

VIÇOSA - MINAS GERAIS
2026

Ficha catalográfica elaborada pela Biblioteca Central da Universidade Federal de Viçosa - Campus Viçosa

T
S729t
2026

Souza, Luana Virgínia, 1987-
Technological potential of *Lactiplantibacillus plantarum* strains focusing on metabolomic analysis and their potential use as bioprotective cultures / Luana Virgínia Souza. – Viçosa, MG, 2026.
1 tese eletrônica (199 f.): il. (algumas color.).

Texto em inglês.
Orientador: Luis Augusto Nero.
Tese (doutorado) - Universidade Federal de Viçosa, Departamento de Medicina Veterinária, 2026.
Inclui bibliografia.
DOI: https://doi.org/10.47328/ufvbbt.2026.418
Modo de acesso: World Wide Web.

1. *Lactiplantibacillus plantarum*. 2. Bactérias do ácido lático. 3. Antifúngico. 4. Genômica - Análise.
5. Metabolômica. I. Nero, Luis Augusto, 1975-. II. Universidade Federal de Viçosa. Departamento de Medicina Veterinária. Programa de Pós Graduação em Medicina Veterinária.
III. Título.

CDD 22. ed. 579.37

Bibliotecário(a) responsável: Bruna Silva CRB-6/2552

LUANA VIRGINIA SOUZA

Technological potential of Lactiplantibacillus plantarum strains focusing on metabolomic analysis and their potential use as bioprotective cultures

Thesis submitted to the Veterinary Medicine Graduate Program of the Universidade Federal de Viçosa in partial fulfillment of the requirements for the degree of *Doctor Scientiae*.

APPROVED: June 10, 2026.

Assent:

Luana Virginia Souza
Author

Luis Augusto Nero
Adviser

First to God,
To Vinicius, my safe place in life and at work. Thank you for celebrating every achievement with me and, even more, for standing by my side through every challenge we faced. To my family, my foundation. Thank you for your unconditional love, your strength, and the unwavering support you have given me over the years. To my tutors Luís Augusto Nero, Antonio Fernandes, Cinzia Caggia, and Cinzia Lucia Randazzo, invaluable leaders and incredible supporters of my pHD training. I am grateful for your guidance, for the enthusiasm with which you foster our professional growth, and for believing in me from the beginning. To coordinator Antonio Biondi for the all support for my pHD in Unict. To my dear friends, my emotional anchors. Thank you for bringing joy to light-hearted moments and for being essential pillars in every endeavor I undertake.To the teams at InspoA and Inovaleite laboratories, who share experiences and accompany me through successes and challenges alike. I am thankful for the coffee time that lightened difficult moments and for the unexpected ones that became turning points in our work. To all who have nurtured, encouraged, and inspired me along the way.
From my heart. Thank you.

ACKNOWLEDGMENTS

This work has been sponsored by the following Brazilian research agencies: Coordination for the Improvement of Higher Education Personnel (CAPES; Financing code 001), Minas Gerais State Foundation for Research Aid (FAPEMIG) and National Council of Scientific and Technological Development (CNPq).

ABSTRACT

SOUZA, Luana Virginia, D.Sc., Universidade Federal de Viçosa, June, 2026. **Technological potential of Lactiplantibacillus plantarum strains focusing on metabolomic analysis and their potential use as bioprotective cultures**. Adviser: Luis Augusto Nero. Co-adviser: Antonio Fernandes de Carvalho.

In the context of food production, where multiple microbial and fungal communities interact within complex matrices, filamentous fungi represent both technological assets and major spoilage risks. Regarding spoilage, these fungi can disperse spores, colonize industrial environments, and produce mycotoxins, posing significant challenges for dairy products and fermented foods. Among strategies to mitigate fungal contamination, lactic acid bacteria (LAB) have gained attention as natural bioprotective cultures while contributing to product sensory enhancement. In this study, *Lactiplantibacillus plantarum* strains isolated from dairy matrices were selected for antifungal activity, technological and safety features. Metabolomic profiling revealed key antifungal compounds, including organic acids, peptides, and aromatic metabolites. Additionally, volatile compounds analysis identified metabolites associated with improved flavor and aroma, highlighting the dual role of these strains as both bioprotective and adjunct cultures. *In vitro* and *in situ* assays in a simulated cheese matrix demonstrated effective inhibition of *Aspergillus niger* and *Penicillium chrysogenum* without compromising sensory quality. Genomic analyses confirmed safety, functional potential, and genetic determinants underlying antifungal activity and metabolite production. These findings support the application of *L. plantarum* strains as multifunctional cultures that enhance food safety, sensory attributes, and align with clean-label trends in dairy and fermented products.

Keywords: bioprotective cultures; *Lactiplantibacillus plantarum*; antifungal activity; metabolome; genomic analysis; clean label

RESUMO

SOUZA, Luana Virginia, D.Sc., Universidade Federal de Viçosa, junho de 2026. **Potencial tecnológico de cepas de Lactiplantibacillus plantarum com foco em análise metabolômica e seus potenciais usos como culturas bioprotetoras**. Orientador: Luis Augusto Nero. Coorientador: Antonio Fernandes de Carvalho.

No contexto da produção de alimentos, onde múltiplas comunidades microbianas e fúngicas interagem em matrizes complexas, os fungos filamentosos desempenham um papel de dualidade, podendo estar associados tanto a aplicações tecnológicas quanto a importantes agentes de deterioração. Do ponto de vista da deterioração, esses microrganismos podem dispersar esporos, colonizar ambientes industriais e produzir micotoxinas, impondo desafios significativos à qualidade e segurança de produtos lácteos e alimentos fermentados. Entre as estratégias para mitigar a contaminação fúngica, as bactérias ácido láticas (BAL) têm ganhado destaque como culturas bioprotetoras naturais, ao mesmo tempo em que contribuem para a melhoria das características sensoriais dos produtos. Neste estudo, cepas de *Lactiplantibacillus plantarum* isoladas de matrizes lácteas foram selecionadas com base em sua atividade antifúngica, características tecnológicas e aspectos de segurança. A análise metabolômica revelou compostos com atividade antifúngica, incluindo ácidos orgânicos, peptídeos e metabólitos aromáticos. Adicionalmente, a análise de compostos voláteis identificou metabólitos associados à melhoria de sabor e aroma, destacando o papel duplo dessas cepas como culturas bioprotetoras e adjuntas. Ensaios *in vitro* e *in situ* em uma matriz de queijo simulada demonstraram inibição eficaz de *Aspergillus niger* e *Penicillium chrysogenum*, sem comprometer a qualidade sensorial. Análises genômicas confirmaram a segurança, o potencial funcional e os determinantes genéticos subjacentes à atividade antifúngica e à produção de metabólitos. Esses achados sustentam a aplicação de cepas de *L. plantarum* como culturas multifuncionais que aumentam a segurança dos alimentos, melhoram atributos sensoriais e estão alinhadas às tendências de inovação em produtos lácteos e fermentados.

Palavras-chave: culturas bioprotetoras; *Lactiplantibacillus plantarum*; atividade antifúngica; metaboloma; análise genômica; clean label

INDEX

CHAPTER 1

1.GENERAL INTRODUCTION

Context

In recent years, the demand for bioprotective cultures with diverse technological applications in food production has intensified. Cultures aimed at food biopreservation are projected to expand significantly in the global market, reaching an estimated value of 2 billion USD by 2025, with an annual growth rate of up to 9.6% expected through 2033(OjDATA, 2025). Such market growth is largely attributed to consumer preference for more natural options in foods, drinks, dairy products, and especially in fermented foods. In response, major multinational companies are investing in the development of new cultures, with a strong emphasis on innovation and effective market applicability.

In the dairy sector, microbial contamination, especially by pathogenic and spoilage microorganisms, remains a major challenge for quality control. Although good manufacturing practices are essential, they are not always sufficient, and contamination can spread across processing facilities (Garnier, Valence and Mounier, 2017). Although good manufacturing practices are essential, they are not always sufficient, and contamination can spread across processing facilities. Bacteria and fungi are the primary contributors to these problems, which are associated with considerable economic losses and public health risks (Pitt and Hocking, 2009).

Filamentous fungi are a particular concern because, despite multiple hurdle technologies in food preservation, they can disperse spores, germinate in industrial products and/or environments, and cause post-processing contamination (Pitt and Hocking, 2009, Souza et al., 2025). Their spores are ubiquitous and, if not controlled, can easily spread within production plants. Fungal spoilage results in visible growth on product surfaces, pigment formation such as black, brown, or reddish spots, strong musty or bitter off-flavors, and, in some cases, the production of mycotoxins, that pose serious public health concerns (Pinches and Apps, 2007; Garnier, Valence and Mounier, 2017).

One traditional strategy to mitigate microbial contamination, particularly filamentous fungi, is the use of fermentation by lactic acid bacteria (LAB). Lactic acid is the main antimicrobial compound produced in this process, but additional metabolites from primary and secondary metabolism are also generated, including organic acids, hydrogen peroxide, acetoin, diacetyl, reuterin, and bacteriocins, many of which exhibit antimicrobial properties. Moreover, these compounds can enhance flavor and improve the sensory quality of the final product (Bintsis, 2018; Salas et al., 2019, Souza et al., 2022).

Among LAB, *Lactiplantibacillus plantarum* is especially notable for producing antimicrobial metabolites, particularly bacteriocins such as plantaricins (types A, F, J, K, N, W, S) (Kareem and Razavi, 2020). In addition, it is recognized for its excellent functional and probiotic properties, with numerous patents registered worldwide (Robertson, 2018; Pesaro et al., 2020; Martoni et al., 2024; Kim et al., 2025). More recently, researchers have also explored its specific antifungal activity against different fungal genera (Wang et al., 2024; Zha et al., 2024; Vasundaradevi et al., 2025)

Considering that *Lactiplantibacillus plantarum* is well established for its diverse technological capabilities, and in light of the growing trend to reduce chemical preservatives in the food industry, this study aimed to conduct a comprehensive evaluation of *L. plantarum* strains as potential bioprotective cultures. The investigation encompassed in vitro and in situ antifungal assessments, characterization of technological and safety traits, and co-culture experiments against filamentous fungi from different genera. Additionally, metabolomic profiling was carried out to elucidate the mechanisms underlying antifungal activity, while genomic analysis of the antifungal strains was performed to confirm their safety and identify potential genetic drivers for future applications in food matrices.

Research hypotheses and aims

The hypothesis was to select strains with proven antifungal activity and conduct an in-depth study of their metabolome and genome in order to assess their safety and potential for future application in the food industry. To test this hypothesis, the following steps were defined:

i. To select *L. plantarum* strains that exhibit primary antifungal activity through an initial screening, evaluating their technological potential, antifungal capacity, and safety aspects.

ii. To investigate the antifungal activity of selected *L. plantarum* strains through co-culture experiments in liquid media, assessment of damage to fungal spores, and in situ evaluation using a simulated cheese matrix.

iii. To assess the metabolome of the antifungal *L. plantarum* strains, characterize the main antifungal compounds derived from primary and secondary metabolism, such as organic acids, peptides, and aromatic compounds.

iv. To analyze the genomes of the *L. plantarum* strains to identify the drivers underlying safety profiles, technological potential, and metabolic capabilities.

Thesis outline

For this thesis, four articles are presented. Three of them have already been published, while the fourth is in the final stages of preparation for submission.

FIRST PAPER (CHAPTER 2) gives a general overview of the world filamentous fungi in the food industry, highlighting their dual role as both beneficial agents in food production and sources of spoilage and mycotoxin-related health risks. While historically important for antibiotic and cheese production and widely used for biomass, enzymes, and biopolymers, filamentous fungi remain a major challenge due to contamination and food safety concerns.

SECOND PAPER (CHAPTER 3) describes published research that describes the technological potential, safety, and antifungal properties of LAB from sicilian dairy products, highlighting their potential as bioprotective cultures in the food industry.

THIRD PAPER (CHAPTER 4) describes the proven antifungal attributes of *Lactiplantibacillus plantarum* strains in vitro and in a simulated cheese matrix, with a focus on evaluating their metabolomic profile to identify compounds potentially responsible for antifungal activity and assessing the damage caused by the cultures to fungal spores.

FOURTH PAPER (CHAPTER 4) describes the genomic analyses of *antifungal L. plantarum* strains, where safety insights, functional annotation, taxonomic comparison, protein clustering, and carbohydrate pathways were analyzed and compared among the strains, along with the assessment of volatile aroma compounds in fermented milk.

References

Bintsis, T. (2018). Lactic acid bacteria as starter cultures: An update in their metabolism and genetics. *AIMS microbiology*, *4*(4), 665. https://doi.org/10.3934/microbiol.2018.4.665

Garnier, L., Valence, F., & Mounier, J. (2017). Diversity and contro gbl of spoilage fungi in dairy products: An update. *Microorganisms*, *5*(3), 42. https://doi.org/10.3390/microorganisms5030042

Kareem, R. A., & Razavi, S. H. (2020). Plantaricin bacteriocins: As safe alternative antimicrobial peptides in food preservation—A review. *Journal of Food Safety*, 40(1), e12735. https://doi.org/10.1111/jfs.12735

Kim, S.K., Seo, H., Lee, H.S., Kim, J.G., Kim, S.B., JU, J.Y, Cheon, H.G. *Novel lactic acid Lactiplantibacillus plantarum SKO* (European Patent Office Korean, 2025). Retrieved September 18, 2025 from https://data.epo.org/publication-server/rest/v1.2/publication-dates/2025-07-09/patents/EP4581940NWA1/document.pdf

Martoni, C. *Lactiplantibacillus plantarum* for use in addressing the symptoms of irritable bowel syndrome (French patente nº WO2024089260A1, 2024). Retrieved September 18, 2025 from https://patents.google.com/patent/WO2024089260A1/en

Ojdata. (2025). Bioprotective Cultures Market Breakthrough: Soaring to $2 billion with Enhanced Demand in the United States, China, and Japan. Market Forecast for Bioprotective Cultures in Q1 and Q2 2025. Retrieved September 18, 2025. https://ojdata.com/market-reports/bioprotective-cultures-market/

Pesaro, M., Stuhlmann, D., Garbe, L., Schmaus, G., Holmgren, K., Larsson, N.. *Lactobacillus plantarum* for skin care (French patente nº WO2020002429A1, 2020). Retrieved September 18, 2025 from https://patents.google.com/patent/WO2020002429A1/en

Pinches, S.E.; Apps, P. Production in Food of 1,3-Pentadiene and Styrene by *Trichoderma* Species. *International Journal of Food Microbiology.* 2007, *116*, 182–185. https://doi.org/10.1016/j.ijfoodmicro.2006.12.001

Pitt, J. I., & Hocking, A. D. (2009). *Fungi and food spoilage* (Vol. 519, p. 388). New York: Springer. https://doi.org/10.1007/978-3-030-85640-3

Robertson, A, K. Therapeutic uses of *Lactobacillus plantarum*. (French patent nº WO2018234255A1, 2018). Retrieved September 18, 2025 from https://patents.google.com/patent/WO2018234255A1/en

Salas, M. L., Mounier, J., Maillard, M. B., Valence, F., Coton, E., & Thierry, A. (2019). Identification and quantification of natural compounds produced by antifungal bioprotective cultures in dairy products. *Food chemistry*, *301*, 125260. https://doi.org/10.1016/j.foodchem.2019.125260

Souza, L. V., Martins, E., Moreira, I. M. F. B., & De Carvalho, A. F. (2022). Strategies for the development of bioprotective cultures in food preservation. *International Journal of Microbiology*, *2022*(1), 6264170. https://doi.org/10.1155/2022/6264170

Souza, L. V., Silva, R. R., Cavicchioli, V. Q., Tavares, R.M, Randazzo, C. L., Caggia, C., CARVALHO, A, F., & NERO, L. A. (2025). The duality of filamentous fungi: Beneficial uses and risks in the food industry. *Food Research International*, 220, 117191. https://doi.org/10.1016/j.foodres.2025.117191

Vasundaradevi, R., Sarvajith, M., Divyashree, S., Deepa, N., Achar, P. N., & Sreenivasa, M. Y. (2025). Tropical fruit-derived *Lactiplantibacillus* as potential probiotic and antifungal agents against *Fusarium oxysporum*. *Scientific Reports*, 15(1), 2144. https://doi.org/10.1038/s41598-025-85190-0

Wang, Q., Zhang, F., Zhang, Y., Zhang, Y., Wang, H., Song, J., & Suo, H. (2024). A novel strain *Lactiplantibacillus plantarum* LPP95 isolated from Chinese pickles: Antifungal effect, mechanism, and potential application in yogurt. *Food Bioscience*, 58, 103640. https://doi.org/10.1016/j.fbio.2024.103640

Zha, M., Li, J., Zhu, S., Huang, W., Sun, Z., Xia, Y., & Chen, Y. (2024). Selection and analysis of the characteristics of *Lactiplantibacillus plantarum* with antifungal activity. *LWT*, 202, 116277. https://doi.org/10.1016/j.lwt.2024.116277

CHAPTER 2

2.FIRST PAPER

FIRST PAPER - The duality of filamentous fungi: beneficial uses and risks in the food industry

Luana Virgínia Souza et al.,

Manuscript published in *Food Research International 2025*

DOI: https://doi.org/10.1016/j.foodres.2025.117191

The duality of filamentous fungi: beneficial uses and risks in the food industry

Luana Virgínia SOUZA[ab], Raiane Rodrigues da SILVA[cd], Valéria Quintana CAVICCHIOLI[e], Rafaela de Melo TAVARES[a], Cinzia Lucia RANDAZZO[b], Cinzia CAGGIA[b], Antonio Fernandes de CARVALHO[c], Luís Augusto NERO[a]

Author affiliations:

[a] InsPOA - Laboratório de Inspeção de Produtos de Origem Animal, Departamento de Veterinária, Universidade Federal de Viçosa, 36570 900, Viçosa, MG, Brazil

[b] Dipartimento di Agricoltura, Alimentazione e Ambiente, Università degli Studi di Catania, 95123, Catania, CT, Italy

[c] INOVALEITE - Department of Food Technology, Federal University of Viçosa (Universidade Federal de Viçosa) (UFV), Avenida Peter Henry Rolfs, s/n - Campus Universitário, Viçosa - MG, 36570-900, Brazil.Di3A -

[d] State research institute Center for Physical Sciences and Technology, Saulėtekio al. 3, Vilnius, Lithuania;

[e] LIP - Innovation and Research Laboratory, School of Veterinary Medicine and Animal Science, Federal University of Goiás (Universidade Federal de Goiás) (UFG), Rod. GO 462, KM 0, Campus Samambaia, Goiânia - GO, 74690-900, Brazil.

*Corresponding author: Luís Augusto Nero

E-mail address: nero@ufv.br (L. A. Nero)

Abstract

Filamentous fungi are ubiquitous microorganisms, comprising approximately 150,000 documented species, although global estimates suggest a total diversity of 11 million species. The role of filamentous fungi in the food supply chain is complex, balancing production benefits with risks of contamination and health concerns. These fungi have both positive and negative impacts on the food industry, reflecting their duality. Historically, fungi have played a key role in the production of antibiotics, such as penicillin, and have revolutionized food production, particularly cheese manufacturing, by contributing to the development of new sensory traits. Currently, filamentous fungi are extensively used in food production for biomass, protein, acid, enzyme, pigment, and biopolymer production, and the commercialization of fungal cultures has been well established. However, research into new fungal strains for food applications continues to emerge. However, filamentous fungi are a major cause of spoilage in the food industry. Due to their reproductive characteristics, they can contaminate food and lead to mold growth, causing flavors and odor. A significant concern is mycotoxin-producing fungi, as the ingestion of mycotoxins has been linked to adverse health effects. Despite ongoing studies, the mechanisms behind mycotoxin production remain incompletely understood. This review aims to explore both the positive and negative aspects of filamentous fungi in the food industry, highlighting the current challenges in their commercialization and the potential negative impacts they have on food safety and quality.

Keywords:

Filamentous fungi, food biotechnology, food control, food safety, mycotoxins, mycology, antifungal.

1. Introduction

Filamentous fungi play an essential role in the environment, acting as primary agents of organic matter decomposition and contributing essential components to the ecosystem for microbial communities. Additionally, they facilitate nutrient absorption in plants (Research Features, 2025). In food production, filamentous fungi are being increasingly utilized, particularly in the large-scale synthesis of ingredients, such as organic acids, pigments, biopolymers, proteins, and enzymes, as well as in the production of specific food products (Siddiqui, 2016; Huq et al., 2022). Similar to bacterial cultures and their by products, fungal cultures and their derivatives can obtain Generally Recognized as Safe (GRAS) or Qualified Presumption of Safety (QPS) status (Singh & Gaur, 2021). Several filamentous fungi, including *Aspergillus*, *Penicillium*, *Fusarium*, *Mucor*, *Trichoderma*, *Rhizopus*, *Monascus*, and *Talaromyces* spp., are used in food production, either directly as food or as components of food products (Takahashi et al., 2020). Numerous fungal-derived products are commercially available for food applications (Table 1).

Despite the widespread commercialization of fungi and their by-products in a continually expanding market, certain factors must be considered. Studies have suggested that the global reduction in crop yields may be linked to the spread of fungal pathogens into previously unexplored geographic regions (Meyer et al., 2020). Additionally, the ongoing search for new fungicides is contributing to the rise of antifungal resistance, exacerbating clinical fungal infections worldwide (Meyer et al., 2020). Lind et al. (2018) identified important genes associated with the pathogenicity of *Aspergillus fumigatus*. Notably, the presence of the brlA gene, which regulates secondary metabolism in this fungus, is controlled by laeA, a major regulator of this metabolic pathway. This regulatory network drives secondary metabolism and plays a direct role in cellular processes linked to the virulence of *A. fumigatus* (Lind et al., 2018). *A. fumigatus* is commonly found in the air and soil and has also been historically isolated from food (Melo dos Santos et al., 2003; Egbuta et al., 2015; Nieminen et al., 2022), but its role in aspergillosis, a clinical disease and its increasing resistance to antifungal treatments have been confirmed (Lestrade et al., 2018).

Table 1.

Commercial fungal derived products available for food applications

Fungi product	Company	Food application	Website
ClearIQ™ Natural Flavor	Myco Technology™	Flavor/Ingredient	https://www.mycoiq.com/
Honey Truffle Sweet Protein	Myco Technology™	Protein/Ingredient	https://www.mycoiq.com/
Quorn Meatless ChiQin Patties	Quorn	Vegan Protein (Burguer)	https://www.quorn.us/products/meatless-burgers
Mycelium	Mycovation	Ingredient/Protein snacks application	https://www.mycovation.asia/
ProteVin™	NextFerm	Vegan Protein	https://nextferm.com/protevin/
BakeZime®	Dsm-firmenich	Glucose oxidase/Enzyme/ Ingredient	https://www.dsm-firmenich.com/en/home.html
Tolerase®	Dsm-firmenich	Lactase Enzyme/ Ingredient	https://www.dsm-firmenich.com/en/home.html
Plant Based Meal	More Foods	Vegan Protein	https://www.more-foods.co/
Chy-Max®	CHR Hansen	Enzyme/ Ingredient for cheese production	https://www.chr-hansen.com/
Portabella Mushroom Jerk	Savory Wild	Flavor/Ingredient	https://savorywild.com/
MonascusRed®	Biotech	Colorant/Ingredient	https://biotech.uplb.edu.ph/technologies/monascus-red-colorant/
Red+	Michroma	Colorant/Ingredient	https://www.michroma.co/our-ingredients
My®BACON	My Forest Foods	Whole cut meat analogues	https://myforestfoods.com/mybacon
Citric acid	Tate&Lyle	Acidulant/Ingredient	https://www.tateandlyle.com/
Mixed mushrooms with Porcini	Nova Funghi	Protein/ ready-to-eat product	https://novafunghi.it/
Promyc® S110	Naplasol	Mycoprotein	https://www.promyc.com/products
Choozit® LYO Cheese cultures	Danisco	*Penicillium roquefort*/ Cheese Production	https://www.iff.com/food-beverage/dairy/

Another important aspect related to filamentous fungi is their impact on food spoilage. This represents a significant challenge for the processing industry, as spoilage agents are undesirable and can lead to several problems, including visible mold growth and flavor and odor productions (Garnier et al., 2017). Recent studies have also highlighted the increasing occurrence of biofilm production by fungi, which can be difficult to remove, especially considering that spores can be disseminated throughout the industrial environment (Miranda et al., 2022). Although studies on fungal biofilm formation have been limited in recent years, it is essential to emphasize the relevance of this topic. A recent study explored the adaptation of methods typically used for bacteria and yeasts to assess fungal biofilm formation, aiming to identify reproducible and reliable methodologies for evaluating fungal performance and biofilm development (Kulišová et al., 2023).

In the food industry, quality control management systems, such as Good Manufacturing Practices (GMP), Hazard Analysis and Critical Control Points (HACCP), and other quality systems must be robust and effective to prevent contamination by pathogenic and spoilage microorganisms, including both bacteria and filamentous fungi (Garnier et al., 2017) Recently, several studies have explored the antifungal activity of bacteria as a potential biocontrol strategy for fungal contaminants in the food industry (Taroub et al., 2019; Zhao et al., 2022; Souza et al., 2023). This approach offers an alternative to traditional chemical preservatives, to which fungi are increasingly developing resistance mechanisms. Furthermore, this aligns with the growing “clean label” trend, which seeks to reduce the use of chemical preservatives in food processing (Guimarães et al., 2018; Ma et al., 2019; Taroub et al., 2019; Zhao et al., 2022, Simões et al., 2023; Souza et al., 2023).

The ability of filamentous fungi to produce mycotoxins in food must also be addressed. Mycotoxins such as ochratoxins, aflatoxins (B1, B2, G1, G2, M1, and M2), patulin, fumonisins, zearalenone, trichothecenes, alternariol, deoxynivalenol and enniatin B represent a significant public health concern. These toxins cause a range of diseases, such as carcinomas, impaired renal function, genotoxic, cytotoxic, DNA damage, and reproductive toxicity. The main symptoms of exposure include apathy, vomiting, and food rejection (Hamad et al., 2022).

Recent studies have identified and quantified mycotoxins in food products, such as fruit, cereal, tea, and dairy products (Carraturo et al., 2018; Ji et al., 2022; Rodríguez-Cañás et al., 2023).

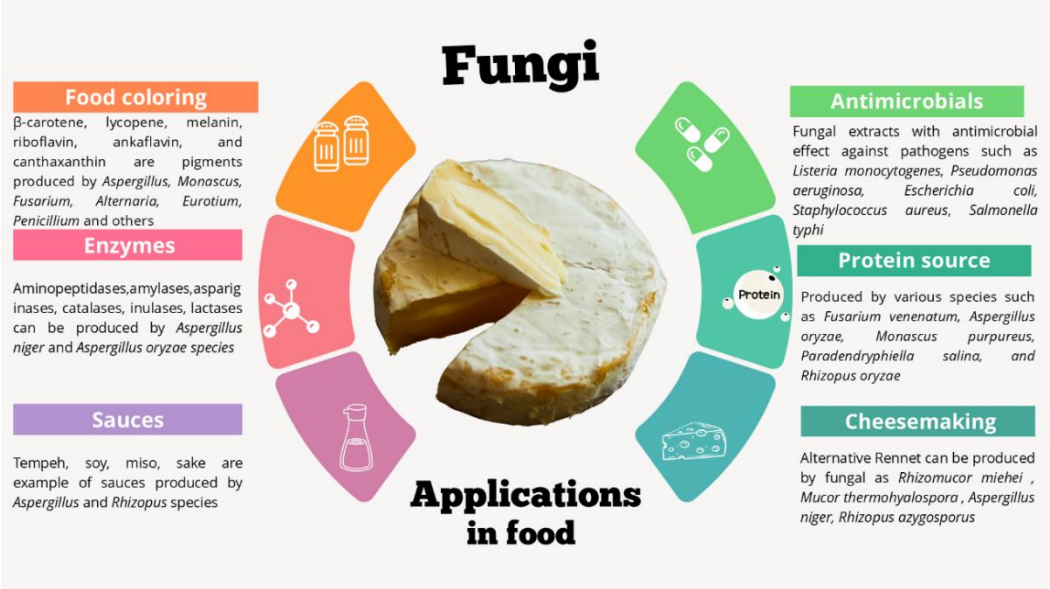
Considering the significant role that fungi play in the food chain, this review aims to explore the pathways through which fungi impact the food industry. The dual nature of fungi´s effects are explored, focusing on their beneficial effect in food applications and providing an overview of fungal cultures for food production in the market, and their detrimental effects, such as spoilage and safety concerns, as mycotoxin producers in food production. This review also examines the implications of fungal contamination on food safety and quality at all stages of food production, from a farm-to-fork perspective.

2. Biotechnological properties of filamentous fungi

The use of filamentous fungi in production is not a recent development. The earliest documented applications of filamentous fungi in history date back to the 18th century, particularly in the production of cheeses, such as Roquefort and Camembert (Labbe & Serres, 2009; Ropars et al., 2017). Initially, these cheeses were not produced through deliberate inoculation but became colonized due to accidental contamination by spores naturally present in the environment. Once the beneficial effects of these fungi in cheese production were discovered, several types, including those found on spoiled bread, were isolated and intentionally introduced during cheese production (Labbe & Serres, 2009; Ropars et al., 2017).

Over time, the use of filamentous fungi spread globally and became key ingredients in producing various cheeses, rather than Roquefort and Camembert, as Brie, St. Nectaire and Reblochon, significantly enhancing their value (Ropars et al., 2020). During this time, the technological application of filamentous fungi expanded in food production, covering a relevant financial market worldwide. Currently, fungi are exploited across diverse foods, such as cheese, beverages, fermented foods, sauces, and essential ingredients that are used in the food industry such as enzymes, pigment, protein, and antimicrobials (Figure 1) (Niego et al., 2023; Kaya et al., 2024; Tu et al., 2025).

Figure 1. Functional roles of fungi in food applications.



Regarding the potential of fungi for protein production in the food industry, one widely known product is Quorn™, produced by Marlow Foods, which has been on the market for over 30 years. This product is made from *Fusarium venenatum*, although other fungal species, such as *Aspergillus oryzae*, *Monascus purpureus*, *Paradendryphiella salina*, and *Rhizopus oryzae*, are also used (Souza Filho et al., 2018; Mapook et al., 2022). The market for fungal-derived proteins alone is expected to reach a value of USD 69.6 billion in 2025, with a growing trend worldwide (Global Market Insights, 2025). A comprehensive review of several fungal-derived protein products can be found in Mapook et al. (2022). In addition to serving as a protein source, fungi also produce a variety of enzymes with relevant applications in the food industry, including aminopeptidases, amylases, aspariginases, catalases, inulases, lactases, and triacylglycerol lipases, which are produced by strains of *Aspergillus niger* and *A. oryzae* (Sidiqui, 2016).

A significant historical development in the use of filamentous fungi took place in Asian countries, particularly China, Japan, and Korea, where edible fungi were first discovered. Today, these fungi play a crucial role in the large-scale production of traditional fermented

foods and beverages. For example, filamentous fungi are widely used in the production of soy sauce, miso, and sake fermentation, with *A. oryzae* being the predominant species involved (Hyde et al., 2019). Similarly, in Indonesia, fungus-fermented products are relevant to local cuisine, with *Rhizopus oligosporus* being the dominant species used in soybean fermentation to produce "tempeh", which is a widely consumed product, known for its distinctive flavor and nutritional properties, undergoing a fermentation process that typically lasts around two days (Nout & Kiers, 2005). In addition, filamentous fungi also participate in the fermentation of dark green tea in several Chinese provinces, where their succession and metabolic activities significantly influence the tea's flavor profile. *Aspergillus* is often the dominant genus in the fermentation process, contributing to the production of key flavor compounds, such as catechins and amino acids, through its metabolic activities (Hu et al., 2021).

Pigments have several applications in the areas of health, textiles, and the food industry (ATCC, 2022). For over a century, researchers have investigated fungi that produce pigments through secondary metabolism, focusing on optimizing large-scale production. Qiu et al. (2010) explored the ability of endophytic fungi to synthesize red pigments, while a more recent study examined the potential of *Aspergillus ustus* to synthesize brown and bright pigments (Zhou et al., 2023). In another study, Gong et al. (2022) evaluated the efficiency of red pigment production by an isolated strain of *Talaromyces aurantiacus* under various conditions and utilizing different favorable substrates. Furthermore, they conclusively demonstrated the antioxidant potential of the pigment produced. Furthermore, Chen et al. (2020) evaluated the ability of *Monascus* to produce yellow and orange pigments under extreme conditions, such as high-salt stress, and reported a notable capacity for pigment production under these conditions. Many other genera are also capable of producing pigments suitable for application in food products, such as *Monascus*, *Eurotium*, *Fusarium*, *Penicillium*, *Alternaria*, *Talaromyces*, *Ashbya*, *Blakeslea*, *Epicoccum*, *Paecilomyces* and others (Dufossé et al., 2014).

The American Type Culture Collection (ATCC), a leading organization of cell culture development and supply, maintains a collection of more than 180 fungal strains with demonstrated potential for pigment production. These strains can be used for scientific research and commercial applications under proper licensing (ATCC, 2022). Lagashetti et al. (2019) and

Kalra et al. (2020) highlighted the essential role of fungi in pigment production, noting that fungal-derived pigments are already widely used in the food industry. These include beta-carotene, lycopene, melanin, riboflavin, ankaflavin, and canthaxanthin, and others already available on the market (Kalra et al., 2020; Rousta et al., 2021).

Filamentous fungi are also recognized as promising sources of bioactive compounds, particularly protein hydrolysates such as bioactive peptides, which can act as physiological modulators. Depending on their residual chemical structure and amino acid sequence, these peptides may exhibit antioxidant, antimicrobial, antithrombotic, hypocholesterolemic, and antihypertensive properties. Examples of bioactive peptides produced by filamentous fungi include Dipeptidyl Peptidase-IV (DPP-IV) inhibitory peptides, as well as peptides containing leucine, valine, lysine, arginine, isoleucine, proline, histidine, tyrosine, tryptophan, methionine, cysteine, glycine, glutamic acid, glutamine, serine, and tyrosine (Rousta et al., 2024).

In addition to peptides, other bioactive compounds synthesized by various fungal species include ergosterol, chitin and chitosan, β-glucans, and fructooligosaccharides. Multiple fungal genera have been associated with the production of these bioactive metabolites, such as *Aspergillus*, *Rhizopus*, *Monascus*, *Fusarium*, *Neurospora*, *Mucor*, *Scopulariopsis*, *Aureobasidium*, *Acremonium*, and *Trichoderma* (Rousta et al., 2024).

Species such as *Aspergillus niger* and *A. oryzae* are widely used in fermentation processes. For instance, a study conducted by Magro et al. (2019) reported the use of these species for the production of phenolic compounds, along with in vitro evaluations of antidiabetic and antioxidant activity. Furthermore, the production of L-carnitine has been explored in species including *A. oryzae*, *Neurospora intermedia*, *Rhizopus oligosporus*, and *Rhizopus oryzae*, as described by Rousta et al. (2021). L-carnitine is a quaternary ammonium that plays an important role in energy metabolism. It functions as a transport molecule across the mitochondrial membrane, facilitating the transfer of long-chain fatty acids for subsequent β-oxidation. The consumption of L-carnitine has been associated with nutraceutical benefits (Walter et al., 2000; Rousta, 2021).

Fermentation products derived from filamentous fungi, such as *A. oryzae* have also been explored for their antimicrobial properties, having potential applications in the food industry. Since *A. oryzae* has already been granted GRAS status, its use in food production is particularly promising. Tu et al. (2025) evaluated the antimicrobial potential of extracts fermented by *A. oryzae* against *Listeria monocytogenes*, showing effective inhibition in both in vitro and in situ tests using a milk matrix. *Penicillium chrysogenum* has also been employed as an antimicrobial agent, as its crude extract has demonstrated inhibitory activity against pathogenic bacteria, such as *Staphylococcus* and *Pseudomonas aeruginosa*, with minimum inhibitory concentrations (MIC) of 12.5 and 25 mg/mL, respectively (Adeoyo & Adewumi, 2024). Similarly, Sharaf et al. (2022) reported antimicrobial activity from crude extracts of fungal species such as *Aspergillus nidulans*, *Aspergillus fumigatus*, and *Aspergillus flavus* against a range of pathogenic microorganisms, including *Staphylococcus aureus*, *Bacillus cereus*, *Bacillus subtilis*, *Escherichia coli*, *Salmonella* typhimurium, *Pseudomonas aeruginosa*, *Klebsiella pneumoniae,* and *Candida albicans*. Other fungal genera have also been investigated for their antimicrobial potential against pathogenic bacteria. These include *Alternaria*, *Engyodontium*, *Fusarium*, *Acremonium*, *Cladosporium*, *Trichoderma*, and *Periconia* (Jakubczyk & Dussart, 2020).

Another compound that can be produced on a large scale by filamentous fungi is chitosan, as the fungal cell wall itself is composed of this polysaccharide. Chitosan has applications in the food industry, such as antimicrobial agents and coating materials (Huq et al., 2022). Similar to bacteria, filamentous fungi can synthesize organic acids with potential of industrial applications in the food industry, including lactic, citric, succinic, aspartic, fumaric, itaconic, malic, and glucaric acid. *Aspergillus* and *Rhizopus* spp. are commonly used for this purpose, with *A. niger* being the most extensively used species (Porro & Branduardi, 2017).

Rennet is another biotechnological product that can be derived from filamentous fungi for use in food production. Traditionally, rennet for cheese manufacturing has been sourced from animals, particularly calves. However, fungal-derived rennet is a cost-effective and sustainable alternative that caters to specific consumer groups, such as vegetarians. Several fungal rennet products are already commercially available, including Fromase® XL, derived

from *Rhizomucor miehei* and produced by the DSM company, and Chy-Max® derived from *A. niger* and produced by the CHR Hansen company. Additionally, studies such as that by Chinmayee et al. (2019), have explored new fungal strains belonging to *Mucor thermohyalospora* and *R. azygosporus*, which have demonstrated high yields in solid-state fermentation (SSF). These strains may provide more efficient enzymatic activity while maintaining the compositional characteristics of cheeses (Chinmayee et al., 2019).

Fungal mycelia have also been utilized as flavor and color modifiers in fermented foods, including blue cheese, red mold rice, soy sauce, green tea and tempeh (Hu et al., 2021; Mapook et al., 2022). Filamentous fungi are known to produce flavor compounds, such as methyl ketones, vanillin, β-carotene, benzaldehyde, umami amino acids, and various organic acids with a special focus on sensory applications (Kinsella et al., 1976; Lesage-Meessen et al., 2002; Zorn et al., 2003; Zhang et al., 2013; Lv et al., 2024). Given the extensive applications of filamentous fungi in the food industry, understanding the mechanisms underlying metabolite production is essential. This knowledge enables industries to develop optimization strategies that enhance production efficiency while ensuring sustainability, practicality and food safety.

2.1. Biosynthesis pathways for industrially relevant fungal metabolites

Fungal metabolism is known for its high versatility. As discussed in the previous section of this review, filamentous fungi produce a wide range of compounds that are relevant to the food industry. However, the complexity of fungal metabolism continues to present challenges for comprehensive understanding. In recent years, significant advancements in DNA sequencing of filamentous fungi, as well as improvements in the accuracy of fungal taxonomy databases, have enabled more in-depth studies on species of interest for food production and genetic engineering for large-scale production. Both the primary and secondary metabolism of fungi are generally utilized to generate compounds of interest for the food industry. Further details on the general metabolism of fungi are presented by Keller (2019) and Cairns et al. (2019).

Primary metabolism involves the biosynthesis of molecules essential for the growth, development, and maintenance of the fungal cells. These molecules are also important in

secondary metabolism, acting as a source of monomeric building blocks (Chroumpi et al., 2020). Organic acid production, a key aspect of primary metabolism, is one of the most significant processes in the food industry. These compounds are used in several applications, such as acidulants, flavoring agents, buffers, preservatives, and technological additives (Copetti, 2019).

Citric acid, predominantly produced by *A. niger*, is the most widely used in food, pharmaceuticals, and several other industries, and it holds a substantial global market value (Cairns et al., 2019). *A. niger* produces citric acid through the fermentation of glucose or sucrose, with both the cytosol and mitochondria serving as sites for this process (Copetti, 2019). Citric acid biosynthesis is an intermediate of the Krebs cycle, in which, during glycolysis, one molecule of D-glucose is converted into two molecules of pyruvate. One pyruvate enters the mitochondrion and is converted to acetyl-CoA, while the other contributes to the tricarboxylic acid (TCA) cycle. Malate produced in this process is transported into the mitochondrion via the malate–citrate antiporter (CTP) and subsequently converted into citrate through TCA cycle activity. Citrate is then exported to the cytosol via counter transport with malate, leading to cytosolic citrate accumulation. The rise in intracellular malate levels, observed in organisms such as *A. niger*, appears to precede and stimulate citrate accumulation, highlighting malate's regulatory role. This pathway has a theoretical maximum yield of 1 mol of citrate per mol of glucose when pyruvate is derived from glycolysis (Chroumpi et al., 2020). The distinction between primary and secondary metabolism is often blurred, as several primary metabolites can exhibit specific features typically associated with secondary metabolism. Historically, secondary metabolism has represented a major turning point due to the biological relevance of many secondary metabolites. These compounds display diverse bioactivities and have significant applications in human health (e.g., pharmaceuticals and cosmetics), agriculture (e.g., biopesticides and plant defense agents), and the food industry (e.g., functional ingredients and additives) (Bills & Gloer, 2016).

In secondary metabolism, metabolites are usually produced during the stationary phase, and the genes that regulate their biosynthesis are often clustered together. These metabolites are commonly low-molecular-weight compounds, including metal chelators, hormones,

pigments, antioxidants, and antimicrobials. They are chemically and metabolically derived from four main classes: non-ribosomal peptides, terpenes, polyketides and alkaloids (Keller et al., 2005). Multiple regulatory mechanisms are involved in the production of these compounds, and certain aspects of these mechanisms are not yet fully understood (Cairns et al., 2019).

In the context of evolutionary taxonomy, particularly with regard to phylogenetic interpretations, a key feature of fungal secondary metabolism is the diversification of biosynthetic gene clusters across fungal lineages. This diversification is driven by several factors, including horizontal gene transfer, gene duplication, and the differential expression of regulatory genes involved in metabolic processes. For example, certain metabolites such as pigments and other bioactive compounds are classified as secondary metabolites. Their production can be optimized through a detailed understanding of the corresponding biosynthetic pathways and the genes involved in their synthesis. Furthermore, the application of specific stimuli and the optimization of growth conditions are essential strategies to enhance the production of these metabolites (Bills & Gloer, 2016).

Extracellular proteins typically contain an N-terminal signal peptide (15–36 amino acids) that directs them to the secretory pathway. Translation of the corresponding mRNA begins on cytosolic ribosomes. As the signal peptide emerges from the ribosome, it is recognized by the signal recognition particle (SRP), which temporarily halts translation and facilitates the targeting of the ribosome–nascent chain complex to the endoplasmic reticulum (ER) membrane. Translation then resumes, and the growing polypeptide is translocated into the ER lumen, where it undergoes initial folding and post-translational modifications (Lübeck & Lübeck, 2022). Nearly mature proteins are subsequently transported in secretory vesicles through the Golgi apparatus and delivered to the cytoplasmic membrane, where they are secreted. A defining characteristic of filamentous fungi, in contrast to yeasts, is their polarized hyphal growth, with protein secretion occurring predominantly at the hyphal tip (Lübeck & Lübeck, 2022). Like bacteria, filamentous fungi require specific nutrients for growth and compound production. Nutrients source of nitrogen, carbon, phosphate, and certain mineral salts are vital for metabolite production (Soccol et al., 2017; Sharif et al., 2021). Consequently, research focuses on manipulating cultivation conditions, including intrinsic and extrinsic

factors, such as temperature, nutrient availability, oxygen levels, pH, water content, and redox potential, to optimize metabolite production on both laboratory and industrial scales.

In the food industry, fungal fermentation can be carried out through two processes: submerged fermentation (SmF) and SSF. In SmF, the process occurs in a liquid medium that contains all the necessary nutrients for fungal growth (Sharif et al., 2021). One of the key advantages of SmF is its capacity for large-scale processing, rapid scalability, reduced fermentation duration, minimal labor requirements, and ease of parameter control (Strong et al., 2022). Cao et al. (2024) utilized almond hull extract as a nutrient source in the SmF process, to enhance the production of biomass of *Aspergillus awamori* in batches with passive aeration. When combined with other nutrients and compared to the potato dextrose broth medium, there was a 19.59% increase in protein production of the biomass, and a reduction in fat content, demonstrating that the nutrients provided to fungi impact the final contents of the biomass, such as protein, fat and ash contents.

In a recent study by Tiwari et al. (2025), SmF cultivation was applied with variations in the liquid medium, using corn steep liquor and defatted soya flour to enhance the production of mycelium and improve the nutritional composition of the edible mushroom *Agaricus bisporus*. Although these by-products are not commonly used for submerged cultivation of fungi, a growing trend toward using by-products and adopting more sustainable strategies has been observed.

Conversely, SSF is a process carried out on an insoluble substrate that serves as both physical support and a nutrient source, without the presence of a free-flowing liquid. In contrast to SmF, SSF requires less water, presents a lower risk of contamination, a greater use of food industrial by-products and lower energy requirements (Verduzco-Oliva & Gutierrez-Uribe, 2020; Strong et al., 2022;). Açaí biomass, a significant agro-food by-product in Brazil, has gained attention for its use in SSF by fungi. Açaí, a palm native to the Amazon, is economically valuable due to its pulp, but it also produces large quantities of waste that are now being repurposed for innovative applications. Lima et al. (2021) demonstrated that açaí seeds enhanced β-mannanase production by *Penicillium citrinum*, highlighting the potential to generate valuable enzymes from waste materials.

Novelli et al. (2016) conducted a comparative study on protease production via SSF using *Aspergillus oryzae, A. flavipes*, and *Penicillium roquefortii* strains employing various agro-industrial substrates (e.g., agro-industrial waste, soybean, and wheat bran). Distinct biochemical differences were observed among the enzymes produced by the different strains. All exhibited thermal stability up to 50 °C and activity across a broad pH range; however, *A. flavipes* showed an optimal activity temperature of 90 °C, while *P. roquefortii* exhibited optimal activity at 50 °C. The lyophilized protease from *A. oryzae* achieved a yield of 1251 U/g (~155,010 U/kg of substrate). Although the yields for *A. flavipes* and *P. roquefortii* were not reported in U/g, their enzyme production levels were comparable to those observed for *A. oryzae*. These findings underscore the critical influence of both process parameters and strain selection on enzymatic production efficiency and biochemical properties.

Thus, it is important to emphasize that fungal metabolism is inherently complex and exhibits specificities in each biosynthetic process relevant to the food industry. Conversely, it is known that this metabolism can be modulated in different ways that contribute to the spoilage of several food matrices. Therefore, within this largely unexplored domain, it is crucial to elucidate the environmental factors, particularly those related to food production, and to identify which fungal species may be beneficial or detrimental within the food chain, in order to address the duality challenge highlighted in this review.

3. Spoilage fungi in the food industry

Food spoilage, loss, and waste are major global concerns, particularly for food safety and security (FAO, 2019). According to the latest Food Waste Index Report from the United Nations (UNEP), the rate of food waste has increased, totaling 1.05 billion tons of food waste—equivalent to 132 kilograms per capita—representing almost one-fifth of all food available to consumers. This alarming fact contradicts the United Nations Sustainable Development Goals (SDGs), which aim to halve the per capita global food waste, confirming that this goal remains a significant global challenge (UNEP, 2024).

Food spoilage occurs through chemical, physical, and microbiological changes. Bacteria and fungi are the primary agents responsible for microbial spoilage. In general, in the food

industry, filamentous fungi, including species of *Aspergillus*, *Penicillium*, *Fusarium*, *Cladosporium*, *Alternaria*, *Mucor*, *Rhizopus*, *Trichoderma*, and others are frequently associated with food degradation, contributing to significant economic losses, quality deterioration, and product recalls (Pitt & Hocking, 2009).

The presence of these fungi demands careful monitoring and control to ensure food safety. Conventional counting methods often classify yeasts and molds together, limiting the resolution required for accurate detection of specific spoilage organisms. In addition, limitations in species-level identification techniques can hinder effective risk assessment, especially when spoilage is caused by toxigenic fungi (Snyder et al., 2024). Several of these species can produce hazardous mycotoxins, such as aflatoxins, ochratoxins, fumonisins, zearalenone, patulin, and citrinin, which may compromise consumer health and further affect the sensory and structural integrity of food products (Pattono et al., 2013; Ráduly et al., 2020). Filamentous fungi are ubiquitous and common contaminants of food, including raw items, such as fruit, vegetables, animal-derived products, and their processed forms (Parussolo et al., 2019; Ráduly et al., 2020). Grains and their products, processed beverages, nuts, teas, and meat products are also associated with fungal spoilage (Gong et al., 2024). In recent years, plant-based meat analogs have experienced significant growth in the market. The market was valued at USD 14,552.69 million in 2024 and is projected to reach USD 20,858.97 million by 2033, reflecting a promising outlook with a compound annual growth rate (CAGR) of 12.75% over the forecast period. However, even these newer products are susceptible to fungal contamination (Global Market Statistics, 2025).

Li et al. (2024) investigated spoilage dynamics in pea-based meat analogues and compared these with the profiles observed in ground beef. The study found that *Geotrichum* species were predominant in the plant-based products, where they caused significant deterioration in aroma, flavor, color, and texture. In ground beef, a different fungal community was identified, primarily composed of *Cladosporium* species, which are associated with surface discoloration and the appearance of black spots due to mycelial development. This comparison clarifies that the objective was to assess differences both in spoilage characteristics and in the composition of fungal communities between plant-based and animal-based products. Including

quantitative data, such as changes in volatile compounds or measurable losses in textural integrity, would provide further insight into the specific impact of *Geotrichum* species during storage. In general, the food production environment constitutes a potential source of contamination, as fungal spores can be aerosolized and dispersed throughout the processing facility (Parussolo et al., 2019).

The impact of fungal contamination can lead to visible and invisible defects in the final product. The most visible defect is fungal growth with the noticeable presence of a thallus, which eventually leads to rotting (Pitt & Hocking, 2009). Black, white, green, pink, and/or yellow spots are commonly associated with fungal development. This often results in elimination, whether at the industrial or consumer level. Some "invisible" defects include gas production, off-flavors, and texture changes (Pinches and Apps, 2007). Recently, compounds with atypical odors, identified as 1-hydroxyoctan-3-one and octane-1,3-diol, were detected in wines contaminated by fungi (Ployon et al., 2025). Several types of volatile organic compounds, particularly C8-alcohol/ketone and terpene, induce mushroom and/or earthy odors in a wide range of food products (Gong et al., 2024).

Regarding dairy products, several studies have covered contamination situations in different types of products, such as cheese, yogurt, butter, cream, and milk (Kure et al., 2004; Pattono et al., 2013; Garnier et al., 2017; Anelli et al., 2019). Many *Penicillium*, *Aspergillus*, *Acremonium*, *Cladosporium*, *Fusarium, Nigrospora, Mucor, Phaeosphaeria, Riopa*, and *Trichoderma* spp. are commonly found in dairy environments and products with the potential to cause spoilage (Pitt & Hocking, 2009; Garnier et al., 2017; Anelli et al., 2019; Souza et al., 2022). Due to the growth capabilities of certain spoilage fungi such as some species of *Penicillium* (e.g., *Penicillium charlesii*, *Penicillium fellutanum*, *Penicillium adametzioides*, and *Penicillium antarcticum*) at low pH values and storage temperatures below 10°C, they can easily contaminate fermented dairy products, producing enzymes that modify the product's sensory characteristics. Additionally, they can cause visible color changes and excessive mustiness (Pitt & Hocking, 2009; Garnier et al., 2017).

Fungal contamination in cereal grains can occur at any stage of the process, whether during harvest or processing. The negative effects of fungal contamination include loss of

germination potential of the grains, yield losses, and changes in the color and production of odorous compounds (Almeida et al., 2023). In bread production, the primary concern related to fungal contamination is the presence of a visible mold. These products often provide ideal conditions for fungal growth, particularly when preservatives are not incorporated. However, even with preservatives, fungal contamination remains a risk due to mycotoxin production, which is further addressed in this review, or resistance development. Additional defects associated with bread include the overproduction of alcohol compounds and the emergence of undesirable odors (Garcia et al., 2019).

Spoilage of fruits and vegetables results from a combination of internal and external factors. Internal factors include physiological processes such as natural ripening, senescence, and the progressive decline in intrinsic resistance mechanisms, including the production of phenolic compounds and the activation of wound-healing responses (Moss, 2008; Alegbeleye et al., 2022). Despite these natural defenses, fruits and vegetables remain susceptible to fungal contamination. External factors, however, play a more decisive role in postharvest spoilage. Mechanical injuries occurring during harvesting or handling provide direct entry points for fungal pathogens. These injuries also accelerate ripening and senescence, further reducing resistance to infection. In addition, inadequate storage and transportation conditions, particularly high humidity and temperature fluctuations, create environments that promote fungal growth and sporulation. Fungal species demonstrate considerable adaptability to these conditions, and some genera exhibit marked specialization in colonizing postharvest produce. Among the most frequently isolated fungi from spoiled fruits and vegetables are *Alternaria*, *Fusarium*, *Botrytis cinerea*, *Rhizopus*, *Penicillium*, *Cladosporium*, and *Aspergillus* (Tournas & Katsoudas, 2005; Al-Najada & Gherbawy, 2015; Hasan & Zanuddin, 2018; Saleh & Al-Thani, 2019).

Overall, the ecological dynamics of fungal growth in foods are modulated by a suite of physicochemical parameters that determine the suitability of a substrate for fungal spoilage. Key determinants include water activity (aw), pH, temperature, gas composition, physical consistency, nutrient profile, solute effects, and the presence of preservatives. Water activity, in particular, is a pivotal factor, as it defines the thermodynamic availability of water necessary

for fungal metabolism. Xerophilic fungi, such as *Xeromyces bisporus*, are capable of germinating and growing at aw values as low as 0.62 in fructose based environments, representing one of the lowest known thresholds among food-associated microorganisms (Pitt & Hocking, 2022).

Penicillium expansum is capable of growth across a moderately wide pH range (approximately 3.5–7.0) and water activity levels typical of fruit surfaces (aw >0.90). The pathogenicity of this fungus and its production of the mycotoxin patulin are significantly affected by the surrounding pH, showing increased toxin synthesis under acidic conditions. These physiological adaptations enable *P. expansum* to effectively colonize fruit substrates, other food products and contribute to spoilage (Jimdjio et al., 2021).

Additionally, similar to bacteria, fungi are increasingly exhibiting traits of resistance to preservatives commonly used in the food industry, which can facilitate the spread of fungi potentially adapted to adverse conditions, thereby enabling their dissemination. Wang et al. (2025) identified dipicolinic acid synthase, relevant components of bacterial spores, in *Paecilomyces* and some strains of *Aspergillus* and *Penicillium*, indicating that this compound may be associated with enhanced resistance to heat, salt content, and pH variations, which are conditions commonly used in the food industry.

Heat resistance significantly influences fungal survival through processing, particularly in acidic food products. The ascospore-forming *Paecilomyces variotii* (formerly *Byssochlamys spectabilis*) can survive pasteurization, with D-values equal to or greater than 10 minutes at 90 °C. This resistance is attributed to cytoplasmic vitrification by trehalose and mannitol matrices, which stabilizes internal structures under thermal stress (Pitt & Hocking, 2022). These resistance mechanisms illustrate the importance of applying multiple preservation strategies in parallel, combining reduced water activity, low pH, thermal processing, and chemical preservatives to control fungal spoilage effectively.

Another extremely important point related to the emergence and predominance of filamentous fungi as spoilage agents in food is associated with climate projections. It is expected to substantially alter fungal global geographic distributions over the years.

Considering the dual role of filamentous fungi within the food chain, as both ecological allies and potential contaminants, climate change may profoundly disrupt their functional balance in ecosystems. Environmental fungi perform essential ecological functions by decomposing organic matter, forming mycorrhizal associations that support plant development, and establishing mutualistic relationships with various insect species (Case et al., 2025).

Global climate change, characterized by rising average temperatures and increasingly frequent extreme weather events such as droughts, floods, wildfires, and storms, has been shown to significantly affect fungal community dynamics and their ecological interactions (Seidel et al., 2024; Case et al., 2025).These disturbances may select for more adaptable and virulent fungal strains, with higher temperatures promoting heat tolerance close to human body temperature and increasing zoonotic risks. Additionally, shifts in humidity and ecosystems alter fungal distributions, enabling new fungal disease outbreaks in different regions and hosts (Fisher et al., 2020; Casadevall, Kontoyiannis, & Robert, 2021). Therefore, these shifts underscore the urgent need to reassess current control strategies and anticipate emerging risks in a changing environment.

3.1. Mycotoxins: A significant public health concern

Mycotoxins are low-molecular-weight metabolites produced by the secondary metabolism of filamentous fungi and pose a potential hazard to humans and animals (El-Sayed et al., 2022; Ji et al., 2022). The main food contaminated by fungi, and associated with potential risks, are cereals, including corn, wheat, soybean, sorghum, peanut, and their by-products. Additionally, the consumption of animal-derived products such as milk, butter, and meat, which may contain mycotoxin residues or metabolites represents a public concern (Table 2) (El-Sayed et al., 2022).

Table 2. Occurrence of mycotoxins in different food matrices

Mycotoxin	Type	Food	Reference
Aflatoxin	M1	Milk and infant milk food	(Rastogi et al., 2004)
	B1	Dry fruit	(Awan et al., 2022)
	B1	Luncheon and hot dog meat	(Algahtani et al., 2020)
	-	Maize	(Akello et al., 2021)
	-	Rice	(Makun et al., 2011)
Citrin	-	Vegetable foodstuffs	(Ostry et al., 2013)
	-	Red yeast rice	(Liao et al., 2014)
	-	Broiler Chicks	(Roberts & Mora, 1978)
Fumonisin	B1	Corn	(Wang et al., 2008)
	B1 and B2	Rice	(Makun et al., 2011)
Ochratoxin	A	Rice	(Makun et al., 2011)
		Semi-hard cheeses	(Pattono et al., 2013)
		Coffee	(Taniwaki et al., 2003)
		Dry-cured ham	(Delfino et al., 2022)
		Dry fruits	(Iamanaka et al., 2005)
Patulin	-	Tomato products and apple juice	(Cunha et al., 2014)
	-	Semi-hard cheeses	(Pattono et al., 2013)
	-	Fruits products	(Ji et al., 2017)
	-	Dry cured ham	(Rodríguez et al., 2012)

Zearalenone	-	Hen's eggs	(Osaili et al., 2022)
	-	Rice	(Makun et al., 2011)

Among the filamentous fungi known to produce mycotoxins, three genera *Aspergillus*, *Penicillium*, and *Fusarium*, are the most prominent in food contamination. Although *Trichoderma*, *Trichothecium*, and *Alternaria* are less frequently related to mycotoxin contamination in food, they also remain important (Reddy et al., 2010; Adeyeye, 2016). The mycotoxins produced by these fungi can cause mycotoxicosis, with a range of adverse reactions, which, depending on their toxicity, may include teratogenic, mutagenic, hemorrhagic, estrogenic, hepatotoxic, nephrotoxic, immunosuppressive, endocrine, and carcinogenic effects (Agriopoulou et al., 2020). Although more than 400 mycotoxins have been reported in the literature, six classes are considered the most significant to public health: aflatoxins, ochratoxins, fumonisins, trichothecenes, zearalenone, and patulin (Agriopoulou et al., 2020; El-Sayed et al., 2022).

Aflatoxin is one of the most hazardous mycotoxins. It is produced by *Aspergillus* spp., mainly by *Aspergillus flavus* and *A. parasiticus* (Massomo, 2020) and has been classified as a Group 1 carcinogen in humans and animals, due to its toxic and mutagenic effects (Jallow et al., 2021; Shabeer et al., 2022). Food contamination by aflatoxin can occur at different stages of the food chain and is influenced by multiple intrinsic factors, such as temperature, water activity, pH, and nutrient composition (Jallow et al., 2021; Shabeer et al., 2022).

Ochratoxin is another important mycotoxin that is a risk to both human and animal health. It is produced by *Aspergillus* and *Penicillium* spp., particularly *A. ochraceus*, *A. carbonarius*, *A. niger*, and *P. verrucosum*. Contamination can occur both pre- and postharvest, enabling these microorganisms to spread into food and animal feed (Ganesan et al., 2022). Similar to aflatoxins, ochratoxins are found in a wide range of food products (Table 2), and their consumption has been linked to neurotoxicity and carcinogenic effects (Kumar et al., 2020; Ganesan et al., 2022).

Fumonisins are mycotoxins produced by several *Fusarium* spp., with *Fusarium verticillioides* and *Fusarium proliferatum* being the most significant contributors to food contamination, particularly in cereals and cereal-based products (Reddy et al., 2010). Fumonisins have hazardous effects, including neurotoxicity, pulmonary toxicity, immunotoxicity, and carcinogenicity (Yang et al., 2024).

Trichothecenes are also produced by *Fusarium* spp.; however, several other genera, such as *Myrothecium*, *Spicellum*, *Stachybotrys*, *Cephalosporium*, *Trichoderma*, and *Trichothecium*, can also synthesize these compounds (McCormick et al., 2011). Similar to other mycotoxins, trichothecene production occurs under specific environmental conditions that favor fungal growth. Recent studies have suggested that mycotoxin production is higher in tropical and subtropical regions due to the elevated humidity and temperature conditions (Polak-śliwińska & Paszczyk, 2021; Nichea et al., 2022). Considering that this group comprises more than 200 toxins, their effects on humans and animals are diverse. Trichotecenes are quickly absorbed by the body, affecting hematologic and progenitor cell systems, the immune system, skin, kidneys, liver, and gastrointestinal tract (McCormick et al., 2011; Polak-śliwińska & Paszczyk, 2021).

Zearalenone is another mycotoxin produced by *Fusarium* spp., mainly *F. graminearum* and *F. culmorum* (Mahato et al., 2021). The effects of this mycotoxin on the human body are dependent on individual conditions, such as immune status. After ingestion, its effects can vary, including teratogenic, carcinogenic, estrogenic, neurotoxic, and immunosuppressive outcomes (Mahato et al., 2021; Ropejko & Twarużek, 2021).

Although *Fusarium* spp. are major producers of mycotoxins affecting cereals and related products, other fungal genera also contribute to mycotoxin contamination in different food matrices. Notably, *Penicillium*, *Aspergillus*, and *Byssochlamys* spp. synthesize patulin, a mycotoxin predominantly found in moldy fruit, vegetables, and their byproducts (Mahato et al., 2021b; Notardonato et al., 2021). Patulin has been associated with carcinogenic effects, but no conclusive scientific evidence has confirmed this relationship (Notardonato et al., 2021).

Considering the increasing number and diversity of newly discovered mycotoxins, many of which pose potential risks to human health, the need for effective control measures has grown significantly. From a legislative perspective, regulatory agencies continuously update regulations to enhance the monitoring of various emerging mycotoxins. Since 2006, the European Commission (EC) of the European Union (EU) has actively monitored and regulated several mycotoxins in food, including aflatoxins (AFB1, B2, G1, G2, and M1), fumonisins (FB1 and FB2), deoxynivalenol (DON), ochratoxin A (OTA), patulin (PAT), and zearalenone (ZEN) (EC, 2006). In 2013, the EU recommended the monitoring of T-2 and HT-2 mycotoxins in cereals and related products (EC, 2013). More recently, in 2022, regulatory standards were established for alternariol (AOH), alternariol methyl ether (AME), and tenuazonic acid (TeA) in foods, such as cereal-based products, legumes, and nuts (EC, 2022).

In the United States, the Food and Drug Administration (FDA) updated its compliance program in 2024 to include mycotoxins, such as zearalenone and T-2 and HT-2 toxins (FDA 2024). Previously, the program focused on evaluating mycotoxins, such as aflatoxins (B1, B2, G1, G2), deoxynivalenol, fumonisins (B1, B2, B3), and ochratoxin A. Although many mycotoxins are already monitored and regulated, several others may still be present in food without regulatory oversight, posing challenges for the food supply chain. For instance, sterigmatocystin (STE) and emodin (EMO) produced by Aspergillus, enniatins (ENNs), beauvericin (BEA), moniliformin (MON), fusaproliferin (FP), fusaric acid (FA), culmorin (CUL), and butenolide (BUT) produced by Fusarium, mycophenolic acid (MPA), produced by *Penicillium*, and alternariol (AOH), alternariol mono-methyl ether (AME), and tenuazonic acid (TeA) produced by *Alternaria* are currently unregulated mycotoxins (Gruber-Dorninger et al., 2017; Rossi et al., 2020). It is expected that monitoring practices will continue to evolve with advancements in analytical methodologies, detection, and quantification techniques.

Although often underestimated, studies on mycotoxins have the potential to provide a deeper understanding of their effects as well as the mechanisms underlying their production. Consequently, the adoption of key strategies to minimize and mitigate mycotoxin production should be increasingly optimized alongside the development of effective and efficient

techniques for their identification and quantification, which may contribute to the control of these metabolites.

3.2. Strategies for the mitigation of fungal and mycotoxin contamination

The ubiquitous presence of fungi in water, soil, and air renders food contamination a persistent and intrinsic challenge within both the agricultural and food industry sectors. In this context, the adoption of comprehensive and scientifically grounded strategies for the control and mitigation of fungal growth and mycotoxin accumulation is essential across the entire food supply chain. To achieve effective outcomes, these strategies must be systematically implemented at each critical stage of the chain. The initial and perhaps most complex point of intervention lies in source prevention, which encompasses pre-harvest practices and soil management. This stage demands careful attention to agronomic conditions, crop rotation, and ecological variables that influence fungal proliferation.

Crop rotation is a widely used preventive measure, as fungal species often rely on specific hosts for their development (Matumba et al., 2021). Drakopoulos et al. (2021) found that intercropping wheat with maize effectively reduced mycotoxin levels in wheat without compromising yield. In addition to preventive strategies such as crop rotation, the fungal mitigation strategies typically rely on the use of fungicides. However, the widespread application of fungicides contradicts global sustainability principles and may also contribute to the emergence of fungicide-resistant fungal strains. For example, in the European Union, five major health and environmental agencies—EFSA (European Food Safety Authority), ECDC (European Centre for Disease Prevention and Control), ECHA (European Chemicals Agency), EEA (European Environment Agency), and EMA (European Medicines Agency)—have conducted studies demonstrating that azole-based fungicides, the most commonly used compounds in agriculture, and their residues in food products, may pose risks to human health (EMA, 2025).

The primary recommendations emphasize a globally coordinated One Health approach involving human health, veterinary medicine, agriculture, food production, and environmental sciences. Key actions include stricter fungicide approval criteria addressing resistance and

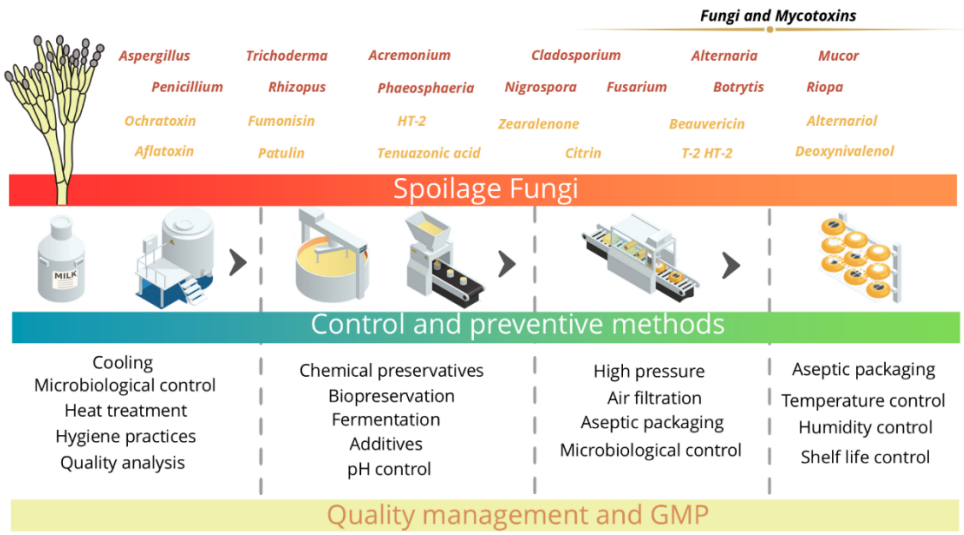
pathogenicity, strengthening good agricultural and manufacturing practices, and advancing research on antifungal agents. Additionally, further exploration of innovative and sustainable strategies is essential to combat antifungal resistance and emerging pathogenic fungal strains (EMA, 2025).

Increased spore dispersal combined with the selection of fungicide-resistant strains undermines food security by reducing crop yields and necessitating greater reliance on chemical pesticides, which in turn pose additional environmental risks. Alongside, advancements in the medical field have led to increasingly regulated and diversified strategies to combat fungal pathogens, including antivirulence therapies, biofilm disruption, immunomodulation, and novel antifungal agents. These developments, while primarily targeting human health, offer a comparative framework that highlights the urgent need for equally robust control strategies in agriculture. The World Health Organization (WHO, 2025) reports that 43 antifungal agents are currently in clinical or preclinical development, with Aspergillus fumigatus identified as a critical priority pathogen due to its high pathogenicity. These medical advances underscore the necessity of integrated, cross-sectoral approaches to mitigate fungal threats, particularly in food systems where prevention and control remain fragmented and underregulated.

Subsequently, proper handling during harvest and the application of effective post-harvest storage practices play a crucial role in minimizing the risk of fungal colonization and subsequent mycotoxin production. Postharvest contamination of grains by mycotoxins is primarily driven by mechanical damage, infestation of insect, harvest timing, inadequate drying, and poor handling and storage infrastructure. Critical environmental parameters such as temperature and moisture content strongly influence fungal proliferation and mycotoxin synthesis. As well reviewed by Neme et al. (2017), effective postharvest mitigation strategies include rapid and uniform drying, pest management, proper packaging and transport, and controlled storage conditions. Additionally, the application of natural or chemical preservatives and physical methods such as irradiation and others can further reduce contamination. Processing steps such as cleaning, sorting, milling, fermentation, roasting, extrusion, and nixtamalization have also been shown to lower mycotoxin levels.

As food progresses through the supply chain, targeted strategies during processing and distribution become equally critical. Given that each food product is subject to specific processing technologies and unit operations, which often involve high levels of complexity and multiple processing stages, mitigation approaches must be tailored to the characteristics of each matrix. Thus, fungi-related food deterioration is a major challenge in the industry. Modern practices, along with preventive and control approaches, are used in combination to reduce the incidence of these fungi in industrial facilities (Garnier et al., 2017). Among the preventive practices, air filtration, aseptic packaging, hygiene protocols, microbiological control, humidity and shelf-life management, as well as the implementation of Good Manufacturing Practices (GMP), can be highlighted. Control strategies, on the other hand, involve the use of additives, chemical preservatives, modified atmosphere packaging, high-pressure processing (HPP), thermal treatments, refrigeration, fermentation, and biopreservation. Prevention methods aim to avoid contamination or recontamination during product processing, while control methods focus on slowing or inhibiting microbial growth (Figure 2). However, when control and prevention methods are not applied effectively, fungal development may deteriorate food quality and lead to fungi dissemination, which may occur both in the processing plant and in the final product (Rahman, 2020).

Figure 2. Control and preventive strategies in food production to reduce or inhibit the growth of spoilage fungi.



Given these challenges, when choosing the most appropriate preventive and control methods, different factors must be considered. These include the product characteristics that need to be maintained (composition and water activity), preservation of microorganisms of interest, and manufacturing, sanitation, and storage conditions (Salas et al., 2017). Consumer perception and acceptance of preservation methods and their impact on hygiene and safety are also important considerations (Garnier et al., 2017; Rahman, 2020). Thus, among the emerging methods used to combat fungi-caused food deterioration, technology-based means have increased as industries aim to reduce the use of chemical preservatives—a development that has attracted considerable interest in the market.

Thus, emerging strategies for the mitigation of fungi and their mycotoxins have been increasingly explored and which can be used at any stage of the food chain and have been widely used, mainly in the processing stages, including physical methods, biopreservation techniques, and the development of novel antifungal products. Among the physical methods, notable examples include ionizing and non-ionizing irradiation (IR), high-pressure processing (HPP), pulsed electric fields (PEF), pulsed light (PL), and cold plasma (CP). Biopreservation approaches involve the utilization of bacteria, yeasts, and even microbial consortia. Regarding the development of new antifungal compounds, attention has been given to the production of salts, natural compounds, bio-based films, peptides, and antibodies (Sánchez Torres, 2024).

Conventional food processing methods, including thermal treatments, are usually ineffective at inactivating mycotoxins, as these compounds exhibit high stability under typical industrial processing conditions (Bullerman & Bianchini, 2007; Piotrowska, 2021). In fact, the optimal approach would not rely on alternative solutions to mitigate mycotoxins; rather, the primary mitigation strategy should focus on controlling fungal growth itself, as effectively inhibiting the fungus prevents mycotoxin production. However, in instances where mycotoxins have already been produced and persist within the food chain, their removal or neutralization becomes a critical objective.

One effective strategy for mitigating mycotoxin production early in the food supply chain is the implementation of robust monitoring systems and rapid diagnostic techniques that facilitate improved agricultural management. Mallmann et al. (2020) assessed the presence of

mycotoxins in corn using near infrared spectroscopy and concluded that such early and rapid diagnostic approaches are instrumental in enabling the adoption of proactive measures to reduce mycotoxin-related risks at any stage of the food supply chain. Given the importance of mitigating filamentous fungi and mycotoxins, most emerging treatments remain insufficient when applied in isolation, highlighting the need for integrated, multi-step approaches aligned with the United Nations (UN) 2030 Sustainable Development Goals (UNEP, 2024).

Applying the emergent method HPP to control and remove fungal and mycotoxins in food, Kalagatur et al. (2018) verified a reduction of 96.73% in the level of deoxynivalenol and 97.04% in the level of ZEN in maize grains. HPP can reduce the fungal population, therefore reducing mycotoxin levels. CP has also been used to remove mycotoxin-producing fungi and mycotoxins. Zhao et al. (2024) evaluated the effect of such a technique on *A. niger* spores and found a reduction of 4.47 log CFU/mL and a consequent reduction in aflatoxin production. In situ studies have also been carried out, such as the one conducted by Soulier et al. (2024), who reported a positive effect of dual-frequency CP on bread wheat. They observed a 1.5 log reduction in grains and a degradation of over 90% of the deoxynivalenol mycotoxin content after just 5 min of CP exposure. Antifungal activity has also been demonstrated through non-thermal techniques such as Intense Pulsed Light (IPL), achieving significant removal rates of up to 90% for biofilms formed by *A. niger* and *Penicillium glaucum*, and up to 99% for planktonic cells (Li et al., 2023).

Strategies such as biopreservation and the development of naturally derived antifungal compounds have gained increasing prominence as sustainable alternatives for fungal control (Souza et al., 2023). Complementing these approaches, omics technologies leveraging high-throughput sequencing have become indispensable for unraveling complex pathogenic mechanisms, metabolic pathways, and fungal genomics. This molecular-level insight is crucial for overcoming key challenges in the agri-food sector, particularly in managing the intricate interactions between fungi and food systems. In parallel, the removal of mycotoxins has been explored through enzymatic and microbiological methods; however, these techniques currently face limitations in scalability and efficacy. Among biological agents, lactic acid bacteria (LAB) stand out due to their demonstrated capacity to reduce mycotoxin concentrations via

transformation and adsorption. This functionality is largely attributed to their structurally complex cell walls, composed of peptidoglycan, teichoic acids, lipoteichoic acids, and exopolysaccharides, which facilitate effective adhesion and binding to mycotoxins and other macromolecules (Zhou et al., 2017; Wafula et al., 2022).

LAB species, such as *Lactobacillus*, *Enterococcus*, and *Streptococcus*, produce exopolysaccharides that contribute to fungal and mycotoxin mitigation. The peptidoglycan layer contains disaccharides that, when modified, affect the bacteria's ability to bind toxins. Detoxification mechanisms involve the interaction of cell wall proteins and exopolysaccharides with mycotoxins, especially after enzymatic treatment, which enhances binding. Positive surface charges, along with calcium and magnesium ions, play a significant role in this binding (Murtaza et al., 2023).

Studies have suggested that the mycotoxin-binding properties of LAB are highly strain-specific and determined by multiple factors, such as cell density, pH value, viability, temperature, and mycotoxin concentration (Nasrollahzadeh et al., 2022; Wafula et al., 2022). Murtaza et al. (2024) demonstrated that the *Lactobacillus consortium* LBC-4 effectively removed 88.7 and 89% of zearalenone (ZEN) in an in vitro model, generating fewer toxic derivatives and exhibiting enhanced adsorption following heat treatment. Structural changes were confirmed by scanning electron microscopy and Fourier transform infrared analysis. The mechanisms of ZEN removal were elucidated, highlighting the roles of the LAB cell wall, viable cells, and culture supernatants (Murtaza et al., 2024). Similarly, Simões et al. (2023) tested strains of *Lacticaseibacillus* spp., *Levilactobacillus* spp., and *Lactiplantibacillus* spp. isolated from naturally fermented Brazilian table olives and observed significant reductions in the production of aflatoxin, ochratoxin A, and patulin, with adsorption rates up to 51, 33, and 24%, respectively. Additionally, Mateo et al. (2022) observed reductions in aflatoxins ranging from 19.0% for aflatoxin B1 to 60.8% for aflatoxin B2, while ochratoxin A reduction varied from 7.3 to 100%, depending on the strains and temperatures tested. In this context, the LAB strains with the highest anti-aflatoxin activity were *Pediococcus pentosaceus* and *Leuconostoc mesenteroides* subsp. *dextranicum*, while the strains with the highest anti-ochratoxin A activity were *Leuconostoc paracasei* subsp. *paracasei* and *L. mesenteroides* subsp. *dextranicum*.

In situ studies have consistently demonstrated the potential of LAB in mycotoxin detoxification across various food matrices. Mischler et al. (2024) achieved up to 90% zearalenone reduction in contaminated grains using *Levilactobacillus brevis* and *Bacillus* spp., while Nahle et al. (2022) observed significant aflatoxin M1 and ochratoxin A removal in Ultra High Temperature (UHT) milk and grape juice through *Lactobacillus rhamnosus* biofilms. Similarly, Bovo et al. (2013) found that heat-killed cells of *L. rhamnosus*, *L. delbrueckii* subsp. *bulgaricus*, and *Bifidobacterium lactis* removed over 33% of AFM1 in phosphate-buffered saline, with Bi. lactis exhibiting the highest efficiency in UHT skim milk at 4°C. Further expanding on LAB applications, *Lactobacillus* sp. RM1, isolated from traditional Egyptian fermented milk, demonstrated antifungal properties, inhibiting aflatoxin B1 and ochratoxin A production and suppressing *Aspergillus parasiticus* growth on wheat grains (Shehata et al., 2019). These findings reinforce the potential of LAB and its metabolites as natural preservatives, contributing to food safety and an extended shelf life.

Despite the implementation of various conventional and emerging techniques at different stages of the food supply chain, the mitigation of filamentous fungi across all links remains a significant challenge. Interventions applied early in the food supply chain, particularly during the pre- and post-harvest phases, play a critical role in reducing the initial fungal load and limiting conditions favorable to mycotoxin synthesis. As food moves through processing, storage, and distribution, the adoption of tailored mitigation strategies becomes increasingly crucial. Accordingly, effective control at each stage not only enhances the overall efficacy of fungal management but also contributes to the safety, quality, and sustainability of the entire food system.

Moreover, regulatory oversight is essential to ensure the effectiveness and consistency of mitigation measures. Competent authorities are responsible not only for enforcing food safety standards and conducting routine inspections but also for adopting continuous improvement strategies within monitoring and control systems, in coordination with other sectors and industries. Coordinated regulatory supervision is, therefore, indispensable for protecting public health and ensuring food safety throughout the production and distribution process.

4. Challenges of duality

The presence of fungi in the food industry represents a duality. As previously discussed, fungi can be utilized in different ways, such as to produce new products or to change the sensorial characteristics of conventional food. However, the same microorganism can also contribute to food spoilage and mycotoxin production. One example is *Penicillium roqueforti*, a filamentous fungus widely used in cheese making and known for producing several desirable compounds in cheese, such as acetic, butyric, caprylic, and caproic acid (Vallone et al., 2014; López-Díaz et al., 2023). However, *P. roqueforti* is also considered a common spoilage agent in foods, such as fruit, bread, and refrigerated products, due to its ability to tolerate cold temperatures, low oxygen concentrations, alkaline preservatives, and weak acids (Ropars et al., 2017).

Another important aspect related to these fungi is their ability to produce mycotoxins, roquefortine C, PR-toxin, patulin, penicillic acid, mycophenolic acid, and isofumigaclavine A and B (Vallone et al., 2014). Their toxicity is low, but they are highly unstable. Therefore, it is essential to protect cheeses against fungal contamination. This is particularly important because many cheese production processes do not rely on the use of defined fungal starter cultures. Instead, they allow the natural colonization of environmental fungi during ripening. As a result, any strain that has not been properly characterized and approved for commercial application should be considered unsuitable for intentional use in food production (Singh & Gaur, 2021; López-Díaz et al., 2023). The potential use of fungal strains in food products, such as cheese, requires careful evaluation prior to any commercial application. This is because, despite possible technological or protective benefits, each strain must be proven safe. Safety assessments typically include testing for the ability to produce mycotoxins, toxicological screening, and genomic analyses to ensure the absence of genes associated with toxin biosynthesis. Only strains that meet these strict safety standards can be considered suitable for use in the food industry (Singh & Gaur, 2021).

Mucor is a ubiquitous microorganism commonly associated with the spoilage of dairy products, particularly cheese, as well as meat and fruit. It can cause off-flavors, anomalous textures, and discoloration (Yegin et al., 2011; Morin-Sardin et al., 2016). Despite these

disadvantages, *Mucor* has recently been explored for its potential in enzyme production for cheesemaking. Bensmail et al. (2020) investigated the potential of certain species in Camembert cheese production and isolated 20 fungal strains from soil, of which 1 demonstrated a strong ability to coagulate milk. This microorganism, identified as *Mucor circinelloides*, exhibited a coagulation capacity comparable to that of vegan rennet. In a sensory panel evaluation, assessors found that cheese produced with *M. circinelloides* was similar to the conventional version, suggesting that the produced enzyme acts as a viable alternative to traditional rennet.

Monascus fungi are widely recognized for red pigment production, which is used as a colorant in the food industry. This beneficial property is already commercially exploited, as shown in Table 1. The pigments produced by *Monascus ruber* have been reported to exhibit antimicrobial activity against pathogenic bacteria, such as *Staphylococcus aureus* and *Escherichia coli*, as demonstrated by Vendruscolo et al. (2013). Additionally, these pigments may possess other biological functions, including antioxidant and anti-inflammatory properties (Qin et al., 2023). However, *Monascus ruber* has been identified as causing spoilage in table olives (Panagou et al., 2002). This duality highlights the need for caution, as certain *Monascus* spp. can produce the mycotoxin citrinin, raising concerns about the biotechnological aspects of pigment production.

Recent studies have aimed to address this challenge through genetic engineering and clustered regularly interspaced short palindromic repeat (CRISPR)/Cas technologies, focusing on understanding and manipulating metabolic pathways to optimize pigment production while minimizing or eliminating citrinin biosynthesis. Zhang et al. (2025) demonstrated that targeted gene editing using CRISPR/Cas9 can effectively knock out or repress key genes involved in citrinin biosynthesis, thereby reducing toxin levels without compromising pigment yield. These strategies offer a promising approach to mitigate the safety concerns associated with *Monascus*-derived products by decoupling pigment production from citrinin formation.

Several *Fusarium* spp. are recognized for their biotechnological potential, particularly in the cultivation of biomass known as mycoproteins, offering a sustainable alternative with diverse applications in various food products (Cheriaparambil & Grossmann, 2025). This includes derived products, such as triacylglycerol lipases from *Fusarium oxysporum*, which

have received GRAS status for use in the production of baked goods, and fungal proteins obtained from Fusarium sp. mycelia (FDA, 2016; FDA, 2021). *Fusarium oxysporum* is also known as a significant plant pathogen, with specificity for over 150 plant host species. Bananas are frequently attacked by *Fusarium oxysporum*, which produces beauvericin. These compounds not only cause physical crop damage but also pose a risk to human health when ingested (Li et al., 2013).

Aspergillus niger is a common black mold found in several types of foods, such as fruit, corn, and nuts (Li et al., 2020). It is a fungus found in air, water, and soil and can develop fast, depending on the food characteristics (Fendiyanto & Dwi Satrio, 2020; Li et al., 2020). In addition to causing food spoilage, there is growing concern regarding the production of the mycotoxins fumonisin and ochratoxin A, which pose significant public health risks (Li et al., 2020). However, similar to the other microorganisms presented, biotechnological studies have been evaluating this organism for the production of important compounds for the food industry, such as citric acid (Xue et al., 2021), amylase (Bellaouchi et al., 2021), cellulase (Bellaouchi et al., 2021; Siva et al., 2022), and lipase (Bellaouchi et al., 2021; Xiang et al., 2021). These show that a single microorganism can perform a variety of functions, maximizing its potential and not only negatively affecting society. To ensure their safe commercialization, fungal strains must obtain GRAS or QPS status (Singh & Gaur, 2021).

Although fungi are commercially available and studies on metabolism are gaining increasing attention, the European Food Safety Authority (EFSA) has excluded filamentous fungi from QPS evaluations. Despite the growing knowledge of their metabolites, this decision was based on information regarding their toxicology, which remains limited. Among the most recent notifications (12 filamentous fungi) submitted, none have been evaluated by this authority to date (EFSA, 2020; EFSA, 2025).

Another important characteristic of fungi is their ability to produce biofilms. Biofilms are microbial communities formed by the adhesion of microorganisms to the surface, followed by exopolysaccharide production. This matrix acts as a protective barrier, enhancing the microorganism’s resistance to environmental stress and antimicrobial agents (Miranda et al., 2022). In the food industry, biofilms are often associated with negative impacts, as they cause

blockages in processing pipelines and contribute to product contamination when biofilm fragments detach from surfaces (Miranda et al., 2022; Becerril et al., 2023). However, in certain areas of food production, biofilm formation can be beneficial, particularly in fermentation processes. For instance, in a study on fumaric acid production, Sebastian et al. (2021) observed a 40% increase in fumaric acid yield when *Rhizopus oryzae* was immobilized on polystyrene foam beads compared to free mycelial fermentation. In addition to improving fermentation efficiency, such techniques can facilitate fungal reuse and simplify fungal mycelium recovery, streamlining the overall process.

All aspects of the duality of filamentous fungi can be linked to the One Health approach, which seeks to sustainably balance human, animal, and environmental health. Within this framework, antimicrobial resistance and food safety must be addressed comprehensively to integrate ecosystems and promote global health. Therefore, advancing research on fungal resistance is crucial. Currently, the most extensively studied aspects of antifungal resistance are related to antifungals commonly used in agriculture and clinical medicine, primarily azole and triazole compounds (Fisher et al., 2022).

Research on antimicrobial resistance has advanced significantly more in bacteria, mainly due to the biological differences between bacterial (prokaryotic) and fungal (eukaryotic) pathogens. Additionally, for a long time, fungi have not been regarded as a major public health threat (Fisher et al., 2022). For example, the resistance of *A. fumigatus* to triazoles in clinical settings has already been reported (Denning et al., 2011). Recently, more in-depth analyses of high-quality nuclear genomes, genome-wide association studies, and closed circular mitogenomes have been conducted to elucidate the pathways involved in the acquisition of resistance in fungi, such as *A. fumigatus*. A key aspect of understanding this process may be linked to mitochondrial functions, which confer resistance to azoles (Delbaje et al., 2025). However, substantial studies utilizing self-definition tools and extrapolating to other fungi and their mechanisms require further investigation. This is particularly important for gaining a comprehensive understanding of competence in acquiring and transmitting resistance and pathogenicity.

In the food industry, studies have been conducted to understand fungal resistance to sanitizers and chemical preservatives traditionally used in the food supply chain. Bernardi et al. (2018) evaluated the efficacy of commercial sanitizers, such as sodium hypochlorite, biguanide, peracetic acid, benzalkonium chloride, and quaternary ammonium compounds, against several fungi, including *Aspergillus*, *Penicillium*, and *Cladosporium*. Despite being recommended for use in the food industry, the authors found that certain sanitizers, such as biguanide, exhibited limited efficacy against the evaluated filamentous fungi. The most effective antifungal sanitizer was sodium hypochlorite. Peracetic acid was ineffective at the lowest recommended concentrations for most species except *Cladosporium cladosporioides*. However, it was effective at the highest concentration against all tested species, with *Aspergillus brasiliensis* being the only species resistant to intermediate concentrations.

The efficacy of a sanitizer can be influenced by various factors including its concentration, recommended usage, temperature, and contact time (Visconti et al., 2022). Furthermore, the diversity of fungi present within an industrial environment must be considered as different species and genera may prevail in different regions. Additionally, emerging fungal strains that are increasingly resistant may exhibit insensitivity to traditionally used sanitizers. These studies are relevant for developing more effective strategies to prevent the spread of undesirable fungi throughout the industry. Such strategies can also support biotechnological processes involving filamentous fungi by minimizing contamination from other fungi that may affect these processes.

In summary, the realm of fungi remains largely unexplored. Compared to bacteria, this gap is evident. A search in the National Center for Biotechnology Information (NCBI) revealed 105,601,236 nucleotide sequences related to bacteria, while only 22,567 sequences were associated with fungi (NCBI, 2024). This disparity highlights the significant gap in our understanding of fungi. Despite their commercial and biotechnological importance, much more research is needed to optimize their use and ensure the safety of fungi and their derived products in the marketplace. Additionally, future studies should focus on addressing issues related to spoilage fungi and mycotoxin production, both of which are major concerns for the food industry and public health.

5. Conflicts of Interest

The authors declare no conflicts of interest.

6. Acknowledgments

The authors are thankful to Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES, Brasília, DF, Brazil), Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq, Brasília, DF, Brazil) and Fundação de Amparo à Pesquisa do Estado de Minas Gerais (FAPEMIG, Belo Horizonte, MG, Brazil).

7. Author's Contribution

Luana Virgínia Souza: Conceptualization, investigation, writing – original draft, writing – review & editing, Visualization. Valéria Quintana Cavicchioli: Writing – original draft, Visualization. Rafaela de Melo Tavares: writing – review & editing, Visualization. Cinzia Lucia Randazzo: Conceptualization, Writing – review & editing, Visualization. Cinzia Caggia: Conceptualization, Writing – review & editing, Visualization. Antonio Fernandes de Carvalho: Conceptualization, Resources, Writing – review & editing, Visualization. Luís Augusto Nero: Conceptualization, Resources, Writing – review & editing, Visualization, Supervision, Project administration, Funding acquisition, All authors read and approved the final manuscript.

8. References

Adeoyo, O., & Adewumi, O. (2024). Characterization of α-amylase and antimicrobial activity of *Penicillium chrysogenum*. Journal of BioScience and Biotechnology, 13(1), 23-28. Retrieved from https://editorial.uni-plovdiv.bg/index.php/JBB/article/view/499

Adeyeye, S. A. O. (2016). Fungal mycotoxins in foods: A review. Cogent Food & Agriculture, 2(1). https://doi.org/10.1080/23311932.2016.1213127

Agriopoulou, S., Stamatelopoulou, E., & Varzakas, T. (2020). Advances in Occurrence, Importance, and Mycotoxin Control Strategies: Prevention and Detoxification in Foods. Foods, 9(2), 137. https://doi.org/10.3390/foods9020137

Alegbeleye, O., Odeyemi, O. A., Strateva, M., & Stratev, D. (2022). Microbial spoilage of vegetables, fruits and cereals. Applied Food Research, 2(1), 100122. https://doi.org/10.1016/j.afres.2022.100122

Algahtani, F. D., Morshdy, A. E., Hussein, M. A., Abouelkheir, E. S., Adeboye, A., Valentine, A., & Elabbasy, M. T. (2020). Biogenic Amines and Aflatoxins in Some Imported Meat Products: Incidence, Occurrence, and Public Health Impacts. Journal of Food Quality, 2020, 1–7. https://doi.org/10.1155/2020/8718179

Almeida, N. A., Freire, L., Carnielli-Queiroz, L., Bragotto, A. P., Silva, N. C., & Rocha, L. O. (2024). Essential oils: An eco-friendly alternative for controlling toxigenic fungi in cereal grains. Comprehensive reviews in food science and food safety, 23(1), e13251. https://doi.org/10.1111/1541-4337.13251

Al-Najada, A. R., & Gherbawy, Y. A. (2015). Molecular Identification of Spoilage Fungi Isolated from Fruit and Vegetables and Their Control with Chitosan. Food Biotechnology, 29(2), 166–184. https://doi.org/10.1080/08905436.2015.1027222

Anastasiou, R., Aktypis, A., Georgalaki, M., et al. (2019). Inhibition of *Clostridium tyrobutyricum* by *Streptococcus macedonicus* ACA-DC 198 under conditions mimicking

Kasseri cheese production and ripening. International Dairy Journal, 19, 330–335. https://doi.org/10.1016/j.idairyj.2008.12.001

Anelli, P., Haidukowski, M., Epifani, F., Cimmarusti, M. T., Moretti, A., Logrieco, A., & Susca, A. (2019). Fungal mycobiota and mycotoxin risk for traditional artisan Italian cave cheese. Food Microbiology, 78, 62-72. https://doi.org/10.1016/j.fm.2018.09.014

American Type Culture Collection (ATCC). Fungal Pigment Production. A potential natural source of colorant. December 07,2022. Shahin Ali, PhD, Anthony Muhle, MS, and Cara Wilder, PhD, ELS. Retrieved from https://www.atcc.org/blogs/2022/fungal-pigmentCC. Accessed February, 2025.

Awan, H. S., Ahmad, K. S., Iram, S., Hanif, N. Q., & Gul, M. M. (2022). Analysis and quantification of naturally occurring aflatoxin B1 in dry fruits with subsequent physical and biological detoxification. Natural Product Research, 36(12), 3100–3104. https://doi.org/10.1080/14786419.2021.1935930

Becerril, R., Precone, M., & Nerin, C. (2023). Antibiofilm activity of LAE (ethyl lauroyl arginate) against food-borne fungi and its application in polystyrene surface coating. Food Microbiology, 113, 104284. https://doi.org/10.1016/j.fm.2023.104284

Bellaouchi, R., Abouloifa, H., Rokni, Y., Hasnaoui, A., Ghabbour, N., Hakkou, A., Bechchari, A., & Asehraou, A. (2021). Characterization and optimization of extracellular enzymes production by *Aspergillus niger* strains isolated from date by-products. Journal of Genetic Engineering and Biotechnology, 19(1), 50. https://doi.org/10.1186/s43141-021-00145-y

Bensmail, S., Boudjema, K., & Naimi-Fazouane, F. (2020). Production of extracellular Rennin-like enzyme by a newly isolate *Mucor circinelloides* (Von Tieghem) and its application in Camembert cheese making. Journal of Applied Biotechnology Reports, 7(1), 16–24. https://doi.org/10.30491/jabr.2020.105914

Bernardi, A. O., Stefanello, A., Garcia, M. V., Parussolo, G., Stefanello, R. F., Moro, C. B., & Copetti, M. V. (2018). Efficacy of commercial sanitizers against fungi of concern in the food industry. LWT, 97, 25-30.https://doi.org/10.1016/j.lwt.2018.06.037

Bills, G. F., & Gloer, J. B. (2016). Biologically active secondary metabolites from the fungi. Microbiology spectrum, 4(6), 10-1128. https://doi.org/10.1128/microbiolspec.funk-0009-2016

Bovo, F., Corassin, C. H., Rosim, R. E., & de Oliveira, C. A. (2013). Efficiency of lactic acid bacteria strains for decontamination of aflatoxin M 1 in phosphate buffer saline solution and in skimmed milk. Food and Bioprocess Technology, 6, 2230-2234. https://doi.org/10.1007/s11947-011-0770-9

Bullerman, L. B., & Bianchini, A. (2007). Stability of mycotoxins during food processing. International journal of food microbiology, 119(1-2), 140-146. https://doi.org/10.1016/j.ijfoodmicro.2007.07.035

Cairns, T. C., Zheng, X., Zheng, P., Sun, J., & Meyer, V. (2019). Moulding the mould: understanding and reprogramming filamentous fungal growth and morphogenesis for next generation cell factories. Biotechnology for Biofuels, 12(1), 77. https://doi.org/10.1186/s13068-019-1400-4

Cao, L., Barzee, T. J., El Mashad, H. M., Pan, Z., & Zhang, R. (2024). Potential of utilizing almond hull extract for filamentous fungi production by submerged cultivation. Food Bioengineering, 3(1), 3-13. https://doi.org/10.1002/fbe2.12079

Carraturo, F., De Castro, O., Troisi, J., De Luca, A., Masucci, A., Cennamo, P., ... & Guida, M. (2018). Comparative assessment of the quality of commercial black and green tea using microbiology analyses. BMC microbiology, 18(1), 1-12. https://doi.org/10.1186/s12866-017-1142-z

Casadevall, A., Kontoyiannis, D. P., & Robert, V. (2021). On the emergence of *Candida auris*: Climate change, azoles, swamps, and birds. mBio, 12(2), e00360-21. https://doi.org/10.1128/mbio.01397-19

Case, N. T., Gurr, S. J., Fisher, M. C., Blehert, D. S., Boone, C., Casadevall, A., ... & Segre, J. A. (2025). Fungal impacts on Earth's ecosystems. Nature, 638(8049), 49-57. https://doi.org/10.1038/s41586-024-08419-4

Chen, G., Yang, S., Wang, C., Shi, K., Zhao, X., & Wu, Z. (2020). Investigation of the mycelial morphology of *Monascus* and the expression of pigment biosynthetic genes in high-salt-stress fermentation. Applied microbiology and biotechnology, 104, 2469-2479. https://doi.org/10.1007/s00253-020-10389-2

Cheriaparambil, R., & Grossmann, L. (2025). Properties and Cultivation of *Fusarium* spp. to Produce Mycoprotein as an Alternative Protein Source. Sustainable Food Proteins, 3(1), e70002.https://doi.org/10.1002/sfp2.70002

Chinmayee, C. V., Vidya, C., Rani, A., & Singh, S. A. (2019). Production of highly active fungal milk-clotting enzyme by solid-state fermentation. Preparative Biochemistry & Biotechnology, 49(9), 858-867. https://doi.org/10.1080/10826068.2019.1630647

Chroumpi, T., Mäkelä, M. R., & de Vries, R. P. (2020). Engineering of primary carbon metabolism in filamentous fungi. Biotechnology Advances, 43, 107551. https://doi.org/10.1016/j.biotechadv.2020.107551

Copetti, M. V. (2019). Fungi as industrial producers of food ingredients. Current opinion in food science, 25, 52-56. https://doi.org/10.1016/j.cofs.2019.02.006

Delbaje, E., Pontes, L., Rhodes, J., Steenwyk, J., Lu, L., Dos Reis, T. F., ... & Goldman, G. H. (2025). *Aspergillus fumigatus* mitogenomes and their influence on azole-resistant and-susceptible populations. Antimicrobials and Resistance, 3(1), 15.https://doi.org/10.1038/s44259-025-00083-6

Denning, D. W., Park, S., Lass-Florl, C., Fraczek, M. G., Kirwan, M., Gore, R., ... & Perlin, D. S. (2011). High-frequency triazole resistance found in nonculturable *Aspergillus fumigatus* from lungs of patients with chronic fungal disease. Clinical infectious diseases, 52(9), 1123-1129.https://doi.org/10.1093/cid/cir179

Drakopoulos, D., Kägi, A., Six, J., Zorn, A., Wettstein, F. E., Bucheli, T. D., Forrer, H.-R., & Vogelgsang, S. (2021). The agronomic and economic viability of innovative cropping systems to reduce Fusarium head blight and related mycotoxins in wheat. Agricultural Systems, 192, 103198. https://doi.org/10.1016/j.agsy.2021.103198

Dufossé, L., Fouillaud, M., Caro, Y., Mapari, S. A., & Sutthiwong, N. (2014). Filamentous fungi are large-scale producers of pigments and colorants for the food industry. Current opinion in biotechnology, 26, 56-61. https://doi.org/10.1016/j.copbio.2013.09.007

European Commission Regulation (EC). (2006). No 118/2006 of 19 December 2006. Setting Maximum Levels for Certain Contaminants in Foodstuffs. Off. J. Eur. Union, 5–24.

EC-European Commission. (2013). Commission recommendation of 27 March 2013 on the presence of T-2 and HT-2 toxin in cereals and cereal products. Off J Eur Comm L, 91, 12-15.

EC-European Commission. (2022). Commission recommendation (EU) 2022/553 of 5 April 2022 on monitoring the presence of Alternaria toxins in food. Off. J. Eur. Communities, 107, 90.

EFSA Panel on Biological Hazards (BIOHAZ), Koutsoumanis, K., Allende, A., Alvarez-Ordóñez, A., Bolton, D., Bover-Cid, S., ... & Herman, L. (2020). Scientific Opinion on the update of the list of QPS-recommended biological agents intentionally added to food or feed as notified to EFSA (2017–2019). EFSA Journal, 18(2), e05966.https://doi.org/10.2903/j.efsa.2020.5966

EFSA Panel on Biological Hazards (BIOHAZ), Allende, A., Alvarez-Ordóñez, A., Bortolaia, V., Bover-Cid, S., De Cesare, A., ... & Herman, L. (2025). Update of the list of qualified presumption of safety (QPS) recommended microbiological agents intentionally added to food or feed as notified to EFSA 21: Suitability of taxonomic units notified to EFSA until September 2024. EFSA Journal, 23(1), e9169. https://doi.org/10.2903/j.efsa.2025.9169

Egbuta, M. A., Mwanza, M., Njobeh, P. B., Phoku, J. Z., Chilaka, C. A., & Dutton, M. F. (2015). Isolation of filamentous fungi species contaminating some Nigerian food commodities. Journal of Food Research, 4(1), 38. http://dx.doi.org/10.5539/jfr.v4n1p38

El-Sayed, R. A., Jebur, A. B., Kang, W., & El-Demerdash, F. M. (2022). An overview on the major mycotoxins in food products: characteristics, toxicity, and analysis. Journal of Future Foods, 2(2), 91–102. https://doi.org/10.1016/j.jfutfo.2022.03.002

European Medicines Agency (EMA). (2025). One Health: EU agencies unite to tackle azole fungicide resistance in Aspergillus fungi. Retrieved from https://www.ema.europa.eu/en/news/one-health-eu-agencies-unite-tackle-azole-fungicide-resistance-aspergillus-fungi. Accessed July 3, 2025.

European Food Safety Authority (EFSA), BIOHAZ Panel (EFSA Panel on Biological Hazards). (2012). Scientific Opinion on the maintenance of the list of QPS biological agents intentionally added to food and feed. EFSA, 10(3020), 84. https://doi.org/10.2903/j.efsa.2012.3020

FAO. 2019. The State of Food and Agriculture 2019. Moving forward on food loss and waste reduction. Rome. Licence: CC BY-NC-SA 3.0 IGO. https://openknowledge.fao.org/server/api/core/bitstreams/11f9288f-dc78-4 171-8d02-92235b8d7dc7/content

FDA. Food and Drug Administration. 2024. The FDA Updates Mycotoxins in Domestic and Imported Human Foods Compliance Program. Constituent Update. September 26, 2024. Retrieved from https://www.fda.gov/food/hfp-constituent-updates/fda-updates-mycotoxins-domestic-and-imported-human-foods-compliance-program. Accessed March 10, 2025.

FDA.Food and Drug Administration. 2016. “GRAS Notice (GRN) No. 904. Retrieved from https://www.hfpappexternal.fda.gov/scripts/fdcc/index.cfm?set=GRASNotices&id=631. Accessed March 10, 2025.

FDA.Food and Drug Administration 2021. “GRAS Notice (GRN) No. 904. Retrieved from https://www.hfpappexternal.fda.gov/scripts/fdcc/index.cfm?set=GRASNotices&id=904. Accessed March 10, 2025.

Fendiyanto, M. H., & Dwi Satrio, R. (2020). Identification, aflatoxin content, and antagonistic test of spoilage fungi in bread to Aspergillus niger. Bioteknology 17(2). https://doi.org/10.13057/biotek/c170202

Fisher, M. C., Gurr, S. J., Cuomo, C. A., & Stukenbrock, E. H. (2020). Threats posed by the fungal kingdom to humans, wildlife, and agriculture. mBio, 11(3), e00449-20. https://doi.org/10.1128/mBio.00449-20

Fisher, M. C., Alastruey-Izquierdo, A., Berman, J., Bicanic, T., Bignell, E. M., Bowyer, P., ... & Verweij, P. E. (2022). Tackling the emerging threat of antifungal resistance to human health. Nature reviews microbiology, 20(9), 557-571.https://doi.org/10.1038/s41579-022-00720-1

Food and Agriculture Organization/World Health Organization (FAO/WHO). (2002). Guidelines for the evaluation of probiotics in food (p. 11). London, Ontario, Canada.

Fungal Protein Market Size. Food and Beverages. Proteins. Animal Based Proteins. By Type, By Application Analysis, Share, Growth Forecast, 2025 – 2034. Global Market Insights. Retrieved from https://www.gminsights.com/industry-analysis/fungal-protein-market#:~:text=Fungal%20Protein%20Market%20size%20was,enthusiasts%20will%20bolster%20industry%20expansion. Accessed February, 13, 2025.

Garcia, M. V., Bernardi, A. O., & Copetti, M. V. (2019). The fungal problem in bread production: Insights of causes, consequences, and control methods. Current Opinion in Food Science, 29, 1-6. https://doi.org/10.1016/j.cofs.2019.06.010

Ganesan, A. R., Mohan, K., Karthick Rajan, D., Pillay, A. A., Palanisami, T., Sathishkumar, P., & Conterno, L. (2022). Distribution, toxicity, interactive effects, and detection of ochratoxin and deoxynivalenol in food: A review. Food Chemistry, 378, 131978. https://doi.org/10.1016/j.foodchem.2021.131978

Garnier, L., Valence, F., & Mounier, J. (2017). Diversity and control of spoilage fungi in dairy products: An update. Microorganisms, 5(3), 42. https://doi.org/10.3390/microorganisms5030042

Global Market Statistics. (2025). Plant Based Meat Market Size, Share, Growth and Industry Analysis, by Type (Soy-based Meat Alternatives, Mycoprotein Meat Alternatives, Wheat-based Meat Alternatives and Others), By Downstream Industry (Vegetarian and Non-Vegetarian), and Regional Forecast to 2033. Retrieved from https://www.globalmarketstatistics.com/market-reports/plant-based-meat-market-11906. Accessed July 4, 2025.

Gong, X., Luo, H., Wu, X., Liu, H., Sun, C., & Chen, S. (2022). Production of red pigments by a newly isolated *Talaromyces aurantiacus* strain with led stimulation for screen printing. Indian Journal of Microbiology, 62(2), 280-292. https://doi.org/10.1007/s12088-022-01008-x

Gong, D., Prusky, D., Long, D., Bi, Y., & Zhang, Y. (2024). Moldy odors in food-a review. Food Chemistry, 140210. https://doi.org/10.1016/j.foodchem.2024.140210

Gruber-Dorninger, C., Novak, B., Nagl, V., & Berthiller, F. (2017). Emerging Mycotoxins: Beyond Traditionally Determined Food Contaminants. Journal of Agricultural and Food Chemistry, 65(33), 7052–7070. https://doi.org/10.1021/acs.jafc.6b03413

Guimarães, A., Venancio, A., & Abrunhosa, L. (2018). Antifungal effect of organic acids from lactic acid bacteria on *Penicillium nordicum*. Food Additives & Contaminants: Part A, 35, 1803–1818. https://doi.org/10.1080/19440049.2018.1500718

Hamad, G. M., Mehany, T., Simal-Gandara, J., Abou-Alella, S., Esua, O. J., Abdel-Wahhab, M. A., & Hafez, E. E. (2022). A review of recent innovative strategies for controlling mycotoxins in foods. Food Control, 109350. https://doi.org/10.1016/j.foodcont.2022.109350

Hasan, N. A., & Zanuddin, N. A. M. (2018). Molecular identification of isolated fungi from banana, mango and pineapple spoiled fruits. Advances in Civil Engineering and Science Technology 020074. https://doi.org/10.1063/1.5062700

Hu, S., He, C., Li, Y., Yu, Z., Chen, Y., Wang, Y., & Ni, D. (2021). Changes of fungal community and non-volatile metabolites during pile-fermentation of dark green tea. Food Research International, 147, 110472.https://doi.org/10.1016/j.foodres.2021.110472

Huq, T., Khan, A., Brown, D., Dhayagude, N., He, Z., & Ni, Y. (2022). Sources, production and commercial applications of fungal chitosan: A review. Journal of Bioresources and Bioproducts. https://doi.org/10.1016/j.jobab.2022.01.002

Hyde, K. D., Xu, J., Rapior, S., Jeewon, R., Lumyong, S., Niego, A. G. T., ... & Stadler, M. (2019). The amazing potential of fungi: 50 ways we can exploit fungi industrially. Fungal Diversity, 97, 1-136. https://doi.org/10.1007/s13225-019-00430-9

Iamanaka, B. T., Taniwaki, M. H., Menezes, H. C., Vicente, E., & Fungaro, M. H. P. (2005). Incidence of toxigenic fungi and ochratoxin A in dried fruits sold in Brazil. Food Additives and Contaminants, 22(12), 1258–1263. https://doi.org/10.1080/02652030500260447

Jakubczyk, D., & Dussart, F. (2020). Selected fungal natural products with antimicrobial properties. Molecules, 25(4), 911. https://doi.org/10.3390/molecules25040911

Jallow, A., Xie, H., Tang, X., Qi, Z., & Li, P. (2021). Worldwide aflatoxin contamination of agricultural products and foods: From occurrence to control. Comprehensive Reviews in Food Science and Food Safety, 20(3), 2332–2381. https://doi.org/10.1111/1541-4337.12734

Ji, X., Li, R., Yang, H., Qi, P., Xiao, Y., & Qian, M. (2017). Occurrence of patulin in various fruit products and dietary exposure assessment for consumers in China. Food Control, 78, 100–107. https://doi.org/10.1016/j.foodcont.2017.02.044

Ji, X., Xiao, Y., Jin, C., Wang, W., Lyu, W., Tang, B., & Yang, H. (2022). Alternaria mycotoxins in food commodities marketed through e-commerce stores in China: Occurrence and risk assessment. Food Control, 140, 109125. https://doi.org/10.1016/j.foodcont.2022.109125

Jimdjio, C. K., Xue, H., Bi, Y., Nan, M., Li, L., Zhang, R., ... & Pu, L. (2021). Effect of ambient pH on growth, pathogenicity, and patulin production of *Penicillium expansum*. Toxins, 13(8), 550. https://doi.org/10.3390/toxins13080550

Kaya, B., Wijayarathna, E. K. B., Yüceer, Y. K., Agnihotri, S., Taherzadeh, M. J., & Sar, T. (2024). The use of cheese whey powder in the cultivation of protein-rich filamentous fungal biomass for sustainable food production. Frontiers in Sustainable Food Systems, 8, 1386519. https://doi.org/10.3389/fsufs.2024.1386519

Kalagatur, N. K., Kamasani, J. R., Mudili, V., Krishna, K., Chauhan, O. P., & Sreepathi, M. H. (2018). Effect of high-pressure processing on growth and mycotoxin production of *Fusarium graminearum* in maize. Food Bioscience, 21, 53–59. https://doi.org/10.1016/j.fbio.2017.11.005

Kalra, R., Conlan, X. A., & Goel, M. (2020). Fungi as a potential source of pigments: harnessing filamentous fungi. Frontiers in Chemistry, 8, 369. https://doi.org/10.3389/fchem.2020.00369

Keller, N. P., Turner, G., & Bennett, J. W. (2005). Fungal secondary metabolism—from biochemistry to genomics. Nature reviews microbiology, 3(12), 937-947.https://doi.org/10.1038/nrmicro1286

Keller, N. P. (2019). Fungal secondary metabolism: regulation, function and drug discovery. Nature Reviews Microbiology, 17(3), 167-180.https://doi.org/10.1038/s41579-018-0121-1

Kinsella, J. E., Hwang, D. H., & Dwivedi, B. (1976). Enzymes of *Penicillium roqueforti* involved in the biosynthesis of cheese flavor. Critical Reviews in Food Science & Nutrition, 8(2), 191-228. https://doi.org/10.1080/10408397609527222

Kulišová, M., Maťátková, O., Brányik, T., Zelenka, J., Drábová, L., & Kolouchová, I. J. (2023). Detection of microscopic filamentous fungal biofilms–Choosing the suitable methodology. Journal of Microbiological Methods, 205, 106676. https://doi.org/10.1016/j.mimet.2023.106676

Kumar, P., Mahato, D. K., Sharma, B., Borah, R., Haque, S., Mahmud, M. M. C., Shah, A. K., Rawal, D., Bora, H., & Bui, S. (2020). Ochratoxins in food and feed: Occurrence and its impact on human health and management strategies. Toxicon, 187, 151–162. https://doi.org/10.1016/j.toxicon.2020.08.031

Kure, C. F., Skaar, I., & Brendehaug, J. (2004). Mould contamination in production of semi-hard cheese. International journal of food microbiology, 93(1), 41-49. https://doi.org/10.1016/j.ijfoodmicro.2003.10.005

Labbe, M., & Serres, J-P. (2009). Chroniques du Roquefort: Des hommes, des entreprises, des marques, période moderne. Graphi Imprimeur.

Lagashetti, A. C., Dufossé, L., Singh, S. K., & Singh, P. N. (2019). Fungal pigments and their prospects in different industries. Microorganisms, 7(12), 604. https://doi.org/10.3390/microorganisms7120604

Lesage-Meessen, L., Lomascolo, A., Bonnin, E., Thibault, J. F., Buleon, A., Roller, M., Asther, M., Record, E., Ceccaldi, B. C., & Asther, M. (2002). A biotechnological process involving filamentous fungi to produce natural crystalline vanillin from maize bran. Applied biochemistry and biotechnology, 102-103(1-6), 141–153. https://doi.org/10.1385/abab:102-103:1-6:141

Lestrade, P. P., Van Der Velden, W. J. F. M., Bouwman, F., Stoop, F. J., Blijlevens, N. M. A., Melchers, W. J. G., ... & Donnelly, J. P. (2018). Epidemiology of invasive aspergillosis and triazole-resistant *Aspergillus fumigatus* in patients with haematological malignancies: a single-centre retrospective cohort study. Journal of Antimicrobial Chemotherapy, 73(5), 1389-1394. https://doi.org/10.1093/jac/dkx527

Li, C., Zuo, C., Deng, G., Kuang, R., Yang, Q., Hu, C., ... & Yi, G. (2013). Contamination of bananas with beauvericin and fusaric acid produced by *Fusarium oxysporum* f. sp. *cubense*. PloS one, 8(7), e70226.https://doi.org/10.1371/journal.pone.0070226

Li, X., Gu, N., Ye, Y., Lan, H., Peng, F., & Peng, G. (2023). Intense pulsed light for inactivating planktonic and biofilm molds in food. Frontiers in Microbiology, 13, 1104875. https://doi.org/10.3389/fmicb.2022.1104875

Li, X., Wang, H., Guo, C., & Wang, L. (2024). Profiling of microbial populations present in ground beef and plant-based meat analogues. LWT, 196, 115845.https://doi.org/10.1016/j.lwt.2024.115845

Li, C., Zhou, J., Du, G., Chen, J., Takahashi, S., & Liu, S. (2020). Developing *Aspergillus niger* as a cell factory for food enzyme production. Biotechnology Advances, 44, 107630. https://doi.org/10.1016/j.biotechadv.2020.107630

Lima, A. C., Silva, D., Silva, V., Godoy, M., Cammarota, M., & Gutarra, M. (2021). β-Mannanase production by *Penicillium citrinum* through solid-state fermentation using açaí residual biomass (Euterpe oleracea). Journal of Chemical Technology & Biotechnology, 96(10), 2744-2754. https://doi.org/10.1002/jctb.6818

Lind, A. L., Lim, F. Y., Soukup, A. A., Keller, N. P., & Rokas, A. (2018). An LaeA-and BrlA-dependent cellular network governs tissue-specific secondary metabolism in the human pathogen *Aspergillus fumigatus*. Msphere, 3(2), e00050-18. https://doi.org/10.1128/msphere.00050-18

López-Díaz, T. M., Alegría, Á., Rodríguez-Calleja, J. M., Combarros-Fuertes, P., Fresno, J. M., Santos, J. A., Flórez, A. B., & Mayo, B. (2023). Blue Cheeses: Microbiology and Its Role in the Sensory Characteristics. Dairy, 4(3), 410–422. https://doi.org/10.3390/dairy4030027

Lübeck, M., & Lübeck, P. S. (2022). Fungal cell factories for efficient and sustainable production of proteins and peptides. Microorganisms, 10(4), 753. https://doi.org/10.3390/microorganisms10040753

Lv, Y., Bai, X., Zhao, H., Xu, Y., Li, J., & Li, X. (2024). Flavor characteristics of large yellow croaker soup served with different dried edible fungi. Food Chemistry: X, 21, 101059. https://doi.org/10.1016/j.fochx.2023.101059

Ma, J., Hong, Y., Deng, L., Yi, L., & Zeng, K. (2019). Screening and characterization of lactic acid bacteria with antifungal activity against *Penicillium digitatum* on citrus. Biological Control, 138, 104044. https://doi.org/10.1016/j.biocontrol.2019.104044

Magro, A. E. A., Silva, L. C., Rasera, G. B., & De Castro, R. J. S. (2019). Solid-state fermentation as an efficient strategy for the biotransformation of lentils: enhancing their antioxidant and antidiabetic potentials. Bioresources and Bioprocessing, 6(1), 1-9. https://doi.org/10.1186/s40643-019-0273-5

Mahato, D. K., Devi, S., Pandhi, S., Sharma, B., Maurya, K. K., Mishra, S., Dhawan, K., Selvakumar, R., Kamle, M., Mishra, A. K., & Kumar, P. (2021). Occurrence, impact on agriculture, human health, and management strategies of zearalenone in food and feed: A review. In Toxins (Vol. 13, Issue 2). MDPI AG. https://doi.org/10.3390/toxins13020092

Mahato, D. K., Kamle, M., Sharma, B., Pandhi, S., Devi, S., Dhawan, K., Selvakumar, R., Mishra, D., Kumar, A., Arora, S., Singh, N. A., & Kumar, P. (2021)b. Patulin in food: A mycotoxin concern for human health and its management strategies. In Toxicon (Vol. 198, pp. 12–23). Elsevier Ltd. https://doi.org/10.1016/j.toxicon.2021.04.027

Mallmann, C. A., Mallmann, A. O., & Tyska, D. (2020). Survey of Mycotoxin in Brazilian Corn by NIR Spectroscopy-Year 2019. Glob. J. Nutri. Food Sci, 3(10.33552).https://doi.org/10.33552/GJNFS.2020.03.000552

Mapook, A., Hyde, K. D., Hassan, K., Kemkuignou, B. M., Čmoková, A., Surup, F., ... & Stadler, M. (2022). Ten decadal advances in fungal biology leading towards human well-being. Fungal Diversity, 116(1), 547-614. https://doi.org/10.1007/s13225-022-00510-3

Massomo, S. M. S. (2020). Aspergillus flavus and aflatoxin contamination in the maize value chain and what needs to be done in Tanzania. Scientific African, 10, e00606. https://doi.org/10.1016/j.sciaf.2020.e00606

Mateo, E. M., Tarazona, A., Jiménez, M., & Mateo, F. (2022). Lactic Acid Bacteria as Potential Agents for Biocontrol of Aflatoxigenic and Ochratoxigenic Fungi. Toxins, 14(11), 807. https://doi.org/10.3390/toxins14110807

Matumba, L., Namaumbo, S., Ngoma, T., Meleke, N., De Boevre, M., Logrieco, A. F., & De Saeger, S. (2021). Five keys to prevention and control of mycotoxins in grains: A proposal. Global Food Security, 30, 100562. https://doi.org/10.1016/j.gfs.2021.100562

McCormick, S. P., Stanley, A. M., Stover, N. A., & Alexander, N. J. (2011). Trichothecenes: from simple to complex mycotoxins. Toxins, 3(7), 802–814. https://doi.org/10.3390/toxins3070802

Melo dos Santos, V., Dorner, J. W., & Carreira, F. (2003). Isolation and toxigenicity of *Aspergillus fumigatus* from moldy silage. Mycopathologia, 156, 133-138. https://doi.org/10.1023/A:1022996911563

Meyer, V., Basenko, E. Y., Benz, J. P., Braus, G. H., Caddick, M. X., Csukai, M., ... & Wösten, H. A. (2020). Growing a circular economy with fungal biotechnology: a white paper. Fungal biology and biotechnology, 7(1), 5. https://doi.org/10.1186/s40694-020-00095-z

Miranda, A. C., Leães, G. F., & Copetti, M. V. (2022). Fungal biofilms: insights for the food industry. Current Opinion in Food Science, 46, 100846. https://doi.org/10.1016/j.cofs.2022.100846

Mischler, S., André, A., Freimüller Leischtfeld, S., Müller, N., Chetschik, I., & Miescher Schwenninger, S. (2024). Potential of lactic acid bacteria and Bacillus spp. in a bio-detoxification strategy for mycotoxin contaminated wheat grains. Applied Microbiology, 4(1), 96-111.https://doi.org/10.3390/applmicrobiol4010007

Morin-Sardin, S., Rigalma, K., Coroller, L., Jany, J.-L., & Coton, E. (2016). Effect of temperature, pH, and water activity on *Mucor* spp. growth on synthetic medium, cheese analog and cheese. Food Microbiology, 56, 69–79. https://doi.org/10.1016/j.fm.2015.11.019

Moss, M. O. (2008). Fungi, quality and safety issues in fresh fruits and vegetables. Journal of Applied Microbiology, 104(5), 1239–1243. https://doi.org/10.1111/j.1365-2672.2007.03705.x

Murtaza, B., Jin, B., Wang, L., Li, X., Saleemi, M. K., Majeed, S., ... & Xu, Y. (2023). Mitigation of zearalenone in vitro using probiotic strains. LWT, 186, 115265.https://doi.org/10.1016/j.lwt.2023.115265

Murtaza, B., Wang, L., Li, X., Ali, A., Haq, S. U., Ji-bin, L., & Xu, Y. (2024). Novel *Lactobacillus consortium* for effective zearalenone adsorption and biodegradation. International Biodeterioration & Biodegradation, 194, 105889. https://doi.org/10.1016/j.ibiod.2024.105889

Nahle, S., El Khoury, A., Assaf, J. C., Louka, N., Chokr, A., & Atoui, A. (2022). A promising innovative technique for mycotoxin detoxification from beverages using biofilms of lactic acid bacteria. Innovative food science & emerging technologies, 82, 103165.https://doi.org/10.1016/j.ifset.2022.103165

Nasrollahzadeh, A., Mokhtari, S., Khomeiri, M., & Saris, P. (2022). Mycotoxin detoxification of food by lactic acid bacteria. International Journal of Food Contamination, 9(1), 1. https://doi.org/10.1186/s40550-021-00087-w

National Center for Biotechnology Information (NCBI). (2024). Genome Browser. Retrieved from https://www.ncbi.nlm.nih.gov/genome/browse/#!/overview/. Accessed January 01, 2025.

Neme, K., & Mohammed, A. (2017). Mycotoxin occurrence in grains and the role of postharvest management as a mitigation strategies. A review. Food Control, 78, 412-425. https://doi.org/10.1016/j.foodcont.2017.03.012

Nichea, M. J., Proctor, R. H., Probyn, C. E., Palacios, S. A., Cendoya, E., Sulyok, M., Chulze, S. N., Torres, A. M., & Ramirez, M. L. (2022). *Fusarium chaquense*, sp. nov, a novel type A trichothecene–producing species from native grasses in a wetland ecosystem in Argentina. Mycologia, 114(1), 46–62. https://doi.org/10.1080/00275514.2021.1987102

Niego, A. G. T., Lambert, C., Mortimer, P., Thongklang, N., Rapior, S., Grosse, M., ... & Stadler, M. (2023). The contribution of fungi to the global economy. Fungal Diversity, 121(1), 95-137. https://doi.org/10.1007/s13225-023-00520-9

Nieminen, S. M., Kärki, R., Auriola, S., Toivola, M., Laatsch, H., Laatikainen, R., ... & Von Wright, A. (2002). Isolation and identification of *Aspergillus fumigatus* mycotoxins on growth medium and some building materials. Applied and environmental microbiology, 68(10), 4871-4875. https://doi.org/10.1128/AEM.68.10.4871-4875.2002

Notardonato, I., Gianfagna, S., Castoria, R., Ianiri, G., De Curtis, F., Russo, M. V., & Avino, P. (2021). Critical review of the analytical methods for determining the mycotoxin patulin in food matrices. In Reviews in Analytical Chemistry (Vol. 40, Issue 1, pp. 144–160). Walter de Gruyter GmbH. https://doi.org/10.1515/revac-2021-0131

Nout, M. R., & Kiers, J. L. (2005). Tempe fermentation, innovation and functionality: update into the third millenium. Journal of applied microbiology, 98(4), 789-805. https://doi.org/10.1111/j.1365-2672.2004.02471.x

Novelli, P. K., Barros, M. M., & Fleuri, L. F. (2016). Novel inexpensive fungi proteases: Production by solid state fermentation and characterization. Food Chemistry, 198, 119-124. https://doi.org/10.1016/j.foodchem.2015.11.089

Panagou, E. Z., Katsaboxakis, C. Z., & Nychas, G. J. E. (2002). Heat resistance of *Monascus ruber* ascospores isolated from thermally processed green olives of the Conservolea variety. International journal of food microbiology, 76(1-2), 11-18.https://doi.org/10.1016/S0168-1605(01)00748-6

Parussolo, G., Bernardi, A. O., Garcia, M. V., Stefanello, A., dos Santos Silva, T., & Copetti, M. V. (2019). Fungi in air, raw materials and surface of dry fermented sausage produced in Brazil. Lwt, 108, 190-198. https://doi.org/10.1016/j.lwt.2019.03.073

Pattono, D., Grosso, A., Stocco, P. P., Pazzi, M., & Zeppa, G. (2013). Survey of the presence of patulin and ochratoxin A in traditional semi-hard cheeses. Food Control, 33(1), 54–57. https://doi.org/10.1016/j.foodcont.2013.02.019

Pinches, S. E., & Apps, P. (2007). Production in food of 1, 3-pentadiene and styrene by *Trichoderma* species. International Journal of Food Microbiology, 116(1), 182-185. https://doi.org/10.1016/j.ijfoodmicro.2006.12.001

Piotrowska, M. (2021). Microbiological Decontamination of Mycotoxins: Opportunities and Limitations. Toxins, 13(11), 819. https://doi.org/10.3390/toxins13110819

Pitt, J. I., & Hocking, A. D. (2009). Fungi and Food Spoilage. Springer US. https://doi.org/10.1007/978-0-387-92207-2

Pitt, J.I., Hocking, A.D. (2022). Ecology of Fungal Food Spoilage. In: Fungi and Food Spoilage. Springer, Cham. https://doi.org/10.1007/978-3-030-85640-3_2

Ployon, S., Suc, L., Le Guernevé, C., Augès, E., Mahé, H., Hervé, M., ... & Roland, A. (2025). High-throughput LC-MS/MS discrimination of musts and wines contaminated by fresh-mushroom-off flavor on the basis of reliable and easily quantifiable analytical markers: 1-hydroxyoctan-3-one and its derivate octane-1, 3-diol. Food Chemistry, 143454. https://doi.org/10.1016/j.foodchem.2025.143454

Polak-śliwińska, M., & Paszczyk, B. (2021). *Trichothecenes* in food and feed, relevance to human and animal health and methods of detection: A systematic review. Molecules, 26(2). https://doi.org/10.3390/molecules26020454

Porro, D., & Branduardi, P. (2017). Production of organic acids by yeasts and filamentous fungi. Biotechnology of yeasts and filamentous fungi, 205-223. https://doi.org/10.1007/978-3-319-58829-2_7

Qin, X., Xie, B., Zong, X., Yu, X., & Feng, Y. (2023). Selective production, relationship and controversy between *Monascus* pigments and citrinin. Food Bioscience, 56, 103233.https://doi.org/10.1016/j.fbio.2023.103233

Qiu, M., Xie, R., Shi, Y., Chen, H., Wen, Y., Gao, Y., & Hu, X. (2010). Isolation and identification of endophytic fungus SX01, a red pigment producer from Ginkgo biloba L. World Journal of Microbiology and Biotechnology, 26, 993-998. https://doi.org/10.1007/s11274-009-0261-6

Ráduly, Z., Szabó, L., Madar, A., Pócsi, I., & Csernoch, L. (2020). Toxicological and medical aspects of *Aspergillus*-derived mycotoxins entering the feed and food chain. Frontiers in microbiology, 10, 2908. https://doi.org/10.3389/fmicb.2019.02908

Rahman, M. S. (Ed.). (2020). Handbook of food preservation. CRC press. https://doi.org/10.1201/9781420017373

Rastogi, S., Dwivedi, P. D., Khanna, S. K., & Das, M. (2004). Detection of Aflatoxin M1 contamination in milk and infant milk products from Indian markets by ELISA. Food Control, 15(4), 287–290. https://doi.org/10.1016/S0956-7135(03)00078-1

Reddy, K., Salleh, B., Saad, B., Abbas, H., Abel, C., & Shier, W. (2010). An overview of mycotoxin contamination in foods and its implications for human health. Toxin Reviews, 29(1), 3–26. https://doi.org/10.3109/15569541003598553

Research Features. (2017). Mycology: unravelling the riddle of the filamentous fungi. June, 01, 2017. Retrieved from https://researchfeatures.com/mycology-unravelling-riddle-filamentous-fungi-2/. Accessed January 01, 2025.

Rodríguez, A., Rodríguez, M., Martín, A., Nuñez, F., & Córdoba, J. J. (2012). Evaluation of hazard of aflatoxin B1, ochratoxin A and patulin production in dry-cured ham and early detection of producing moulds by qPCR. Food Control, 27(1), 118–126. https://doi.org/10.1016/j.foodcont.2012.03.009

Rodríguez-Cañás, I., González-Jartín, J. M., Alvariño, R., Alfonso, A., Vieytes, M. R., & Botana, L. M. (2023). Detection of mycotoxins in cheese using an optimized analytical method based on a QuEChERS extraction and UHPLC-MS/MS quantification. Food Chemistry, 408, 135182. https://doi.org/10.1016/j.foodchem.2022.135182

Ropars, J., Caron, T., Lo, Y. C., Bennetot, B., & Giraud, T. (2020). La domestication des champignons Penicillium du fromage. Comptes Rendus. Biologies, 343(2), 155-176. https://doi.org/10.5802/crbiol.15

Ropars, J., López-Villavicencio, M., Snirc, A., Lacoste, S., & Giraud, T. (2017). Blue cheese-making has shaped the population genetic structure of the mould *Penicillium roqueforti*. PLoS One, 12(3), e0171387. https://doi.org/10.1371/journal.pone.0171387

Ropejko, K., & Twarużek, M. (2021). Zearalenone and Its Metabolites—General Overview, Occurrence, and Toxicity. In Toxins (Vol. 13, Issue 1). MDPI. https://doi.org/10.3390/TOXINS13010035

Rossi, F., Gallo, A., & Bertuzzi, T. (2020). Emerging mycotoxins in the food chain. Mediterranean Journal of Nutrition and Metabolism, 13(1), 7–27. https://doi.org/10.3233/MNM-190345

Rousta, N., Ferreira, J. A., & Taherzadeh, M. J. (2021). Production of L-carnitine-enriched edible filamentous fungal biomass through submerged cultivation. Bioengineered, 12(1), 358-368. https://doi.org/10.1080/21655979.2020.1863618

Rousta, N., Aslan, M., Yesilcimen Akbas, M., Ozcan, F., Sar, T., & Taherzadeh, M. J. (2024). Effects of fungal based bioactive compounds on human health. Critical reviews in food science and nutrition, 64(20), 7004-7027. https://doi.org/10.1080/10408398.2023.2178379

Salas, M. L., Mounier, J., Maillard, M. B., et al. (2019). Identification and quantification of natural compounds produced by antifungal bioprotective cultures in dairy products. Food Chemistry, 301, 125260. https://doi.org/10.1016/j.foodchem.2019.125260

Saleh, I., & Al-Thani, R. (2019). Fungal food spoilage of supermarkets' displayed fruits. Veterinary World, 12(11), 1877–1883. https://doi.org/10.14202/vetworld.2019.1877-1883.

Sánchez-Torres, P. (2024). Emerging alternatives to control fungal contamination. Current Opinion in Food Science, 101255. https://doi.org/10.1016/j.cofs.2024.101255

Sebastian, J., Dominguez, K. V., Brar, S. K., & Rouissi, T. (2021). Fumaric acid production using alternate fermentation mode by immobilized *Rhizopus oryzae* - a greener production strategy. Chemosphere, 281, 130858. https://doi.org/10.1016/j.chemosphere.2021.130858

Seidel, D., Wurster, S., Jenks, J. D., Sati, H., Gangneux, J. P., Egger, M., ... & Kontoyiannis, D. P. (2024). Impact of climate change and natural disasters on fungal infections. The Lancet Microbe, 5(6), e594-e605. https://doi.org/10.1016/ S2666-5247(24)00039-9

Shabeer, S., Asad, S., Jamal, A., & Ali, A. (2022). Aflatoxin Contamination, Its Impact and Management Strategies: An Updated Review. Toxins, 14(5), 307. https://doi.org/10.3390/toxins14050307

Sharaf, M. H., Abdelaziz, A. M., Kalaba, M. H., Radwan, A. A., & Hashem, A. H. (2022). Antimicrobial, antioxidant, cytotoxic activities and phytochemical analysis of fungal endophytes isolated from *Ocimum basilicum*. Applied biochemistry and biotechnology, 1-19. https://doi.org/10.1007/s12010-021-03702-w

Sharif, M., Zafar, M. H., Aqib, A. I., Saeed, M., Farag, M. R., & Alagawany, M. (2021). Single cell protein: Sources, mechanism of production, nutritional value and its uses in aquaculture nutrition. Aquaculture, 531, 735885. https://doi.org/10.1016/j.aquaculture.2020.735885

Shehata, M. G., Badr, A. N., El Sohaimy, S. A., Asker, D., & Awad, T. S. (2019). Characterization of antifungal metabolites produced by novel lactic acid bacterium and their potential application as food biopreservatives. Annals of Agricultural Sciences, 64(1), 71-78. https://doi.org/10.1016/j.aoas.2019.05.002

Siddiqui, S. (2016). Protein Production: Quality Control and Secretion Stress Response. In New and future developments in microbial Biotechnology and Bioengineering (pp. 257-266). Elsevier. https://doi.org/10.1016/B978-0-444-63505-1.00024-5

Simões, L., Fernandes, N., Teixeira, J., Abrunhosa, L., & Dias, D. R. (2023). Brazilian table olives: a source of lactic acid bacteria with antimycotoxigenic and antifungal activity. Toxins, 15(1), 71. https://doi.org/10.3390/toxins15010071

Singh, N., & Gaur, S. (2021). GRAS fungi: a new horizon in safer food product. Fungi in sustainable food production, 27-37. https://doi.org/10.1007/978-3-030-64406-2_3

Siva, D., Srivethi, G., Vasan, P. T., Rajesh, D., Alfarhan, A., & Rajagopal, R. (2022). Enhanced cellulase enzyme production by *Aspergillus niger* using cellulase/iron oxide magnetic nano-composites. Journal of King Saud University - Science, 34(1), 101695. https://doi.org/10.1016/j.jksus.2021.101695

Snyder, A. B., Martin, N., & Wiedmann, M. (2024). Microbial food spoilage: impact, causative agents and control strategies. Nature Reviews Microbiology, 1-15. https://doi.org/10.1038/s41579-024-01037-x

Soccol, C. R., Da Costa, E. S. F., Letti, L. A. J., Karp, S. G., Woiciechowski, A. L., & de Souza Vandenberghe, L. P. (2017). Recent developments and innovations in solid state fermentation. Biotechnology Research and Innovation, 1(1), 52-71. https://doi.org/10.1016/j.biori.2017.01.002

Soulier, M., Maho, T., Lo, J., Guillot, P., & Muja, C. (2024). Bread wheat (*Triticum aestivum* L.) fungal and mycotoxin contamination control enhanced by a dual-frequency cold plasma. Food Control, 163, 110477. https://doi.org/10.1016/j.foodcont.2024.110477

Souza, L. V., da Silva, R. R., Falqueto, A., Fusieger, A., Martins, E., Caggia, C., ... & de Carvalho, A. F. (2023). Evaluation of antifungal activity of lactic acid bacteria against fungi in simulated cheese matrix. LWT, 182, 114773.https://doi.org/10.1016/j.lwt.2023.114773

Souza, L. V., Rodrigues, R. D. S., Fusieger, A., da Silva, R. R., de Jesus Silva, S. R., Martins, E., ... & de Carvalho, A. F. (2022). Diversity of filamentous fungi associated with dairy processing environments and spoiled products in Brazil. Foods, 12(1), 153. https://doi.org/10.3390/foods12010153

Souza Filho, P. F., Nair, R. B., Andersson, D., Lennartsson, P. R., & Taherzadeh, M. J. (2018). Vegan-mycoprotein concentrate from pea-processing industry byproduct using edible filamentous fungi. Fungal biology and biotechnology, 5(1), 1-10. https://doi.org/10.1186/s40694-018-0050-9

Strong, P. J., Self, R., Allikian, K., Szewczyk, E., Speight, R., O'Hara, I., & Harrison, M. D. (2022). Filamentous fungi for future functional food and feed. Current Opinion in Biotechnology, 76, 102729. https://doi.org/10.1016/j.copbio.2022.102729

Takahashi, J. A., Barbosa, B. V., Martins, B. D. A., P. Guirlanda, C., & AF Moura, M. (2020). Use of the versatility of fungal metabolism to meet modern demands for healthy aging, functional foods, and sustainability. Journal of Fungi, 6(4), 223. https://doi.org/10.3390/jof6040223

Taroub, B., Salma, L., Manel, Z., et al. (2019). Isolation of lactic acid bacteria from grape fruit: Antifungal activities, probiotic properties, and in vitro detoxification of ochratoxin A. Annals of Microbiology, 69, 17–27. https://doi.org/10.1007/s13213-018-1359-6

Tiwari, P., Kumar, R., Srivastava, A., Sharma, S., & Sahu, J. K. (2025). Submerged cultivation of Agaricus bisporus: An alternate approach to utilize corn steep liquor and defatted soya flour to produce novel food. Innovative Food Science & Emerging Technologies, 100, 103922.https://doi.org/10.1016/j.ifset.2025.103922

Tournas, V. H., & Katsoudas, E. (2005). Mould and yeast flora in fresh berries, grapes and citrus fruits. International Journal of Food Microbiology, 105(1), 11–17. https://doi.org/10.1016/j.ijfoodmicro.2005.05.002

Tu, Z., Choi, D., Chen, Y., Yu, J. H., & Huynh, T. N. (2025). The food fermentation fungus *Aspergillus oryzae* is a source of natural antimicrobials against Listeria monocytogenes. Journal of Dairy Science.https://doi.org/10.3168/jds.2024-25719

UNEP 2024. United Nations Environment Programme. Food Waste Index Report 2024. Retrieved from https://www.unep.org/resources/publication/food-waste-index-report-2024. Accessed January 01, 2025.

Vallone, L., Giardini, A., & Soncini, G. (2014). Secondary metabolites from *Penicillium roqueforti,* a starter for the production of Gorgonzola cheese. Italian Journal of Food Safety, 3(3). https://doi.org/10.4081/ijfs.2014.2118

Vendruscolo, F., Tosin, I., Giachini, A. J., Schmidell, W., & Ninow, J. L. (2014). Antimicrobial activity of *Monascus* pigments produced in submerged fermentation. Journal of Food Processing and Preservation, 38(4), 1860-1865. https://doi.org/10.1111/jfpp.12157

Verduzco-Oliva, R., & Gutierrez-Uribe, J. A. (2020). Beyond Enzyme Production: Solid State Fermentation (SSF) as an Alternative Approach to Produce Antioxidant Polysaccharides. Sustainability, 12(2), 495. https://doi.org/10.3390/su12020495

Visconti, V., Rigalma, K., Coton, E., & Dantigny, P. (2022). Impact of temperature application and concentration of commercial sanitizers on inactivation of food-plant fungal spores. International Journal of Food Microbiology, 366, 109560.https://doi.org/10.1016/j.ijfoodmicro.2022.109560

Wafula, E. N., Muhonja, C. N., Kuja, J. O., Owaga, E. E., Makonde, H. M., Mathara, J. M., & Kimani, V. W. (2022). Lactic acid bacteria from african fermented cereal-based products: Potential biological control agents for mycotoxins in Kenya. Journal of Toxicology, 2022(1), 2397767. https://doi.org/10.1155/2022/2397767

Walter, P., Schaffhauser, A. O., Zurbriggen, E., Siebrecht, S., Fuhrmann, M., Krähenbühl, S., ... & Owen, K. Q. (2000). L-Carnitine, a 'vitamin-like substance'for functional food. Annals of Nutrition & Metabolism, 44(2), 75-95. https://doi.org/10.1159/000012825

Wang, S. Y., Zhang, Y. J., Chen, L. X., Pang, Y. B., Herrera-Balandrano, D. D., Daly, P., ... & Laborda, P. (2025). Discovery of the dipicolinic acid synthase in the spoilage fungus *Paecilomyces* reveals its role in spore heat, salt, and alkaline pH resistance. Food Research International, 115994. https://doi.org/10.1016/j.foodres.2025.115994

Wang, J., Zhou, Y., Liu, W., Zhu, X., Du, L., & Wang, Q. (2008). Fumonisin level in corn-based food and feed from Linxian County, a high-risk area for esophageal cancer in china. Food Chemistry, 106(1), 241–246. https://doi.org/10.1016/j.foodchem.2007.05.076

World Health Organization (WHO). (2025). Antifungal preclinical and clinical pipeline review. Retrieved from https://www.who.int/observatories/global-observatory-on-health-

research-and-development/monitoring/antifungal-agents-in-development. Accessed July 3, 2025.

Xiang, ma, Zhang, K., Yonggang, W., Ebadi, A. G., & Toughani, M. (2021). Optimization of Low-Temperature Lipase Production Conditions and Study on Enzymatic Properties of *Aspergillus niger*. In J. Chem. Chem. Eng. Research Article (Vol. 40, Issue 4).

Xue, X., Bi, F., Liu, B., Li, J., Zhang, L., Zhang, J., Gao, Q., & Wang, D. (2021). Improving citric acid production of an industrial *Aspergillus niger* CGMCC 10142: identification and overexpression of a high-affinity glucose transporter with different promoters. Microbial Cell Factories, 20(1), 168. https://doi.org/10.1186/s12934-021-01659-3

Yang, D., Ye, Y., Sun, J., Wang, J.-S., Huang, C., & Sun, X. (2024). Occurrence, transformation, and toxicity of fumonisins and their covert products during food processing. Critical Reviews in Food Science and Nutrition, 64(12), 3660–3673. https://doi.org/10.1080/10408398.2022.2134290

Yegin, S., Fernandez-Lahore, M., Jose Gama Salgado, A., Guvenc, U., Goksungur, Y., & Tari, C. (2011). Aspartic proteinases from *Mucor* spp. in cheese manufacturing. Applied Microbiology and Biotechnology, 89(4), 949–960. https://doi.org/10.1007/s00253-010-3020-6

Zhang, X., Chen, W., & Wang, C. (2025). Regulation of citrinin biosynthesis in *Monascus purpureus*: Impacts on growth, morphology, and pigments production. Food Microbiology, 127, 104698.https://doi.org/10.1016/j.fm.2024.104698

Zhang, K., Zhang, B., & Yang, S. T. (2013). Production of citric, itaconic, fumaric, and malic acids in filamentous fungal fermentations. Bioprocessing technologies in biorefinery for sustainable production of fuels, chemicals, and polymers, 375-398. https://doi.org/10.1002/9781118642047.ch20

Zhao, S., Hao, X., Yang, F., Wang, Y., Fan, X., & Wang, Y. (2022). Antifungal activity of *Lactobacillus plantarum* ZZUA493 and its application to extend the shelf life of Chinese steamed buns. Foods, 11(2), 195. https://doi.org/10.3390/foods11020195

Zhao, X., Wang, N., Lu, Y., Li, Y., Zhao, T., Xu, J., ... & Yue, T. (2024). Effects of cold plasma on the growth and aflatoxin production of *Aspergillus flavus.* Food Bioscience, 61, 104552. https://doi.org/10.1016/j.fbio.2024.104552

Zhou, M., Chen, Y., Fang, X., Wu, L., & Zhang, Y. (2023). Isolation and identification of pigment-producing filamentous fungus DBFL05 and its pigment characteristics and chemical structure. CyTA-Journal of Food, 21(1), 374-385. https://doi.org/10.1080/19476337.2023.2207613

Zhou, L. H., Wang, Y. L., Qiu, M., Shi, Q., Sun, L. J., Liao, J. M., ... & Gooneratne, R. (2017). Analysis of T-2 toxin removal factors in a *Lactococcus* fermentation system. Journal of Food Protection, 80(9), 1471-1477. https://doi.org/10.4315/0362-028X.JFP-17-051

Zorn, H., Langhoff, S., Scheibner, M., & Berger, R. G. (2003). Cleavage of β, β-carotene to flavor compounds by fungi. Applied Microbiology and Biotechnology, 62, 331-336. https://doi.org/10.1007/s00253-003-1309-4

CHAPTER 3

3.SECOND PAPER

SECOND PAPER - Selection of Lactic Acid Bacteria from Dairy Matrices with Antifungal Activity to be used as Biopreservative Adjunct Cultures

Luana Virgínia Souza et al.,

Manuscript published in *Food Biotechnology 2025*

DOI: https://doi.org/10.1080/08905436.2025.2501215

Selection of Lactic Acid Bacteria from Dairy Matrices with Antifungal Activity to be used as Biopreservative Adjunct Cultures

Luana Virgínia Souza[a,b], Rafaela da Silva Rodrigues[a], Andressa Fusieger[c], Luís Otávio Toledo Ribeiro[a], Fabrizio Domenico Nicosia[b], Valéria Quintana Cavicchioli[d], Nunziatina Russo[b], Cinzia Caggia[b], Cinzia Lucia Randazzo[b], Antonio Fernandes de Carvalho [b,c], Luís Augusto Nero [a,b]

[a] InsPOA - Laboratório de Inspeção de Produtos de Origem Animal, Departamento de Veterinária, Universidade Federal de Viçosa, 36570 900, Viçosa, MG, Brazil

[b] Di3A - Dipartimento di Agricoltura, Alimentazione e Ambiente, Università degli Studi di Catania, 95123, Catania, CT, Italy

[c] InovaLeite - Laboratório de Pesquisa em Leites e Derivados, Departamento de Tecnologia de Alimentos, Universidade Federal de Viçosa, 36570 900, Viçosa, MG, Brazil

[d] LIP - Laboratório de Inovação e Pesquisa, Centro de Pesquisa em Alimentos, Escola de Medicina Veterinária e Zootecnia, Universidade Federal de Goiás, 74690-900, Goiânia, Goiás, Brazil

*Corresponding author: Luís Augusto Nero. InsPOA - Laboratório de Inspeção de Produtos de Origem Animal, Departamento de Veterinária, Universidade Federal de Viçosa, 36570 900, Viçosa, MG, Brazil.E-mail address: nero@ufv.br (L. A. Nero)

Abstract

This study aimed to assess the technological, safety, antibiotic resistance, and antifungal properties of 35 lactic acid bacteria (LAB) from Sicilian dairy products. Sixteen *Lactiplantibacillus plantarum* isolates produced diacetyl, and 30 LAB isolates showed proteolytic activity, suggesting technological potential. All isolates presented weak milk acidification capacity. Aminopeptidase activity varied, with PepX (5/35) and PepN (2/35) showing better activity on *Lactiplantibacillus fermentum*, *Lactobacillus* sp. and *L. plantarum* strains, indicating species-specificity. None of the isolates showed hemolytic, gelatinase, DNase, or coagulase activity. Most were resistant to oxacillin, tetracycline, rifampicin, and vancomycin. Regarding antifungal activity, *L. plantarum* (n = 10) and *Lactiplantibacillus* sp. (n = 1) isolates inhibited *Aspergillus niger* and *Penicillium chrysogenum*, while *L. rhamnosus* (n = 5) strains were active against *P. chrysogenum*. Overall, *Lactiplantibacillus* isolates, particularly *L. plantarum* from donkey milk, showed promising technological traits, safety, and potential as bioprotective cultures. These findings support the search for new strains with improved characteristics and technological applications.

Keywords: antifungal; lactic acid bacteria; technological properties; safety; bioprotective culture.

1. Introduction

Lactic acid bacteria (LAB) present several traits indicating their potential use in technological application in the food chain, especially in dairy industry. The market of microbial cultures is increasing. A financial turnover of approximately 2.17 billion dollars is expected for 2025, driving this sector to predict even greater growth of up to 4.73% by 2030 (Mordor Intelligence, 2025). Recently, many studies have been conducted in the search for new isolates of LAB with technological potential for application in food, concerning the development of starter and adjunct cultures with bioprotective and/or functional characteristics (Li et al., 2025; Sethi et al., 2024; Souza et al., 2023; Hadef et al., 2023; Kayacan Çakmakoğlu et al., 2023; Nicosia et al., 2023;; Srikham et al., 2021).

LAB produce several metabolites, such as acids like organic acids, protein derived compounds, alcohols, hydrogen peroxide, peracetic acid, bacteriocins, reuterin, diacetyl, among others, which exhibit beneficial appropriate effects for use in food industry (Ibrahim et al., 2021). Some of LAB are already widely used in traditional fermented food and have been granted QPS (Qualified Preservative of Safety) and/or GRAS (Generally Regarded as Safe) status by the European Food Safety Authority (EFSA) and the US Food and Drug Administration (FDA), respectively (Souza et al., 2022). However, technological and safety characterization are essential to achieve this status and even to afford new cultures on the market (Souza et al., 2022).

Recently, bioprotective cultures have attained notoriety mainly due to the clean label claim, and consequently, the industries intend to reduce the use of chemical preservatives by replacing them with the addition of cultures with a preservative effect (Souza et al., 2022). Studies have shown several LAB of the genera *Lactiplantibacillus* and *Lactobacillus* with bioprotective effects, mainly against filamentous fungi (Fernandez et al., 2017; Wang et al., 2024). The effects are due by the produced metabolites, such as lactic acid, acetic, propionic, 2,3-dimethyl-5-ethylpyrazine, 2,5-dimethyl-3-propylpyrazine, palmitic, stearic, butyric, leucic acid, and others, which can be produced by *L. plantarum* (Kanjan & Sakpetch, 2023).

Filamentous fungi are widely recognized for their versatility and their ability to spoilage a variety of food products. These fungi can easily grow on dairy products, which present

nutritionally rich compositions, and causing noticeable or imperceptible issues, including gas production and bitter flavor production (Pitt & Hocking, 2022), as well as mycotoxins that can pose human health risks (Ráduly et al., 2020; Wu et al., 2023). Several genera of fungi can be found in dairy products and environments, such as *Penicillium*, *Aspergillus*, *Cladosporium*, *Fusarium*, *Nigrospora*, *Riopa*, *Hipoxylon, Bipolaris, Didymella, Epicoccum, Clonostachys, Paecilomyces, Talaromyces*, *Phoma*, *Mucor*, *Rhizopus,* among others (Garnier et al., 2017; Souza, Rodrigues et al., 2022).

Some filamentous fungi can tolerate several adverse conditions to which dairy products are subjected to such as high temperature, acidification, cooling and additives (Garnier et al., 2017). Thus, it is necessary to emphasize the importance to adopt prevention practices, especially those regarding the application of quality management systems, such as Good Manufacturing Practices (GMP). However, failure to implement these practices can result in fungal contamination of environment and processing plant, subsequently spreading throughout final products, mainly because the physical and chemical characteristics of various types of ripened cheeses can provide the growth of fungi. Thus, the application of bioprotective cultures represents an additional prevention strategy to avoid problems associated with filamentous fungi in dairy industry. Considering the importance of developing new potential cultures focusing on bioprotection for food applications, our study aimed to evaluate the technological, safety, antibiotic resistance and antifungal features of LAB isolates sourced from Sicilian dairy matrices.

2. Materials and methods

2.1. Isolates and growth parameters

LAB isolates (n = 35) belonging to microbial culture collection of the Food Agricultural Microbiology Laboratory (Department of Agriculture, Food and Environment, University of Catania, Sicily, Italy) were included in this study. The isolates were obtained from Sicilian dairy farms producers of artisanal cheeses (Pecorino Siciliano cheese, Donkey milk and Caciocavallo Ragusano Cheese) and previously were identified at the species or genus level by molecular methods (unpublished data) (Table 1). The isolates were preserved in at -80 ºC, supplemented

with glycerol at 20% (v/v) and in Man Rogosa and Sharpe (MRS) broth (Kasvi, São José dos Pinhais, PR, Brazil). For this study, stock isolates were cultivated in MRS broth and incubated at 37 ºC for 24 h, before the tests.

Table 1. Identification and origin of 35 lactic acid strains isolated from Sicilian dairy matrices (16S rRNA)

Code	**Identification**	**Origin**
D55	*L. rhamnosus*	Pecorino Siciliano Cheese
E26	*L. rhamnosus*	Pecorino Siciliano Cheese
H65	*L. rhamnosus*	Pecorino Siciliano Cheese
E32	*L. rhamnosus*	Pecorino Siciliano Cheese
P26	*L. rhamnosus*	Pecorino Siciliano Cheese
R9.1	*L. plantarum*	Donkey milk
R3.5	*L. plantarum*	Donkey milk
R5.3	*L. plantarum*	Donkey milk
M5.2	*L. plantarum*	Donkey milk
M3.6	*L. plantarum*	Donkey milk
M5.1	*L. plantarum*	Donkey milk
M3.5	*L. plantarum*	Donkey milk
R3.8	*L. plantarum*	Donkey milk
M3.3	*L. plantarum*	Donkey milk
R5.1	*L. plantarum*	Donkey milk
R5.4	*L. plantarum*	Donkey milk
R3.4	*L. plantarum*	Donkey milk
M3.4	*L. plantarum*	Donkey milk
R3.2	*L. plantarum*	Donkey milk
R3.3	*L. plantarum*	Donkey milk
M3.7	*L. plantarum*	Donkey milk
M3.2	*L. plantarum*	Donkey milk
R3.6	*L. plantarum*	Donkey milk
R3.7	*L. plantarum*	Donkey milk
M3.1	*L. plantarum*	Donkey milk
R3.1	*L. plantarum*	Donkey milk
3B	*Limosilactobacillus fermentum*	Caciocavallo Ragusano Cheese
4B	*Limosilactobacillus fermentum*	Caciocavallo Ragusano Cheese
2B	*Lactobacillus delbrueckii*	Caciocavallo Ragusano Cheese

9B	*Lactiplantibacillus* sp.	Caciocavallo Ragusano Cheese
4C	*Limosilactobacillus fermentum*	Caciocavallo Ragusano Cheese
C160	*Limosilactobacillus fermentum*	Caciocavallo Ragusano Cheese
C560	*Lactobacillus* sp.	Caciocavallo Ragusano Cheese
C360	*Limosilactobacillus fermentum*	Caciocavallo Ragusano Cheese
B160	*Lactobacillus* sp.	Caciocavallo Ragusano Cheese

2.2. Rep-PCR fingerprinting of isolates

After initial cultivation, the isolates were centrifuged at 10,000 g for 10 min at 4 °C. The collected pellets were then processed for DNA extraction through the DNA Purification Wizard® Genomic Kit (Promega, Madison, WI, USA). Rep-PCR was carried out following the method outlined by Dal Bello et al. (2010), utilizing a single universal primer (GTG)5 (5′-GTGGTGGTGGTGGTG-3′). The PCR combination of the mixture consisted of 12.5 µL of GoTaq Green Master Mix 2x (Promega, Madison, WI, USA), 50 pmol of the primer, 1 µL of extracted DNA, and ultra-pure water (Promega, Madison, WI, USA) for a final volume of 25 µL. The PCR amplification conditions were conducted in a thermocycler as follows: initial denaturation at 95 °C for 5 min, followed by 30 cycles of denaturation at 95 °C for 30 s, annealing at 40 °C for 30 s, and extension at 65 °C for 8 min. The parameter used for final extension was 65 °C for 16 min. The PCR products were then separated by electrophoresis (1.2%, w/v agarose) for 80 min at voltage of 75 V, using 0.5x TBE buffer. The gels stained with GelRed (Biotium Inc., Fremont, CA, USA) and examined using an LPIX transilluminator (Loccus Biotechnology, São Paulo, Brazil). The resulting fingerprints were analyzed with BioNumerics version 6.6 (Applied Maths, Kortrijk, Belgium), employing the Unweighted Pair Group Method with Arithmetic Mean (UPGMA) and Pearson's correlation coefficient to generate the dendrogram. A similarity threshold of 90% was applied to compare the clusters.

2.3. Technological potential

Phenotypic analyses were performed to determine the following tests: diacetyl production, proteolysis and acidifying activity. Before each assay, isolates were standardized to a concentration of approximately 3×10^8 CFU/mL.

To assess diacetyl production, 1 mL of the cultures was mixed to 0.5 mL of α-naphthol (1% w/v) and KOH (16% w/v) then incubated at 30 °C for 10 min: The presence of diacetyl was indicated by the formation of a ring at the top of the tube, with different intensities of colors between pink and red. (Franciosi et al., 2009).

To reveal the extracellular proteolytic activity, a screening assay was performed as described by Franciosi et al., (2009): The isolates were inoculated on Plate Count Agar (PCA, HiMedia, Mumbai, India) added to skim milk (10% w/v, Nestlé, Araçatuba, SP, Brazil), then incubated at 30 °C for 48 to 96 h. The presence of proteolytic activity was identified by the formation of a halo surrounding the colonies.

Acidification in milk was evaluated by adding 1% of the culture to 10% (w/v) skim milk (Nestlé, Araçatuba, Brazil), then the mixture of each isolate was incubated at 30 °C for 24 h. pH measurements were taken at the start (T0), as well as after 6 (T6), 12 (T12), and 24 hours (T24) using a digital pH meter (Hanna Instruments, São Paulo, SP, Brazil). Additionally, titratable acidity was quantified (T0, T6, T12 and T24) and reported as lactic acid (Dal Bello et al., 2012).

Results were reported as the mean values of three independent repetitions. *Pseudomonas fluorescens* 07A (Alves et al., 2016) was used as positive control in all experiments.

2.4. Aminopeptidase analysis

2.4.1. Elaboration of Cell Free Extract

LAB isolates were cultured in MRS broth (Oxoid, Milan, Italy) at 37°C until they reached the exponential growth. Following centrifugation (10,000 g, 10 min/4°C), the cells were washed twice with sodium phosphate buffer (0.05 M, pH 7.0) and then resuspended to a

concentration of 10^9 CFU/mL in the same buffer. To prepare the cell-free extract (CFE), cell lysis was achieved using the bead-beating technique with the Precellys system (Bertin Technologies, Düsseldorf, Germany). A Zirconium beads (0.1 mm in diameter) were used for the disruption process, which was repeated twice at 10,000 × g for 40 seconds. During each cycle, the samples were placed on ice for 4 min, followed for 10 minutes of rest. Afterward, the zirconium beads, along with the cell debris and intact cells, were removed via centrifugation (10,000 × g, 10 min/4°C), yielding the CFE. The concentration of the protein was measured using the Pierce™ BCA Protein Assay Kit (Thermo Fisher, Waltham, USA).

2.4.2. Aminopeptidase N and X analysis

Aminopeptidase N (PepN) and Aminopeptidase X (PepX) activities were assessed using spectrophotometric techniques, with minor modifications in substrate and buffer compositions according to the methodologies of Requena et al. (1993) and Nicosia et al. (2023), respectively. For PepN, 100 µL of cell-free extract (CFE) was combined with 80 µL of phosphate buffer (50 mM, pH 7.0) and 20 µL of reaction buffer containing Lys-p-nitroanilide dihydrobromide (20 mM in methanol). For PepX, 50 µL of CFE was mixed with 600 µL of phosphate buffer (50 mM, pH 7.2), followed by the addition of 50 µL of reaction buffer containing H-Ala-Pro-p-nitroanilide HCl (20 mM in methanol). Reactions were incubated in their respective buffers at 37°C for 30 minutes, or until the development of a yellow color. The reactions were subsequently halted by the addition of 10% (v/v) glacial acetic acid. Optical density (OD) was recorded at 410 nm using an iMark™ Microplate Absorbance Reader (Biorad, Hercules, California, USA). The assays were performed in triplicate, with a blank (reaction buffer and substrate without CFE) serving as a reference. Enzymatic activity was expressed in units (U) per mg of protein, with one unit corresponding to the enzyme quantity that releases 1 µmol of p-nitroaniline (p-NA) per minute under the given experimental conditions.

2.5. Evaluation of safety aspects

The isolates were analyzed using phenotypic tests to assess their virulence potential, evaluating hemolytic activity, gelatinase production, and DNase activity as described by Barbosa et al. (2010) and coagulase activity as described by Ferrari et al. (2016).

The hemolytic activity test was performed on trypticase soy agar (TSA, Oxoid, Milan, Italy) and supplemented with 5% defibrinated horse blood. The isolates were streaked on the agar and incubated at 25°C/37°C for 24 h. Hemolysis was classified as absent (γ-hemolysis), partial (α-hemolysis) or complete (β-hemolysis).

The gelatinase production test was performed on Luria Bertani Agar (LB, Becton Dickinson, Holdrege, Nebrasca, USA) with added gelatin (3%). Then, 1 ml of the cultured isolates were added on LB and incubated at 25°C/37°C for 48 h. The indication of gelatinase was verified through the formation of opaque halos.

DNase activity was performed on DNase methyl green agar (BD, Becton Dickinson, Holdrege, Nebrasca, USA) supplemented with 1% toluidine blue. Then, 1 ml of the cultured isolates were added on the agar, incubated at 25/37 °C for 48 h. DNase positive was verified by the formation of a pink halo.

Coagulase production was verified by mixing 300 µL of LAB suspension with 300 µL of rabbit plasma. Then, each suspension was incubated at 37°C for 6h to 24h. A positive result for coagulase was measured by the formation of a firm clot in the tube.

All experiments were realized in duplicates and in three repetitions. As control, the strain *Staphylococcus aureus* subps. *aureus* ATCC 25932 was used.

2.6. Antibiotic resistance

The resistance against antibiotics of the isolates was tested using Oxoid® antibiotic discs (Unipath Ltd, Basingstoke, UK). The LAB suspensions were adjusted to the equivalent of 0.5 McFarland turbidity using 0.85% NaCl, then evenly distributed on MRS agar plates. Each tested antibiotic discs were placed on the surface of the media and incubated at 37°C for 24 h. The important antibiotics evaluated were ampicillin (10 µg/disk), oxacillin (1 µg/disk), imipenem (10 µg/disk), erythromycin (15 µg/disk), chloramphenicol (30 µg/disk), clindamycin (2 µg/disk), sulpha/trimethoprim (23.75/1.25 µg/disk), tetracycline (30 µg/disk), vancomycin (30 µg/disk) and rifampicin (5 µg/disk). The inhibition zones on discs were measured and categorized as resistant (R), intermediate resistance (IR), or susceptible (S) according to the

European Committee on Antimicrobial Susceptibility Testing (EUCAST, 2023). The strains *S. aureus* subps. *aureus* ATCC 25932 and *Escherichia coli* ATCC 25922 were used as controls.

2.7. Antifungal activity

Two reference fungi, *Aspergillus niger* IOC 207 and *Penicillium chrysogenum* IOC 132, sourced by Fundação Oswaldo Cruz (Fiocruz, Rio de Janeiro, RJ, Brazil), were used to assess the antifungal properties of LAB isolates. Fungal spore suspensions were elaborated following the protocol of Roy et al. (1996). The fungi were cultured at 25 °C on Potato Dextrose Agar (PDA, Merck, Darmstadt, Germany) plates, which were tilted until spore formation occurred. Afterward, the spores were collected by rinsing the plates with sterile 0.1% Tween-80 solution (v/v), and the suspension was vortexed to disperse any spore clumps. A Neubauer counting chamber under an optical microscope (Olympus, Melville, NY, USA) was used to determine the spore count. A final adjustment of 1.5×10^7 spores/mL, was performed on the spore suspension and for its stock the suspension was added with 10% (v/v) glycerol, stored at -80 °C.

LAB cell cultures were subjected to centrifugation (Thermo Fisher Scientific, Waltham, MA, USA) at 3,260 × g for 15 min at 7 ºC, followed by filtration using a 0.22 µm pore size filter (Kasvi), yielding a cell-free supernatant (CFS). The antifungal effect was assessed using a microtiter plate assay according to Zhao et al. (2022), with slight modifications. 100 µL of the spore suspension of each fungus was diluted (4X and 16X in sterile water) and distributed to a 96-well microtiter plate. To each well, 100 µL of CFS of LAB was introduced, and the microplate was incubated at 30 ºC for 48 h. Fungal growth monitoring was performed using a microplate spectrophotometer (Multiskan™ GO Spectrophotometer, Thermo Fisher Scientific, WI, USA) by measuring optical density (OD) every hour (λ = 600 nm). Positive control wells received 100 µL of the target fungal culture plus MRS broth. And as a negative control, wells received 200 µL of MRS broth only. Each inhibition assay was performed in triplicate and repeated independently twice. The antifungal effect of CFS of LAB was measured through the inhibition percentage, according to the following equation:

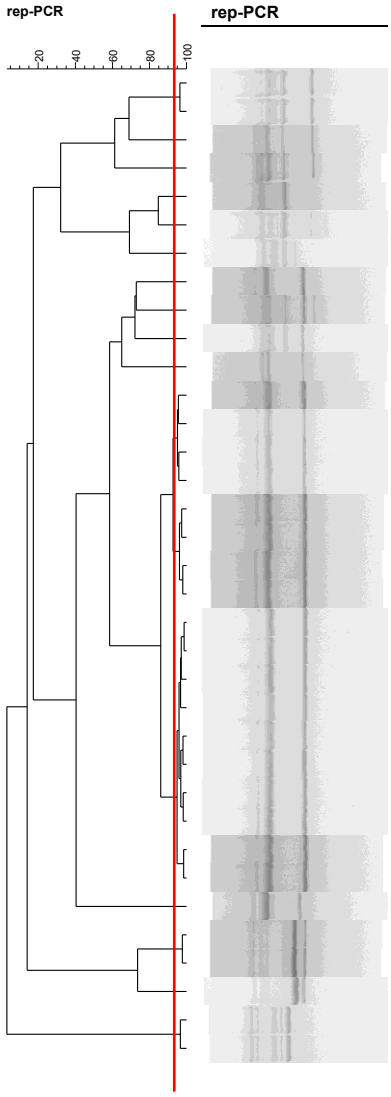
$$\text{Antifungal activity } \% = 1 - \left(\frac{OD \text{ LAB}}{OD \text{ CONTROL}} \right) \text{x } 100$$

Here, OD_{LAB} refers to the optical density (OD) of the fungal suspension treated with CFS, while $OD_{CONTROL}$ represents the OD of the fungal suspension in MRS broth. The recorded OD values were adjusted using the negative control readings, and the average OD values were calculated over the course of the measurement period. The results are represented as inhibition percentage. The experiments were conducted in triplicate.

3. Results and Discussion

3.1. Rep-PCR fingerprinting

The thirty-five LAB isolates subjected to $(GTG)_5$-PCR fingerprint analysis resulted in five distinct clusters and ten individual fingerprints, with similarity indices of at least 90% (Figure 1). The largest clusters, comprising ten and eight isolates, contained exclusively isolates identified as *L. plantarum*. The remaining three *L. plantarum* isolates were identified as unique fingerprints. However, an uncommon cluster included isolates from different genera (namely *Lactobacillus* sp., and *Limosilactobacillus fermentum*). The similarity between the strains, studied by $(GTG)_5$-PCR fingerprint analysis, the low discriminatory power between isolates may be attributed to the closely related genera, as *Limosilactobacillus* is a new genus created from the reclassification of the genus *Lactobacillus*, as well as *Lactiplantibacillus*, *Lacticaseibacillus*, and *Levilactobacillus* (Zheng et al., 2020), also investigated in the present study. Therefore, the rep-PCR fingerprinting technique using $(GTG)_5$ primer could not reveal a high genotypic heterogeneity among most isolates included in this study.



Code	Identification	Origin
4C	Limosilactobacillus fermentum	Cheese Ragusano (Caciocavallo)
C160	Limosilactobacillus fermentum	Cheese Ragusano (Caciocavallo)
4B	Limosilactobacillus fermentum	Cheese Ragusano (Caciocavallo)
D55	L. rhamnosus	Cheese Pecorino Siciliano
3B	Limosilactobacillus fermentum	Cheese Ragusano (Caciocavallo)
C560	Lactobacillus sp.	Cheese Ragusano (Caciocavallo)
H65	L. rhamnosus	Cheese Pecorino Siciliano
R3.1	L. plantarum	Donkey milk
R3.7	L. plantarum	Donkey milk
R3.4	L. plantarum	Donkey milk
2B	Lactobacillus delbrueckii	Cheese Ragusano (Caciocavallo)
M3.1	L. plantarum	Donkey milk
M3.5	L. plantarum	Donkey milk
M3.6	L. plantarum	Donkey milk
R3.8	L. plantarum	Donkey milk
M3.2	L. plantarum	Donkey milk
R3.6	L. plantarum	Donkey milk
M3.4	L. plantarum	Donkey milk
R3.3	L. plantarum	Donkey milk
M5.2	L. plantarum	Donkey milk
R5.3	L. plantarum	Donkey milk
M5.1	L. plantarum	Donkey milk
M3.3	L. plantarum	Donkey milk
R5.1	L. plantarum	Donkey milk
R5.4	L. plantarum	Donkey milk
R3.5	L. plantarum	Donkey milk
R9.1	L. plantarum	Donkey milk
M3.7	L. plantarum	Donkey milk
R3.2	L. plantarum	Donkey milk
9B	Lactiplantibacillus sp.	Cheese Ragusano (Caciocavallo)
E32	L. rhamnosus	Cheese Pecorino Siciliano
P26	L. rhamnosus	Cheese Pecorino Siciliano
E26	L. rhamnosus	Cheese Pecorino Siciliano
B160	Lactobacillus sp.	Cheese Ragusano (Caciocavallo)
C360	Limosilactobacillus fermentum	Cheese Ragusano (Caciocavallo)

Figure 1. Dendrogram obtained by cluster analysis of $(GTG)_5$-based rep-PCR fingerprints of LAB isolated from dairy matrices from Sicily-Italy. The dendrogram is based on Pearson's correlation coefficient with the UPGMA method. The vertical red line indicates the cut-off value of 90% similarity.

3.2. Technological potential

Among the 35 isolates, 16 (45.7 %) identified as *L. plantarum* produced diacetyl, an important aromatic compound for dairy industry (Table 2). Similar results were found by Metrouh et al. (2022) where they detected medium intensity production of diacetyl by a strain of *L. Plantarum*. Diacetyl produced by microbial cultures is an important compound derived from bacterial metabolism according to the relevant percentage produced in our study. The

production of diacetyl by LAB can occur through the degradation of citrate into diacetyl and acetoin *via* the diacetyl pathway (Wang et al., 2019).

These compounds are very interesting, as they can add value and flavor to several dairy products, even if produced at low concentrations, showing a low flavor threshold. Several genera are able to produce diacetyl, such as *Lactobacillus, Streptococcus*, *Leuconostoc*, *Pediococcus*, and *Oenococcus* (Wang et al., 2019). Thus, in the future, exploring the metabolome of the strains found in our study may be interesting to track the strains that have improved characteristics regarding the production of diacetyl, acetoin and other volatile compounds that can incorporate flavor and taste into dairy products.

Concerning the proteolytic activity, it was observed in 30 (85.7%) isolates, the results are presented in Table 2. All *L. plantarum* strains in our study exhibited proteolytic activity. In contrast, the study conducted by Grujović et al. (2024) found that out of 27 *L. plantarum* isolates, only 11 (40.74%) demonstrated proteolytic activity.

The proteolytic activity of starter and adjunct cultures are essential in the production of cheese in the dairy chain. In addition, proteolytic capacity is one of the main technological features to be evaluated and mainly, as already stated, in milk and in dairy products, it can help in gel formation, mainly in yogurt and some types of cheese (Kuerman et al., 2024).

The gelatinization, from a physico-chemical point of view, is due to the property of proteins to form a three-dimensional network, resulting in a balance of repulsive and gravitational forces whose structure can be influenced by some bacteria. Kuerman et al. (2024) evaluated nine strains of proteolytic *L. plantarum* and, according to rheological analyses, confirmed that these bacteria can positively affect the gel formation and improve texture of dairy products. Thus, our results show that the strains evaluated have potential for application as adjunct cultures, mainly for cheese ripening production.

Regarding acidification capacity, all isolates presented a low ability to acidify, with a weak pH decrease after 24 h (Figure 2). However, it was expected that the isolates would grow in milk, being obtained from dairy matrices, the acidification capacity was considered low,

because in 24 h the pH decreased of 0.38-0.91 point and the percentage of lactic acid produced varied between 0.14 and 0.18% among isolates (Table 3).

Table 2. Technological characterization and safety assessment of LAB isolates

Code	Identification	Technological features*		Safety*			
		Diacetyl[a]	Proteolysis	DNase	Gelatinase	Coagulase	Hemolysis
D55	*L. rhamnosus*	-	+	-	-	-	-
E26	*L. rhamnosus*	-	+	-	-	-	-
H65	*L. rhamnosus*	-	+	-	-	-	-
E32	*L. rhamnosus*	-	+	-	-	-	-
P26	*L. rhamnosus*	-	-	-	-	-	-
R9.1	*L. plantarum*	+++	+	-	-	-	-
R3.5	*L. plantarum*	+++	+	-	-	-	-
R5.3	*L. plantarum*	+++	+	-	-	-	-
M5.2	*L. plantarum*	+++	+	-	-	-	-
M3.6	*L. plantarum*	+	+	-	-	-	-
M5.1	*L. plantarum*	+++	+	-	-	-	-
M3.5	*L. plantarum*	-	+	-	-	-	-
R3.8	*L. plantarum*	+	+	-	-	-	-
M3.3	*L. plantarum*	+++	+	-	-	-	-
R5.1	*L. plantarum*	+++	+	-	-	-	-
R5.4	*L. plantarum*	-	+	-	-	-	-
R3.4	*L. plantarum*	-	+	-	-	-	-
M3.4	*L. plantarum*	-	+	-	-	-	-
R3.2	*L. plantarum*	-	+	-	-	-	-
R3.3	*L. plantarum*	++	+	-	-	-	-
M3.7	*L. plantarum*	++	+	-	-	-	-
M3.2	*L. plantarum*	++	+	-	-	-	-
R3.6	*L. plantarum*	++	+	-	-	-	-
R3.7	*L. plantarum*	++	+	-	-	-	-
M3.1	*L. plantarum*	+++	+	-	-	-	-
R3.1	*L. plantarum*	+++	+	-	-	-	-
3B	*Limosilactobacillus fermentum*	-	-	-	-	-	-

4B	*Limosilactobacillus fermentum*	-	+	-	-	-	-
2B	*Lactobacillus delbrueckii*	-	-	-	-	-	-
9B	*Lactiplantibacillus* sp.	-	+	-	-	-	-
4C	*Limosilactobacillus fermentum*	-	-	-	-	-	-
C160	*Limosilactobacillus fermentum*	-	+	-	-	-	-
C560	*Lactobacillus* sp.	-	+	-	-	-	-
C360	*Limosilactobacillus fermentum*	-	+	-	-	-	-
B160	*Lactobacillus* sp.	-	-	-	-	-	-

[a] (+++), strong production; (++), medium production; (+), weak production; (-), no production; * (+) positive; (-) negative.

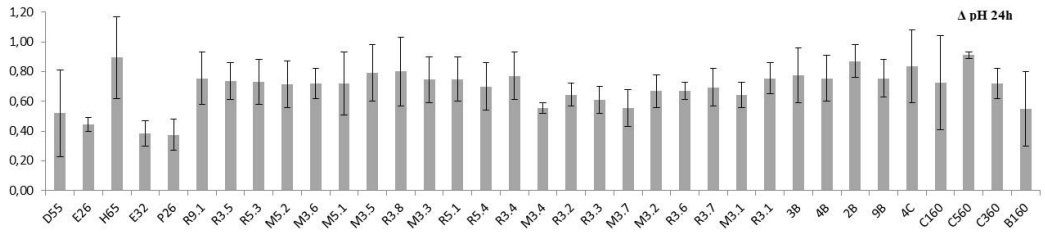


Figure 2. Changes of pH (ΔpH) in milk by LAB isolates after 24 hours (initial pH of 7.30). Error bars represent the standard deviation of two independent experiments.

Table 3. Acidifying activity of LAB isolates in milk.

Code	Identification	pH				% lactic acid		
		T6	T12	T24	ΔpH	T6	T12	T24
D55	*L. rhamnosus*	7.13± 0.22	6.94± 0.06	6.78± 0.16	0.52± 0.29	0.10± 0.00	0.13± 0.01	0.15± 0.01
E26	*L. rhamnosus*	7.22± 0.23	7.12± 0.14	6.86± 0.19	0.45± 0.04	0.10± 0.00	0.10± 0.00	0.14± 0.02
H65	*L. rhamnosus*	7.01± 0.07	6.74± 0.26	6.41± 0.13	0.90± 0.28	0.11± 0.00	0.15± 0.04	0.16± 0.00
E32	*L. rhamnosus*	7.19± 0.30	7.10± 0.19	6.92± 0.13	0.39± 0.09	0.10± 0.00	0.11± 0.01	0.14± 0.01
P26	*L. rhamnosus*	7.22± 0.23	7.15± 0.18	6.93± 0.11	0.38± 0.11	0.10± 0.00	0.10± 0.00	0.14± 0.01
R9.1	*L. plantarum*	7.04± 0.19	6.86± 0.06	6.55± 0.01	0.76± 0.18	0.10± 0.00	0.10± 0.02	0.18± 0.02
R3.5	*L. plantarum*	7.02± 0.18	6.84± 0.06	6.57± 0.08	0.74± 0.13	0.12± 0.02	0.14± 0.02	0.17± 0.02
R5.3	*L. plantarum*	7.00± 0.16	6.84± 0.05	6.57± 0.04	0.73± 0.15	0.12± 0.01	0.15± 0.01	0.16± 0.01
M5.2	*L. plantarum*	7.06± 0.21	6.90± 0.01	6.59± 0.04	0.72± 0.16	0.11± 0.01	0.14± 0.02	0.17± 0.03
M3.6	*L. plantarum*	7.00± 0.25	6.85± 0.04	6.58± 0.11	0.72± 0.10	0.11± 0.01	0.14± 0.02	0.15± 0.01
M5.1	*L. plantarum*	7.05± 0.25	6.88± 0.01	6.58± 0.04	0.72± 0.21	0.12± 0.02	0.14± 0.01	0.15± 0.01
M3.5	*L. plantarum*	7.05± 0.18	6.87± 0.04	6.51± 0.01	0.79± 0.19	0.12± 0.02	0.14± 0.01	0.15± 0.00
R3.8	*L. plantarum*	7.03± 0.25	6.84± 0.00	6.50± 0.07	0.80± 0.23	0.12± 0.03	0.14± 0.01	0.18± 0.01
M3.3	*L. plantarum*	7.06± 0.18	6.89± 0.06	6.56± 0.04	0.75± 0.16	0.12± 0.01	0.15± 0.03	0.16± 0.00
R5.1	*L. plantarum*	7.04± 0.22	6.88± 0.00	6.55± 0.04	0.75± 0.15	0.12± 0.02	0.14± 0.04	0.16± 0.00
R5.4	*L. plantarum*	7.04± 0.22	6.88± 0.01	6.60± 0.03	0.70± 0.16	0.13± 0.04	0.16± 0.01	0.17± 0.01
R3.4	*L. plantarum*	7.08± 0.21	6.89± 0.06	6.53± 0.03	0.77± 0.16	0.12± 0.02	0.16± 0.03	0.15± 0.01
M3.4	*L. plantarum*	7.03± 0.21	6.95± 0.10	6.75± 0.21	0.56± 0.04	0.12± 0.01	0.15± 0.01	0.15± 0.00
R3.2	*L. plantarum*	7.05± 0.18	6.96± 0.05	6.66± 0.15	0.65± 0.08	0.12± 0.01	0.13± 0.01	0.16± 0.01
R3.3	*L. plantarum*	7.04± 0.18	6.91± 0.01	6.69± 0.13	0.61± 0.09	0.12± 0.02	0.12± 0.01	0.15± 0.01
M3.7	*L. plantarum*	7.05± 0.19	6.92± 0.02	6.75± 0.08	0.56± 0.13	0.12± 0.01	0.14± 0.01	0.16± 0.01
M3.2	*L. plantarum*	7.03± 0.18	6.90± 0.01	6.63± 0.10	0.67± 0.11	0.12± 0.03	0.14± 0.01	0.16± 0.01
R3.6	*L. plantarum*	7.02± 0.18	6.92± 0.04	6.63± 0.17	0.67± 0.06	0.12± 0.02	0.14± 0.01	0.17± 0.01

Rapid acidification is desirable in LAB to be used as starter cultures, as these bacteria can accelerate and direct the fermentation process. Furthermore, they can also contribute to the development of taste and texture in fermented dairy products, since, through metabolism, the intermediate products of lactic acid production can give rise to flavor compounds (Santos et al., 2015). On the other hand, low acidification, as found in our study, is an interesting property of adjunct cultures, demonstrating an appropriate technological capacity without altering the centesimal composition or the sensory traits of product, especially in the ripening of soft and semi-hard cheeses or if they are going to be included in a culture with starter strains (Briggiler-Marcó et al., 2007). In general, traditional Sicilian cheeses are an excellent source for capturing new strains with improved characteristics. Caggia et al. (2015) even studied the potential of *Lactiplantibacillus* strains on several technological properties, specifically probiotic potential, with excellent results in these traits. Therefore, our findings may provide valuable support for future analyses concerning technological capabilities, particularly in the identification of novel starter cultures and adjuncts with antimicrobial and/or functional properties.

3.3. Aminopeptidase analysis

PepN and PepX values varied among all isolates from 9.33 to 245.66 and 1.12 to 35.23 U/mg, respectively (Figure 3). The best PepN activity performances were given by isolates C360 (*L. fermentum)*, B160 (*Lactobacillus* sp.), R3.5 (*L. plantarum)*, R9.1 (*L. plantarum*), and H65 (*L. rhamnosus)* with values of 245.66, 204.42, 178.66, 119.19, and 244.75 U/mg, respectively. The best performances of PepX activity were given by isolates C360 (*L. fermentum)* and B160 (*Lactobacillus* sp.*)* with values of 35.23 and 29.51 U/mg, respectively. According to aminopeptidase analysis, in the present study a large difference among aminopeptidase values has been found, suggesting that the activity is strain dependent (Pérez et al., 2003). Carafa et al., (2015) for example, found large variations in aminopeptidases among strains belonging to the same species of *L. paracasei*, with the lowest value being 17 (U/mg) and the highest being 641 (U/mg) for Lys-pNA substrate. Differently, Pérez et al. (2003) highlighted the relevance of the high activity of strains of *Lactiplantibacillus*, *Lacticaseibacillus,* and *Latilactobacillus* that showed >40(U/g) lysyl-aminopeptidase-specific activity. Among the aminopeptidases produced by LAB on different substrates, more than 20,

the mostly evaluated are PepN and PepX, that are the main involved in cheese ripening, are the ones that perform first on oligopeptides (Savijoki et al., 2006).

Other proteolytic activities are investigated with different aims. In our study, we observed that casein proteolysis was presumed in 85.7% (n= 30) of the isolates (Table 2). Aminopeptidases, on the other hand, varied in value among the isolates, but both give us an important insight into the technological potential of LAB. Both analyses indicate that the tested LAB showed the potential to release amino acids, playing an important role in the hydrolysis of bitter peptides, contributing to the production of acetoin/diacetyl and, as a consequence, improving aroma and flavor of cheeses during the ripening (Carafa et al., 2015; McSweeney, 2004).

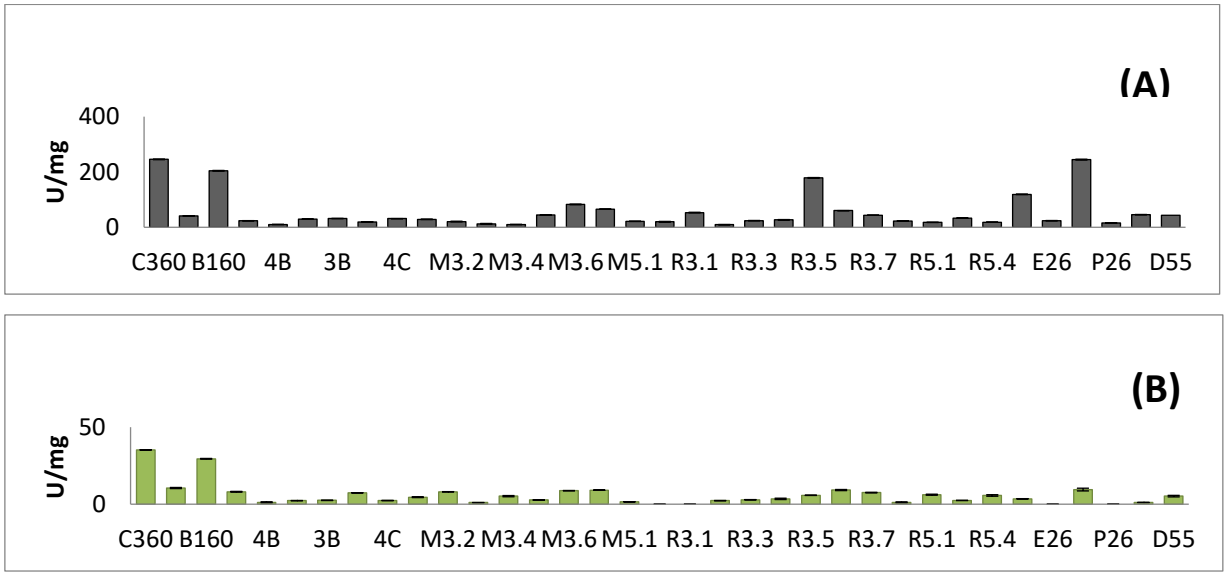


Figure 3. (A) Aminopeptidase N (PepN) activity and **(B)** Aminopeptidase X (PepX) activity exhibited by the tested isolates. Aminopeptidase activity was expressed as the number of activity units per milligram of protein per minute (U/mg). One unit of aminopeptidase activity was considered as the amount of enzyme required to release 1 _mol of p-NA per minute under the assay conditions. Results are reported as mean and standard deviation of three independent repetitions.

3.4. Evaluation of safety aspects and susceptibility to antibiotics

None of the investigated LAB presented hemolytic activity, gelatinase, DNase or coagulase activities (Table 2). Our results are in agreement with other studies focused on LAB isolated from dairy matrices and environments (Colombo et al., 2020; Margalho et al., 2020). Colombo et al. (2020) found no positive results from phenotypic tests of hemolytic activity, gelatinase, DNase and lipase for 15 LAB isolated from dairy environments. Margalho et al. (2020) did not report any positive isolate for hemolytic activity. Domingos-Lopes et al. (2017) found all negative results for DNase. Otherwise, 64% (n=54) of LAB were positive for gelatinase, these being isolates of *Enterococcus*, *Lactococcus,* and *Lactiplantibacillus*. The authors also found hemolytic activity on 2 isolates of *L. paracasei*. The evaluation of these safety aspects for LAB is an extremely important criteria for selecting safe strains to either acquire their GRAS or QPS status and/or be applied as starter, adjunct, probiotic, or bioprotective cultures (Souza et al., 2022).

Regarding susceptibility to antibiotics, all isolates showed some antibiotic resistance (Figure 4 and Supplementary material). Among 35 isolates, 34 (97.14%) were resistant to oxacillin, 32 (91.43%) were resistant to tetracycline, 31 (88.57%) to vancomycin and 28 (80%) to rifampicin, these being the highest frequencies. Four isolates stood out in terms of susceptibility to the majority of antibiotics, these being resistant to only 3 antibiotics: E32 (*L. rhamnosus*) resistant to oxacillin, vancomycin, and tetracycline; 2B (*L. delbrueckii*) resistant to sulpha/trimethoprim, vancomycin, and clindamycin; 4C (*L. fermentum*) resistant to oxacillin, vancomycin, and rifampicin and C160 resistant to oxacillin, vancomycin, and tetracycline. Similar results were found by Colombo et al. (2020) that described all strains of LAB as resistant to oxacillin and the majority to vancomycin and rifampicin. The most antibiotic resistance found in this study, such as vancomycin and oxacillin, it is already well-studied that several strains of LAB can present intrinsic resistance to these antibiotics, which is a non-transferable resistance (Goldstein et al., 2015; Campedelli et al., 2019; Das et al., 2020). However, the tetracycline and rifampicin may come from the transfer of resistance genes. However, additional studies are required to prove whether these genes can be transferred from

bacteria to human gut microbiota and if there is any concern for human consumption (Egervärn et al., 2010; Abriouel et al., 2015).

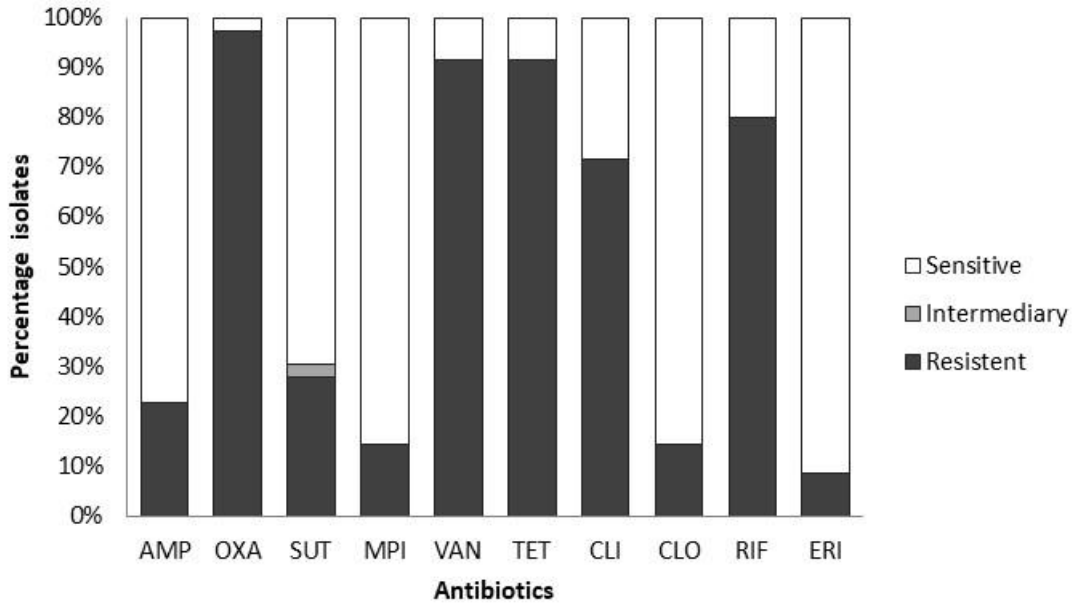


Figure 4. Susceptibility of LAB isolates to antibiotics in the disk diffusion test. AMP, ampicillin; OXA, oxacillin; SUT, sulfamethoxazole/trimethoprim; MPI, imipenem; VAN, vancomycin; TET, tetracycline; CLI, clindamycin; CLO, chloramphenicol; RIF, rifampicin; ERI, erythromycin. Halo (inhibition zone) formation around the discs was measured and classified as presenting resistance (R), intermediate resistance (IR), or sensitivity (S) according to the European Committee on Antimicrobial Susceptibility Testing (EUCAST, 2023).

3.5. Antifungal activity

Overall results showed high percentages of antifungal activity against the tested fungi, especially against *P. chrysogenum* IOC 132 (Table 4). Among the 35 isolates, 11 showed more than 62% antifungal activity against the two targets, even at the least dilute concentration of fungal spore. The isolates *L. plantarum* (R3.5, M3.6, M3.3, M3.4, R3.2, R3.3, R3.6, R3.7, M3.1, R3.1) and *Lactiplantibacillus* sp. (9B) exhibited the best effects against the targets. It was also observed that the antifungal activity showed a small increase when fungal suspension was diluted from 4X to 16X.

Also, according to our results, all *L. rhamnosus* isolates (D55, E26, P26, E32, and H65), some *L. plantarum* isolates (M5.1, R3.8, M3.7, and M3.2), all *L. fermentum* (3B, 4B, 4C, C160, C360), *Lactobacillus delbrueckii* (2B), *Lactiplantibacillus* sp. (9B) and *Lactobacillus* sp. (C560 and B160) showed a relevant antifungal effect against *P. chrysogenum,* and a low effect against *A. niger,* with values below 50% in one and/or both spore suspension concentrations. Recent studies have investigated the antifungal activity of LAB strains with relevant inhibition percentages, which makes these cultures potential choices for the development of bioprotective cultures for the food industry as an alternative solution for foods subject to fungal contamination. In a previous study, Souza et al. (2023) found that strains of *Weissella confusa*, *Weissella paramesenteroides*, *Weissella cibaria*, and *L. plantarum* showed more than 70% antifungal activity against the same targets and the antifungal activity remained even against undiluted spore suspensions (approximately 10^7 spores/mL). Peng et al. (2023) evaluated the antifungal activity of *L. plantarum* from naturally fermented yogurt and found a good effect against *Aspergillus flavus*, *Aspergillus niger*, *Penicillium* sp., and *Rhizopus delemar*.

Studies have reported the importance of several *Lactiplantibacillus* species with broad antifungal effects both *in vitro* and in food matrices (Wu et al., 2025; Vasundaradevi et al., 2025; Souza et al., 2023; Zhao et al., 2022). The studies carried out by Souza et al. (2023), Prabawati et al. (2023), and Wang et al. (2024) proved the antifungal effect of strains of *L. plantarum* against P. *chrysogenum* and *A. niger* in cheese model; *P. commune* in cheddar cheese; and *Penicillium* sp. in yogurt, respectively. As described by Li et al. (2023), the antifungal effect of *L. plantarum* can be mediated by several pathways. One of these pathways is linked to the bacterial metabolism, which is facultative heterofermentative. *L. plantarum* can then produce a wide range of compounds, such as organic acids, protein derived compounds, and volatile compounds (VOCs). Vasundaradevi et al. (2025) investigated the antagonistic potential of a *L. plantarum* strain against *Fusarium oxysporum*. Additionally, the authors assessed the production of organic acids such as lactic, malic, and citric acids, as well as the probiotic properties exhibited by the strain.

Therefore, the antifungal effect detected in *L. plantarum* may be related to several compounds, among which organic acids, as lactic acid, produced by primary metabolism, but

the main antifungal effect may be related to other acids such as phenylactic, oleic, vanillic, decanoic, oxalic, benzoic, citric acids, among others (Leyva Salas et al., 2019; Li et al., 2023). Antifungal protein derived compounds, like linear or cyclic dipeptides, and volatile compounds, such as alcohols, pyrazines, benzenes, and diacetyl also can be involved in an antifungal activity (Li et al., 2023). Such a versatility of *L. plantarum* means that the antifungal activity could be performed both individually and in synergy. The low antifungal activity of some strains against *Aspergillus*, as found in our results are in agreement with those reported by Souza et al. (2023) and Peng et al. (2023) who also observed weaker antifungal activity of LAB against *Aspergillus* species and stronger against *Penicillium* species, that can be justified by the spore structural differences. For example, *A. niger* presents a layer composed of chitin and glucans, covered by an outer layer of hydrophobins and melanin pigments: this structure makes spores highly hydrophobic and more prone to acquire certain resistance against certain types of metabolites at specific concentrations (Beauvais et al., 2014; Cortesão et al., 2020; Geoghegan et al., 2020).

Additionally, some studies have report the ability of *L. plantarum* isolates to inhibit the production of mycotoxins or even to remove mycotoxins already produced (Cruz et al., 2020; Guimarães et al., 2018). Thus, isolates with potential antifungal activity tested in the present study could be further investigated to address issues associated with products already contaminated with fungi or mycotoxins. Some studies have reported the presence of mycotoxins in dairy products, such as patulin and ochratoxin A in semi-hard cheeses, and the latter in artisanal cheeses (Marcelão et al., 2024; Pattono et al., 2013).

According to our results, all the LAB isolates that showed antifungal activity also showed the ability to produce diacetyl and/or proteolysis, which are interesting technological properties for commercial applications of strains. In this sense, further studies aiming to characterize the complete metabolome to trace and better understand the origin of the antifungal activity should be carried out.

Table 4. Inhibitory potential of the cell-free supernatant of LAB isolates against target fungi.

Codes	Identification	4X		16X	
		A. niger IOC 207	*P. chrysogenum* IOC 132	*A. niger* IOC 207	*P. chrysogenum* IOC 132
D55	*L. rhamnosus*	0.52±0.18	79.12±0.02	40.33±0.39	78.33±0.02
E26	*L. rhamnosus*	23.21±0.13	84.13±0.01	61.30±0.40	78.59±0.09
H65	*L. rhamnosus*	45.53±0.23	73.08±0.01	76.71±0.10	76.76±0.01
E32	*L. rhamnosus*	42.43±0.29	57.40±0.08	64.31±0.27	66.54±0.10
P26	*L. rhamnosus*	33.11±0.14	62.26±0.05	52.77±0.23	67.25±0.22
R9.1	*L. plantarum*	56.72±0.37	77.68±0.02	79.24±0.02	79.90±0.07
R3.5	*L. plantarum*	63.24±0.03	86.38±0.03	70.76±0.14	89.62±0.01
R5.3	*L. plantarum*	57.54±0.32	83.92±0.02	74.68±0.09	86.09±0.02
M5.2	*L. plantarum*	56.25±0.28	78.46±0.05	73.77±0.11	79.94±0.01
M3.6	*L. plantarum*	72.06±0.07	78.36±0.02	78.95±0.02	76.40±0.01
M5.1	*L. plantarum*	19.94±0.15	87.96±0.03	77.25±0.02	90.85±0.01
M3.5	*L. plantarum*	52.88±0.04	79.56±0.01	76.12±0.02	82.66±0.06
R3.8	*L. plantarum*	49.23±0.24	72.34±0.02	81.27±0.08	76.71±0.01
M3.3	*L. plantarum*	72.87±0.03	77.03±0.15	79.02±0.02	82.34±0.05
R5.1	*L. plantarum*	56.89±0.23	80.51±0.11	75.64±0.16	86.07±0.01
R5.4	*L. plantarum*	58.49±0.24	78.37±0.03	70.56±0.27	80.16±0.03
R3.4	*L. plantarum*	50.92±0.20	80.97±0.08	83.02±0.01	78.21±0.01
M3.4	*L. plantarum*	62.93±0.24	76.95±0.04	83.04±0.02	78.80±0.04
R3.2	*L. plantarum*	72.94±0.07	81.24±0.06	82.90±0.04	86.83±0.02
R3.3	*L. plantarum*	71.71±0.09	71.35±0.06	84.03±0.01	76.96±0.01
M3.7	*L. plantarum*	29.94±0.24	75.51±0.04	69.70±0.18	78.00±0.01
M3.2	*L. plantarum*	34.60±0.22	87.39±0.05	67.70±0.23	86.07±0.02
R3.6	*L. plantarum*	74.13±0.02	88.04±0.03	85.43±0.01	84.17±0.02
R3.7	*L. plantarum*	63.01±0.15	77.46±0.04	82.52±0.03	82.87±0.09
M3.1	*L. plantarum*	71.66±0.12	77.60±0.06	83.60±0.02	81.67±0.06
R3.1	*L. plantarum*	69.00±0.11	79.83±0.02	83.80±0.02	81.78±0.06
3B	*Limosilactobacillus fermentum*	52.78±0.07	65.64±0.24	79.10±0.07	67.62±0.30
4B	*Limosilactobacillus fermentum*	11.60±0.10	79.08±0.07	43.53±0.21	83.41±0.02
2B	*Lactobacillus delbrueckii*	29.35±0.30	78.70±0.02	67.51±0.24	77.44±0.07
9B	*Lactiplantibacillus* sp.	74.44±0.04	75.75±0.01	83.77±0.01	76.25±0.08
4C	*Limosilactobacillus fermentum*	27.26±0.16	72.63±0.02	54.37±0.37	78.15±0.04
C160	*Limosilactobacillus fermentum*	33.67±0.04	80.23±0.09	52.35±0.17	77.60±0.01

C560	*Lactobacillus* sp.	24.60±0.09	79.97±0.03	48.76±0.15	80.15±0.01
C360	*Limosilactobacillus fermentum*	26.39±0.06	73.43±0.03	77.57±0.17	78.92±0.01
B160	*Lactobacillus* sp.	23.40±0.14	72.98±0.04	70.48±0.24	78.37±0.01

The inhibition is expressed as percentage (%) = 100 – (mean $OD_{600\ nm}$ / mean $OD_{600\ nm}$ target strain) * 100.
4X and 16X mean the diluted suspension of fungal spores.

4. Conclusions

Our study demonstrated that *Lactiplantibacillus* isolates, mainly from sicilian donkey milk present interesting and promising potential for technological application and a safe profile to be used as adjunct cultures in food industry. Sixteen *L. plantarum* isolates exhibited the ability to produce diacetyl and thirty LAB isolates demonstrated proteolytic activity. None of the isolates presented virulence features and resistance was to few antibiotics. Based on the antifungal characterization, 10 isolates of *L. plantarum* and 1 *Lactiplantibacillus* sp. present an excellent antifungal activity against *A. niger* IOC 207 and *P. chrysogenum* IOC 132. Continued studies of the technological traits of the strains in situ and on a large scale can be carried out to prove their effectiveness, as well as tests with different food matrices of interest. Our results demonstrated that the antifungal effect of LAB isolates from can guide new studies to apply selected strains as bio-protective cultures in the food industry.

Acknowledgments

The authors are thankful to Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES, Brasília, DF, Brazil, Financial code 001), Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq, Brasília, DF, Brazil) and Fundação de Amparo à Pesquisa do Estado de Minas Gerais (FAPEMIG, Belo Horizonte, MG, Brazil). This study was conducted within a PhD research program in Agricultural, Food and Environmental Science (XXXVIII Ciclo) by Luana Virginia Souza Department of Agriculture, Food and Environment with cotutelle on PhD course in Veterinary Medicine – University of Viçosa, Minas Gerais. Brazil.

Declaration of competing interest

The authors have no competing interests to declare.

5.References

Abriouel, H., Casado Muñoz, M. del C., Lavilla Lerma, L., Pérez Montoro, B., Bockelmann, W., Pichner, R., Kabisch, J., Cho, G.-S., Franz, C. M. A. P., Gálvez, A., & Benomar, N. (2015). New insights in antibiotic resistance of *Lactobacillus* species from fermented foods. *Food Research International*, *78*, 465–481. https://doi.org/10.1016/j.foodres.2015.09.016

Alves, M. P., Salgado, R. L., Eller, M. R., Vidigal, P. M. P., & Fernandes de Carvalho, A. (2016). Characterization of a heat-resistant extracellular protease from *Pseudomonas fluorescens* 07A shows that low temperature treatments are more effective in deactivating its proteolytic activity. *Journal of Dairy Science*, *99*(10), 7842–7851. https://doi.org/10.3168/jds.2016-11236

Barbosa, J., Gibbs, P. A., & Teixeira, P. (2010). Virulence factors among enterococci isolated from traditional fermented meat products produced in the North of Portugal. *Food Control*, *21*(5), 651–656. https://doi.org/10.1016/j.foodcont.2009.10.002

Beauvais, A., Fontaine, T., Aimanianda, V., & Latgé, J.-P. (2014). *Aspergillus* cell wall and biofilm. *Mycopathologia*, *178*, 371–377. https://doi.org/10.1007/s11046-014-9766-0

Briggiler-Marcó, M., Capra, M. L., Quiberoni, A., Vinderola, G., Reinheimer, J. A., & Hynes, E. (2007). Nonstarter *Lactobacillus* strains as adjunct cultures for cheese making: *in vitro* characterization and performance in two model cheeses. *Journal of Dairy Science*, *90*(10), 4532–4542. https://doi.org/10.3168/jds.2007-0180

Busetta, G., Garofalo, G., Claps, S., Sardina, M. T., Franciosi, E., Alfonzo, A., Francesca, N., Moschetti, G., Settanni, L., & Gaglio, R. (2024). The wooden shelf surface and cheese rind mutually exchange microbiota during the traditional ripening process. *International Journal of Food Microbiology*, *409*, 110478. https://doi.org/10.1016/j.ijfoodmicro.2023.110478

Caggia, C., De Angelis, M., Pitino, I., Pino, A., & Randazzo, C. L. (2015). Probiotic features of Lactobacillus strains isolated from Ragusano and Pecorino Siciliano cheeses. *Food Microbiology*, *50*, 109-117. https://doi.org/10.1016/j.fm.2015.03.010

Campedelli, I., Mathur, H., Salvetti, E., Clarke, S., Rea, M. C., Torriani, S., Ross, R. P., Hill, C., & O'Toole, P. W. (2019). Genus-wide assessment of antibiotic resistance in *Lactobacillus* spp. *Applied and Environmental Microbiology*, *85*(1), e01738-18. https://doi.org/10.1128/AEM.01738-18

Carafa, I., Nardin, T., Larcher, R., Viola, R., Tuohy, K., & Franciosi, E. (2015). Identification and characterization of wild lactobacilli and pediococci from spontaneously fermented Mountain Cheese. *Food Microbiology*, *48*, 123–132. https://doi.org/10.1016/j.fm.2014.12.003

Colombo, M., Nero, L. A., & Todorov, S. D. (2020). Safety profiles of beneficial lactic acid bacteria isolated from dairy systems. *Brazilian Journal of Microbiology*, *51*, 787–795. https://doi.org/10.1007/s42770-020-00227-y

Cortesão, M., de Haas, A., Unterbusch, R., Fujimori, A., Schütze, T., Meyer, V., & Moeller, R. (2020). *Aspergillus niger* spores are highly resistant to space radiation. *Frontiers in Microbiology*, *11*, Article 560. https://doi.org/10.3389/fmicb.2020.00560

Cruz, P. O. da, Matos, C. J. de, Nascimento, Y. M., Tavares, J. F., Souza, E. L. de, & Magalhães, H. I. F. (2020). Efficacy of potentially probiotic fruit-derived *Lactobacillus fermentum*, *L. paracasei* and *L. plantarum* to remove aflatoxin M1 in vitro. *Toxins*, *13*(1), 4. https://doi.org/10.3390/toxins13010004

Dal Bello, B., Cocolin, L., Zeppa, G., Field, D., Cotter, P. D., & Hill, C. (2012). Technological characterization of bacteriocin producing *Lactococcus lactis* strains employed to control *Listeria monocytogenes* in Cottage cheese. *International Journal of Food Microbiology*, *153*(1–2), 58–65. https://doi.org/10.1016/j.ijfoodmicro.2011.10.016

Dal Bello, B., Rantsiou, K., Bellio, A., Zeppa, G., Ambrosoli, R., Civera, T., & Cocolin, L. (2010). Microbial ecology of artisanal products from North West of Italy and antimicrobial activity of the autochthonous populations. *LWT - Food Science and Technology*, *43*(7), 1151–1159. https://doi.org/10.1016/j.lwt.2010.03.008

Das, D. J., Shankar, A., Johnson, J. B., & Thomas, S. (2020). Critical insights into antibiotic resistance transferability in probiotic *Lactobacillus*. *Nutrition*, *69*, 110567.

https://doi.org/10.1016/j.nut.2019.110567

Domingos-Lopes, M. F. P., Stanton, C., Ross, P. R., Dapkevicius, M. L. E., & Silva, C. C. G. (2017). Genetic diversity, safety and technological characterization of lactic acid bacteria isolated from artisanal Pico cheese. *Food Microbiology*, *63*, 178–190. https://doi.org/10.1016/j.fm.2016.11.014

Egervärn, M., Lindmark, H., Olsson, J., & Roos, S. (2010). Transferability of a tetracycline resistance gene from probiotic *Lactobacillus reuteri* to bacteria in the gastrointestinal tract of humans. *Antonie van Leeuwenhoek*, *97*(2), 189–200. https://doi.org/10.1007/s10482-009-9401-0

EUCAST. (2023). *European Committee on Antimicrobial Susceptibility Testing. Breakpoint tables for interpretation of MICs and zone diameter. Version13.1*.

Fernandez, B., Vimont, A., Desfossés-Foucault, É., Daga, M., Arora, G., & Fliss, I. (2017). Antifungal activity of lactic and propionic acid bacteria and their potential as protective culture in cottage cheese. *Food Control*, *78*, 350–356. https://doi.org/10.1016/j.foodcont.2017.03.007

Ferrari, I. da S., de Souza, J. V., Ramos, C. L., da Costa, M. M., Schwan, R. F., & Dias, F. S. (2016). Selection of autochthonous lactic acid bacteria from goat dairies and their addition to evaluate the inhibition of Salmonella typhi in artisanal cheese. *Food Microbiology*, *60*, 29–38. https://doi.org/10.1016/j.fm.2016.06.014

Franciosi, E., Settanni, L., Cavazza, A., & Poznanski, E. (2009). Biodiversity and technological potential of wild lactic acid bacteria from raw cows' milk. *International Dairy Journal*, *19*, 3–11. https://doi.org/10.1016/j.idairyj.2008.07.008

Garnier, L., Valence, F., & Mounier, J. (2017). Diversity and control of spoilage Fungi in dairy products: an update. *Microorganisms*, *5*(3), 42. https://doi.org/10.3390/microorganisms5030042

Geoghegan, I. A., Stratford, M., Bromley, M., Archer, D. B., & Avery, S. V. (2020). Weak acid resistance A (WarA), a novel transcription factor required for regulation of weak-acid resistance and spore-spore heterogeneity in *Aspergillus niger*. *MSphere*, *5*(1), e00685-19.

https://doi.org/10.1128/mSphere.00685-19

Goldstein, E. J. C., Tyrrell, K. L., & Citron, D. M. (2015). *Lactobacillus* species: Taxonomic complexity and controversial susceptibilities. *Clinical Infectious Diseases*, *60*(suppl_2), S98–S107. https://doi.org/10.1093/cid/civ072

Grujović, M. Ž., Marković, K. G., Morais, S., & Semedo-Lemsaddek, T. (2024). Unveiling the Potential of Lactic Acid Bacteria from Serbian Goat Cheese. *Foods*, *13*(13), 2065. https://doi.org/10.3390/foods13132065

Guimarães, A., Santiago, A., Teixeira, J. A., Venâncio, A., & Abrunhosa, L. (2018). Anti-aflatoxigenic effect of organic acids produced by *Lactobacillus plantarum*. *International Journal of Food Microbiology*, *264*, 31–38. https://doi.org/10.1016/j.ijfoodmicro.2017.10.025

Hadef, S., Idoui, T., Sifour, M., Genay, M., & Dary-Mourot, A. (2023). Screening of wild lactic acid bacteria from Algerian traditional cheeses and goat butter to develop a new probiotic starter culture. *Probiotics and Antimicrobial Proteins*, *15*, 387–399. https://doi.org/10.1007/s12602-022-10000-2

Ibrahim, S. A., Ayivi, R. D., Zimmerman, T., Siddiqui, S. A., Altemimi, A. B., Fidan, H., Esatbeyoglu, T., & Bakhshayesh, R. V. (2021). Lactic acid bacteria as antimicrobial agents: Food safety and microbial food spoilage prevention. *Foods*, *10*(12), 3131. https://doi.org/10.3390/foods10123131

Kanjan, P., & Sakpetch, P. (2023). Effect of antifungal compounds secreted by *Lactiplantibacillus plantarum* 124 against *Aspergillus flavus* and *Penicillium* sp. and its application in Kaeng-Tai-Pla-Haeng to extend the shelf life. *International Journal of Food Science & Technology*, *58*(10), 5376–5387. https://doi.org/10.1111/ijfs.16656

Kayacan Çakmakoğlu, S., Vurmaz, M., Bezirci, E., Kaya, Y., Dikmen, H., Göktaş, H., Demirbaş, F., Encu, B., Acar Soykut, E., Alemdar, F., Çakır, İ., Durak, M. Z., Arıcı, M., Sağdıç, O., Türker, M., & Dertli, E. (2023). Isolation and characterization of yogurt starter cultures from traditional yogurts and growth kinetics of selected cultures under lab-scale fermentation. *Preparative Biochemistry & Biotechnology*, *53*(4), 454–463.

https://doi.org/10.1080/10826068.2022.2098325

Kuerman, M., Shi, R., Zhang, Y., Liu, Y., Hou, B., Li, B., Yi, H., Zhang, L., & Liu, T. (2024). *Lactiplantibacillus plantarum* strains with proteolytic abilities showed diverse effects on casein gel formation during fermentation. *Food Hydrocolloids*, *148*(PA), 109406. https://doi.org/10.1016/j.foodhyd.2023.109406

Leyva Salas, M., Mounier, J., Maillard, M.-B., Valence, F., Coton, E., & Thierry, A. (2019). Identification and quantification of natural compounds produced by antifungal bioprotective cultures in dairy products. *Food Chemistry*, *301*, 125260. https://doi.org/10.1016/j.foodchem.2019.125260

Li, Q., Zeng, X., Fu, H., Wang, X., Guo, X., & Wang, M. (2023). *Lactiplantibacillus plantarum*: A comprehensive review of its antifungal and anti-mycotoxic effects. *Trends in Food Science & Technology*, *136*, 224–238. https://doi.org/10.1016/j.tifs.2023.04.019

Li, L., Liu, J., Wang, D., Kwok, L. Y., Li, B., Guo, S., & Chen, Y. (2025). Enhancing storage stability, antihypertensive properties, flavor and functionality of fermented milk through co-fermentation with Lactobacillus helveticus H11 adjunct culture. *Food Chemistry*, *470*, 142574. https://doi.org/10.1016/j.foodchem.2024.142574

Marcelão, C. V. P., Souza, M. C., Silva, J. J., Couto, F. A., Lacorte, G. A., Pinto, U. M., Maffei, J. T., Zacarchenco, P. B., Iamanaka, B. T., & Taniwaki, M. H. (2024). Unveiling ochratoxin A and ochratoxigenic fungi in Brazilian artisanal Cheeses: Insights from production to consumption. *Food Research International*, *183*, 114214. https://doi.org/10.1016/j.foodres.2024.114214

Margalho, L. P., Feliciano, M. D. E., Silva, C. E., Abreu, J. S., Piran, M. V. F., & Sant'Ana, A. S. (2020). Brazilian artisanal cheeses are rich and diverse sources of nonstarter lactic acid bacteria regarding technological, biopreservative, and safety properties—Insights through multivariate analysis. *Journal of Dairy Science*, *103*(9), 7908–7926. https://doi.org/10.3168/jds.2020-18194

Metrouh, R., Fares, R., Mechai, A., Debabza, M., & Menasria, T. (2022). Technological properties and probiotic potential of Lactiplantibacillus plantarum SJ14 isolated from

Algerian traditional cheese “Jben”. *Journal of Food Processing and Preservation*, *46*(4), e16482. https://doi.org/10.1111/jfpp.16482

McSweeney, P. L. H. (2004). Biochemistry of cheese ripening. *International Journal of Dairy Technology*, *57*(2–3), 127–144. https://doi.org/10.1111/j.1471-0307.2004.00147.x

Mordor Intelligence. (2025). *Microbiology Culture Market Size & Share analysis Growth Trend & Forecasts (2025-2030)*. https://www.mordorintelligence.com/industry-reports/microbial-culture-market

Nicosia, F. D., Pino, A., Maciel, G. L. R., Sanfilippo, R. R., Caggia, C., de Carvalho, A. F., & Randazzo, C. L. (2023). Technological characterization of lactic acid bacteria strains for potential use in cheese manufacture. *Foods*, *12*(6), 1154. https://doi.org/10.3390/foods12061154

Parussolo, G., Bernardi, A. O., Garcia, M. V., Stefanello, A., Silva, T. dos S., & Copetti, M. V. (2019). Fungi in air, raw materials and surface of dry fermented sausage produced in Brazil. *LWT*, *108*, 190–198. https://doi.org/10.1016/j.lwt.2019.03.073

Pattono, D., Grosso, A., Stocco, P. P., Pazzi, M., & Zeppa, G. (2013). Survey of the presence of patulin and ochratoxin A in traditional semi-hard cheeses. *Food Control*, *33*(1), 54–57. https://doi.org/10.1016/j.foodcont.2013.02.019

Peng, Q., Yang, J., Wang, Q., Suo, H., Hamdy, A. M., & Song, J. (2023). Antifungal effect of metabolites from a new strain *Lactiplantibacillus plantarum* LPP703 isolated from naturally fermented yak yogurt. *Foods*, *12*(1), 181. https://doi.org/10.3390/foods12010181

Pérez, G., Cardell, E., & Zárate, V. (2003). Technological characterization of lactic acid bacteria from Tenerife cheese. *International Journal of Food Science & Technology*, *38*(5), 537–546. https://doi.org/10.1046/j.1365-2621.2003.00696.x

Pitt, J. I., & Hocking, A. D. (2022). Ecology of Fungal Food Spoilage. In *Fungi and Food Spoilage* (pp. 3–12). Springer International Publishing. https://doi.org/10.1007/978-3-030-85640-3_2

Prabawati, E. K., Turner, M. S., & Bansal, N. (2023). *Lactiplantibacillus plantarum* as an adjunct culture exhibits antifungal activity in shredded Cheddar cheese. *Food Control*, *144*, 109330. https://doi.org/10.1016/j.foodcont.2022.109330

Ráduly, Z., Szabó, L., Madar, A., Pócsi, I., & Csernoch, L. (2020). Toxicological and medical aspects of Aspergillus-derived mycotoxins entering the feed and food chain. *Frontiers in microbiology*, *10*, 2908. https://doi.org/10.3389/fmicb.2019.02908

Requena, T., Pelaez, C., & Fox, P. F. (1993). Peptidase and proteinase activity of *Lactococcus lactis*, *Lactobacillus casei* and *Lactobacillus plantarum*. *Zeitschrift Für Lebensmittel-Untersuchung Und Forschung*, *196*(4), 351–355. https://doi.org/10.1007/BF01197934

Roy, U., Batish, V. K., Grover, S., & Neelakantan, S. (1996). Production of antifungal substance by *Lactococcus lactis* subsp. *lactis* CHD-28.3. *International Journal of Food Microbiology*, *32*(1–2), 27–34. https://doi.org/10.1016/0168-1605(96)01101-4

Santos, K. M. O. dos, Vieira, A. D. S., Buriti, F. C. A., do Nascimento, J. C. F., de Melo, M. E. S., Bruno, L. M., de Fátima Borges, M., Rocha, C. R. C., de Souza Lopes, A. C., de Melo Franco, B. D. G., & Todorov, S. D. (2015). Artisanal Coalho cheeses as source of beneficial *Lactobacillus plantarum* and *Lactobacillus rhamnosus* strains. *Dairy Science & Technology*, *95*, 209–230. https://doi.org/10.1007/s13594-014-0201-6

Savijoki, K., Ingmer, H., & Varmanen, P. (2006). Proteolytic systems of lactic acid bacteria. *Applied Microbiology and Biotechnology*, *71*(4), 394–406. https://doi.org/10.1007/s00253-006-0427-1

Sethi, M., OK, A., Dash, J., Parida, D., Kar, S., Mishra, S., Minz, A. P., Padhi, A., Das, K. R., Pradhan, B., Prakash, T., & Senapati, S. (2024). Whole genome mining and characterization of a new probiotic strain *Levilactobacillus brevis* ILSH3 from Handia: An ethnic fermented beverage of Odisha, India. *Probiotics and Antimicrobial Proteins*. https://doi.org/10.1007/s12602-024-10217-3

Souza, L. V., Martins, E., Moreira, I. M. F. B., & de Carvalho, A. F. (2022). Strategies for the development of bioprotective cultures in food preservation. *International Journal of Microbiology*, *1*, 6264170. https://doi.org/10.1155/2022/6264170

Souza, L. V., Rodrigues da Silva, R., Falqueto, A., Fusieger, A., Martins, E., Caggia, C., Randazzo, C. L., & Carvalho, A. F. de. (2023). Evaluation of antifungal activity of lactic acid bacteria against fungi in simulated cheese matrix. *LWT*, *182*, 114773. https://doi.org/10.1016/j.lwt.2023.114773

Souza, L. V., Rodrigues, R. da S., Fusieger, A., da Silva, R. R., de Jesus Silva, S. R., Martins, E., Machado, S. G., Caggia, C., Randazzo, C. L., & de Carvalho, A. F. (2022). Diversity of filamentous fungi associated with dairy processing environments and spoiled products in Brazil. *Foods*, *12*(1), 153. https://doi.org/10.3390/foods12010153

Srikham, K., Daengprok, W., Niamsup, P., & Thirabunyanon, M. (2021). Characterization of *Streptococcus salivarius* as new probiotics derived from human breast milk and their potential on proliferative inhibition of liver and breast cancer cells and antioxidant activity. *Frontiers in Microbiology*, *12*, Article 797445. https://doi.org/10.3389/fmicb.2021.797445

Vasundaradevi, R., Sarvajith, M., Divyashree, S., Deepa, N., Achar, P. N., & Sreenivasa, M. Y. (2025). Tropical fruit-derived Lactiplantibacillus as potential probiotic and antifungal agents against Fusarium oxysporum. *Scientific Reports*, *15*(1), 2144. https://doi.org/10.1038/s41598-025-85190-0

Wang, Q., Zhang, F., Zhang, Y., Zhang, Y., Wang, H., Song, J., & Suo, H. (2024). A novel strain *Lactiplantibacillus plantarum* LPP95 isolated from Chinese pickles: Antifungal effect, mechanism, and potential application in yogurt. *Food Bioscience*, *58*, 103640. https://doi.org/10.1016/j.fbio.2024.103640

Wang, S., Chen, P., & Dang, H. (2019). Lactic Acid Bacteria and γ-Aminobutyric Acid and Diacetyl. In *Lactic Acid Bacteria* (pp. 1–19). Springer Singapore. https://doi.org/10.1007/978-981-13-7283-4_1

Wu, X., Liu, Y., Guo, Z., Ji, N., Sun, Q., Liu, T., & Li, Y. (2025). Antifungal activities of Pediococcus pentosaceus LWQ1 and Lactiplantibacillus plantarum LWQ17 isolated from sourdough and their efficacies on preventing spoilage of Chinese steamed bread. *Food Control*, *168*, 110940. https://doi.org/10.1016/j.foodcont.2024.110940

Wu, Q., You, L., Wu, W., Long, M., & Kuca, K. (2023). Mycotoxins: Emerging toxic mechanisms, and unanswered research questions. *Food and Chemical Toxicology*, *174*, 113673. https://doi.org/10.1016/j.fct.2023.113673

Zhao, S., Hao, X., Yang, F., Wang, Y., Fan, X., & Wang, Y. (2022). Antifungal activity of *Lactobacillus plantarum* ZZUA493 and its application to extend the shelf life of Chinese steamed buns. *Foods*, *11*(2), 195. https://doi.org/10.3390/foods11020195

Zheng, J., Wittouck, S., Salvetti, E., Franz, C. M. A. P., Harris, H. M. B., Mattarelli, P., O'Toole, P. W., Pot, B., Vandamme, P., Walter, J., Watanabe, K., Wuyts, S., Felis, G. E., Gänzle, M. G., & Lebeer, S. (2020). A taxonomic note on the genus *Lactobacillus*: Description of 23 novel genera, emended description of the genus *Lactobacillus* Beijerinck 1901, and union of *Lactobacillaceae* and *Leuconostocaceae*. *International Journal of Systematic and Evolutionary Microbiology*, *70*(4), 2782–2858. https://doi.org/10.1099/ijsem.0.004107

CHAPTER 4

4.THIRD PAPER

THIRD PAPER - Metabolomic profiling of antifungal *Lactiplantibacillus plantarum* strains and evaluation of *Aspergillus niger* and *Penicillium chrysogenum* inhibition in a cheese matrix

Luana Virgínia Souza et al.,

Manuscript published in *International Journal of Food Microbiology 2026*

DOI: https://doi.org/10.1016/j.ijfoodmicro.2026.111658

Metabolomic profiling of antifungal *Lactiplantibacillus plantarum* strains and their inhibition of *Aspergillus niger* and *Penicillium chrysogenum* in a cheese matrix

Luana Virgínia Souza[a,b], Andressa Falqueto[ab], Valéria Quintana Cavicchioli[d], Almir Custodio Batista Junior[e], João Victor Ataíde Oliveira[e], Andréa Rodrigues Chaves[e], Cíntia Silvia Minafra[d], Cinzia Caggia[b], Cinzia Lucia Randazzo[b], Antonio Fernandes de Carvalho [b,c], Luís Augusto Nero [a,b]

[a] InsPOA - Laboratório de Inspeção de Produtos de Origem Animal, Departamento de Veterinária, Universidade Federal de Viçosa, 36570 900, Viçosa, MG, Brazil

[b] Di3A - Dipartimento di Agricoltura, Alimentazione e Ambiente, Università degli Studi di Catania, 95123, Catania, CT, Italy

[c] InovaLeite - Laboratório de Pesquisa em Leites e Derivados, Departamento de Tecnologia de Alimentos, Universidade Federal de Viçosa, 36570 900, Viçosa, MG, Brazil

[d] LIP - Laboratório de Inovação e Pesquisa, Centro de Pesquisa em Alimentos, Escola de Medicina Veterinária e Zootecnia, Universidade Federal de Goiás, 74690-900, Goiânia, Goiás, Brazil

[e] LACEM – Laboratório de Cromatografia e Espectrometria de Massas, Instituto de Química, Universidade Federal de Goiás, 74690-900, Goiânia, Goiás, Brazil

*Corresponding author: Luís Augusto Nero. InsPOA - Laboratório de Inspeção de Produtos de Origem Animal, Departamento de Veterinária, Universidade Federal de Viçosa, 36570 900, Viçosa, MG, Brazil. E-mail address: nero@ufv.br (L. A. Nero)

Abstract

The dairy industry has increasingly seeking natural strategies to enhance food safety and quality. Fungal contamination remains a major challenge due to spoilage, economic losses, and mycotoxin production, making bioprotective microbial cultures a promising alternative. This study aimed to investigate the antifungal potential of five *Lactiplantibacillus plantarum* strains (M3.1, M3.3, M3.6, R3.2, and R3.6) against *Aspergillus niger* and *Penicillium chrysogenum*. All strains significantly inhibited fungal growth, as demonstrated by in vitro assays and reductions in fungal biomass. Scanning electron microscopy (SEM) revealed severe morphological damage to fungal spores, including surface disruption, deformation, and shrinkage. Metabolomic analyses identified the production of organic acids, including lactic, acetic, succinic, malic, propionic, butyric, and formic acids, using high-performance liquid chromatography (HPLC), as well as 35 additional metabolites, mainly amino acids, fatty acids, cyclic dipeptides, phenolic compounds, and esters, using high-performance liquid chromatography–mass spectrometry (HPLC-MS), revealing a synergistic and multifactorial antifungal mechanism. In a cheese model system, all strains completely inhibited *P. chrysogenum*, while strains M3.3, M3.6, and R3.2 fully inhibited *A. niger*. These findings demonstrate the strong antifungal activity of *L. plantarum* strains and support their application as bioprotective cultures in dairy products.

Keywords: antifungal; *Lactiplantibacillus plantarum*; metabolites; bioprotective culture.

1. Introduction

Filamentous fungi exhibit remarkable adaptability and resilience, allowing them to thrive under the challenging conditions often encountered in dairy environments, including fluctuations in water activity and temperature during processing or storage, as well as exposure to low pH, refrigeration, and the presence of preservatives, salt, or sugar (Pitt and Hocking, 2022). These traits make them prominent contaminants in food production, particularly within the dairy sector. Under favorable conditions, they can contaminate a wide range of food matrices, leading to spoilage, shortened shelf life, and economic losses. Despite the implementation of preventive strategies such as Good Manufacturing Practices (GMP) and quality management systems, filamentous fungi persist as recurrent contaminants in dairy processing facilities, posing a continuous risk of cross-contamination to finished products (Garnier et al., 2017).

Modern dairy production systems have increasingly adopted strategies centered on the use of microbial cultures with enhanced functional properties and diverse technological capabilities, primarily to enhance product preservation (Graham and Ledesma-Amaro, 2023). As the variety of dairy products continues to expand, global research on innovative microbial cultures for food applications has grown substantially. Data from Dimensions (Digital Science, 2025) indicate that related publications increased from 1,456 in 2014 to 5,531 in 2024, with notable emphasis on the topics “microbial cultures” and “food.”

L. plantarum has been widely investigated for its roles in food fermentation, antimicrobial activity, antioxidant capacity, flavor enhancement, and probiotic potential (Zheng et al., 2020; Yilmaz et al., 2022; Echegaray et al., 2023). Although its antifungal properties are recognized, they are highly strain-specific, largely due to variations in metabolomic profiles. Therefore, elucidating its metabolic pathways and identifying antifungal metabolites remain essential to understand its bioprotective potential. *L. plantarum* synthesizes a broad spectrum of antifungal compounds derived from both primary and secondary metabolism, including organic acids, fatty acids, bacteriocins, cyclic dipeptides, and phenolic compounds, which may act independently or synergistically (Ming et al., 2018; Sadiq et al., 2019; Vanitha et al., 2023).

These compounds may be characterized as a diverse array of metabolites, including lactic acid, citric acid, acetic acid, pyruvic acid, malonic acid, maleic acid, succinic acid, fumaric acid, malic acid, tartaric acid, hydroxycitric acid, shikimic acid, propanoic acid, oxalic acid, and formic acid. Additionally, various other metabolites have been identified, such as 2-nonanol, 7-methyl-1-octene, 2-tridecanol, 1-octen-3-one, 3-amino-5-methylhexanoic acid, 3-phenylpropionic acid, phenyllactic acid (PLA), indolelactic acid (ILA), 3-hydroxyphenylacetic acid (OH-PLA), indoleacetic acid (IAA), vanillic acid, coumaric acid, pyroglutamic acid, and pelargonic acid, among many others (Vanitha et al., 2023; De Simone et al., 2024; Zha et al., 2024; Kaddouri et al., 2025). This metabolic diversity is exemplified by the study conducted by Zha et al. (2024), which reported the identification of 374 distinct metabolites produced by the species under investigation.

The metabolic and antifungal activities of microbial cultures are governed by multifactorial mechanisms. Effective development requires consideration of both intrinsic and extrinsic factors, along with variability across food matrices (Li et al., 2023). As the behavior of compounds may differ between systems, studies in simulated or large-scale matrices are essential. In dairy systems, the antifungal potential of *L. plantarum* strains has been demonstrated (Souza et al., 2023; Prabawati et al., 2023; Wang et al., 2024). However, broader evaluations across diverse food types remain necessary to confirm their wide-spectrum potential. Thus, the present study aims to investigate the antifungal properties of *L. plantarum*, focusing on fungi-bacteria interactions, effects on fungal spore integrity, and the identification of antifungal metabolites. Subsequently, the efficacy of selected strains was further assessed in a cheese-based model to simulate real conservation conditions.

2. Materials and methods

2.1. Isolates and growth parameters

L. plantarum (M3.1, M3.3, M3.6, R3.2 and R3.6) antifungal strains isolated from Donkey milk from the Food Agricultural Microbiology Laboratory (Department of Agriculture, Food and Environment, University of Catania, Sicily, Italy) were used in this study. These isolates had already been identified and characterized according to a study conducted by Souza

et al., (2025). The isolates from the stock were cultured in MRS broth (Kasvi, São José dos Pinhais, PR, Brazil) and incubated at 37 ºC for 24 h, prior the experiments.

The fungi used in the screening and antifungal characterization were *Aspergillus niger* (IOC 207) and *Penicillium chrysogenum* (IOC 132) obtained from Fundação Oswaldo Cruz (Fiocruz, Rio de Janeiro, RJ, Brazil). The standardization of the spore suspension for subsequent analyses was conducted following the protocol described by Roy et al. (1996), with minor modifications. Fungal mycelia were cultivated on Potato Dextrose Agar (Kasvi, São José dos Pinhais, PR, Brazil) at 25°C for five days, allowing their sporulation. Subsequently, the spores were gently harvested and resuspended in sterile distilled water. Spore quantification was performed using a Neubauer chamber under an optical microscope (Olympus, Melville, NY, USA), and the spore suspension was adjusted to a final concentration of 1.5×10^7 spores/mL. The standardized suspension was then stored under refrigeration until further use.

2.2. Antifungal activity dual agar overlay

The antifungal activity was evaluated using the dual overlay agar method (Magnusson et al., 2003). Bacterial strains were grown from stock cultures and incubated overnight. Subsequently, two linear streaks were inoculated onto MRS agar plates and incubated at 37 °C for 24 hours. Then, 100 µL of a standardized spore suspension (approximately 10^6 spores/mL) of *A. niger (*IOC 207) and *P. chrysogenum* (IOC 132) were added to 3 mL of an overlay medium composed of 1.5% yeast extract (Kasvi, São José dos Pinhais, PR, Brazil) and 0.7% bacteriological agar (Kasvi, São José dos Pinhais, PR, Brazil). This overlay was poured over the MRS plates and incubated for 3 to 5 days. After incubation, antifungal activity was assessed by measuring the inhibition zones (in millimeters) between the LAB strains and the fungal growth using a vernier caliper. The results were measured according to the size of the inhibition halo, and follows: (-) no inhibition, (+) mild inhibition (1–3 mm) and (+++) strong inhibition (>3 mm), respectively, based on the score used by De Simone et al. (2024). All assays were conducted in duplicate and repeated in three independent experiments.

2.3. Biomass inhibition test by co-inoculation

Co-inoculation was performed according to the methodology described by Vasundaradevi et al. (2025) with minor modifications. Flasks containing 50 mL of sterile MRS broth were inoculated with 100 µL of *L. plantarum* (~ 10^6 CFU/mL) strains and 100 µL *A. niger* (IOC 207) and *P. chrysogenum* (IOC 132) (~10^6 spores/mL). Then, the flasks were incubated for 3 to 12 days at 24°C. The fungal biomass was collected at 3, 7 and 12 days and filtered and dried at 80 °C for 2 h. The viability of *L. plantarum* strains in each treatment was assessed through serial dilutions and plating on MRS agar and expressed as CFU/mL.

2.4. Scanning electron microscopy (SEM)

The changes in the morphologies of *A. niger* and *P. chrysogenum* spores caused by co-inoculation of *L. Plantarum* (M3.1, M3.3, M3.6, R3.2 and R3.6) were visualized using SEM according to Wu et al. (2025) with modifications. The co-inoculation was performed according to the method previously described and a filter paper was submerged. After incubation, the filter paper was removed with the aid of tweezers and inserted into the stub, which had been fixed with 2.5% (v/v) glutaraldehyde at 4°C overnight. No dehydration step was performed, and the stubs were sputter coated with gold (Quorum – Q150TES Plus, East Sussex, England). The samples were observed using a SEM (JSM-IT700HR, JEOL, Tokyo, Japan) operating at 10 kV and 18000 ×magnification.

2.5. **Metabolomic profiling of *Lactiplantibacillus plantarum* strains**

The characterization and quantification of antifungal metabolites produced by *L. plantarum* strains were performed using a combination of High-Performance Liquid Chromatography (HPLC) and High-Performance Liquid Chromatography coupled with mass spectrometry (HPLC-MS). Prior to both analyses, the cell-free supernatant (CFS) of the strains was obtained by centrifugation (Heraeus Megafuge 8 R, Thermo Fisher Scientific, Waltham, MA, USA) at 3260×g for 15 min at 7 ºC, followed by filtration (Ø = 0.22 µm pore size, Merck). The resulting CFS was then subjected to analysis.

2.5.1. HPLC

Organic acids metabolites were quantified by HPLC in a SHIMADZU chromatograph (SHIMADZU do Brasil, São Paulo-SP), coupled to a refractive index detector (RID), model RID-20A. The conditions used in the analysis involved an HPX 87H column (Aminex®) (300 mm x 7.8 mm) and a pre-column of the same phase (Bio-Rad Laboratórios Brasil, Rio de Janeiro-RJ). The flow rate was 0.7 mL/min, the run lasted 30 min, and the oven temperature 45 ºC. The injection volume used was 10 µL and the mobile phase consisted of acidified water (0.005 M sulfuric acid). Data were obtained using the Lab Solutions software, Shimadzu Corporation (2013).

2.5.2. HPLC-MS

General metabolites were identified by HPLC-MS using an Agilent 1260 Infinity system (Agilent, Santa Clara, USA) coupled to a Q-Exactive Orbitrap mass spectrometer (Thermo Scientific, Bremen, Germany). Chromatographic separation was achieved on a C18 column (100 × 4 mm, 5 µm) maintained at 25 °C, with a 10 µL injection volume. The mobile phase consisted of water (A) and methanol (B) under isocratic conditions, with a flow rate of 0.300 $mL \cdot min^{-1}$. The total run time was 40 min. Mass spectrometric data was acquired in both positive and negative ion modes using an electrospray ionization (ESI) source, with a capillary voltage of 3.5 kV, capillary temperature of 250 °C, and a spray current of 3.5 µA. High-resolution full-scan MS spectra were acquired over an m/z range of 100–1200 at a resolution of 140,000. MS/MS spectra were obtained through data-dependent acquisition, fragmenting the top five most abundant ions using a collision energy of 20–35 eV.

2.5.2.1 Data analysis

ESI (−) and ESI (+) HPLC-MS data were converted in .cdf using Xcalibur software (Thermo Scientific, USA) and imported to Matlab (MathWorks, USA) using personal algorithms. Firstly, the data were centroided and then a matrix were built. For ESI (−) LC-MS chemometrics analyses, a matrix containing 10 rows and 12,303 columns, which each row represents a sample and each column represents a m/z variable. For ESI (+) LC-MS, 10 samples

and 60,981 variables. For exploratory proposals, ESI (−) and ESI (+) HPLC-MS data matrices were submitted to hierarchical cluster analysis (HCA), aiming to explore association between samples (Murtagh & Contreras, 2012). Principal component analysis (PCA) also was performed, to assess the association between samples and to investigate m/z variables and their influences samples distribution (Granato et al., 2018). For annotation, the data were converted from raw to. mzML using MSConvert software (ProteoWizard, USA). Molecular network was created using Global Natural Products Social Molecular Networking (GNPS) workflow (http://gnps.ucsd.edu) (Wang et al., 2016). The precursor ion mass tolerance was set to 2.0 Da and a MS/MS fragment ion tolerance of 0.5 Da. A cosine scores above 0.7 with more than 4 matched peaks were set as filters. Additionally, an error of less than 5 ppm was used to the identification of the ions.

2.6. In situ antifungal activity

2.6.1. Preparation of the cheese matrix

The cheese matrix used in the in situ antifungal analysis was prepared according to Rouxel et al. (2020), with adaptations. One liter of pasteurized milk (90°C/5min) was prepared with a concentration of 20% (w/v) of skim milk powder (Nestlé). Then, 1.5 mL/L of rennet (Maxiren 180; DSM Food Specialties), diluted 5× and filtered (0.22µm, Kasvi) was added to milk. After homogenization, the milk was distributed into 6-well plates (5 mL / well) and *L. plantarum* strains (M3.1, M3.3, M3.6, R3.2 and R3.6) were added to the wells in triplicate (final concentration of approximately 10^5 CFU/mL per well). Wells containing only milk and wells containing a commercial bioprotective culture (Delvo® Guard 201; DSM Food Specialties) were used as controls. In each well, to perform a fermented "mini-matrix-cheese" the plates were incubated at 30 ºC for 24 h.

2.6.2. Inhibition of Aspergillus niger and Penicillium chrysogenum in cheese matrix

The antifungal efficacy against *A. niger* (IOC 207) and *P. chrysogenum* (IOC 132) within a cheese matrix was assessed through the observation of mycelial growth. Following the preparation of mini cheese matrices, residual exudate was carefully removed using a sterile

micropipette. Subsequently, 10^2 spores/mL of each fungal suspension were individually inoculated into designated wells (one fungal species per well, in triplicate) and incubated at 30°C for 7 days. Fungal growth was monitored visually to evaluate antifungal activity. Comparative analyses were conducted using two controls: a positive control comprising wells treated with a commercial bioprotective culture, and a negative control consisting of wells without fungal spore inoculation. Furthermore, wells containing only the fungal inoculum were used to monitor baseline fungal growth. Antifungal activity was qualitatively classified according to visible fungal development: total inhibition was defined as the complete absence of fungal growth, whereas partial inhibition was characterized by the presence of initial or limited mycelial growth.

3. Statistical analysis

The experiments were generally conducted in triplicate, and the data are presented as mean ± SD (standard deviation). The effect of *L. plantarum* in co-inoculation against *A. niger* and *P. chrysogenum* was first assessed for normality using Shapiro-Wilk test ($p>0.05$) and then subjected to analysis of variance (ANOVA), followed by Dunnett's test to compare the treatments with the control using RStudio software (Rstudio Team, 2019). The viability of *L. plantarum* was assessed using the Shapiro-Wilk normality test, followed by ANOVA and Tukey's post-hoc test to evaluate significant differences between time points.

4. Results and Discussion

4.1. Antifungal activity dual agar overlay

All *L. plantarum* strains exhibited antifungal activity in dual agar overlay assays against *Penicillium* and *Aspergillus*, except for strain R3.2, which showed no inhibitory effect against *A. niger* (Table 1). Strains (M3.1, M3.3, M3.6, and R3.6) exhibited mild inhibition against *A. niger*, with inhibition zones measuring less than 3 mm. In contrast, all strains displayed strong antifungal activity against *Penicillium*, characterized by inhibition halos greater than 3 mm. Similar results were reported by De Simone et al. (2024), who used a dual agar overlay to perform antifungal screening based on the measurement of inhibition zones, distinguishing between mild (1–3 mm) and strong (>3 mm) activity of *L. plantarum* strains against *A. niger.*

	Aspergillus niger (IOC 207)		*Penicillium chrysogenum* (IOC 132)	
Strain	Zone inhibition (mm)	Screening[a]	Zone inhibition (mm)	Screening
M3.1	2,60 ±0,45	+	7,77±0,34	++
M3.3	1,04 ±0,04	+	8,34±0,60	++
M3.6	1,25±0,31	+	6,85±0,25	++
R3.2	No inhibition	-	9,10±0,38	++
R3.6	1,30 ±0,17	+	8,28±0,31	++

Table 1. Zone of inhibition of *L. plantarum* against *Aspergillus niger* (IOC 207) and *Penicillium chrysogenum* (IOC 132)

[a] Size of the inhibition halo which means (-) no inhibition, (+) and (++) stand for mild (1–3 mm) and strong (>3 mm) inhibition, respectively. Values are means and standard deviation of three biological replicates

Antifungal activity can be influenced by various factors, such as the availability of nutrients, the cell density of the antagonistic bacterium, and the initial fungal contamination level, among others. In the study conducted by Wu et al. (2025), the authors observed antifungal activity of *Pediococcus pentosaceus* and *Lactobacillus plantarum* against *Aspergillus niger* and *Penicillium polonicum*. Higher bacterial concentrations resulted in a more pronounced antifungal effect, which also altered the phenotypic expression of the fungi, delaying the development of black and green pigments in *A. niger* and *P. polonicum*, respectively.

In contrast, in an initial screening of 88 strains conducted by Russo et al. (2017), it was found that between 60 to 80% of *Lactobacillus plantarum* strains were ineffective against more resistant fungi such as *Penicillium roqueforti*, *Aspergillus niger*, *A. flavus*, and *Cladosporium* spp. Moreover, 75% of *L. plantarum* strains were effective against *P. chrysogenum*, which led the authors to conduct further investigations with nine isolates that showed antifungal activity against at least three of the tested fungi. Thus, it is possible to infer that even though the strains may exhibit considerable antifungal activity against some types of fungi, they may not show the same activity against others. These differences may be related to the specificity of the fungal species or genus, as well as their adaptive capacities to various adverse conditions, such as extreme temperatures (low and high), high concentrations of salt and sugar, a wide pH range, and, most notably, fungal resistance (Souza et al., 2025b). Additionally, the variability among *L. plantarum* strains can also be influenced by their ecological origin and the environmental conditions during isolation, which affect their metabolic profiles and production of antifungal metabolites. Since the antifungal effect is multifactorial, depending on the secretion of organic acids, peptides, volatile compounds, and phenolic derivatives, differences in gene expression and metabolic regulation among strains may determine their inhibitory potential. Understanding this variability supports the selection of LAB strains capable of maintaining consistent antifungal activity under industrial fermentation conditions, where control of spoilage fungi remains a major challenge.

4.2. Biomass inhibition test by co-inoculation

As shown in Figure 1 (A and B), a progressive increase in fungal biomass was observed in the control groups over the 12-day evaluation period, reaching up to 1.0 g and 0.4 g of dry weight for *A. niger* and *P. chrysogenum*, respectively. These results reflect fungal growth under conditions without any bacterial interference. In contrast, under co-inoculation conditions with bacterial suspensions of strains M3.1, M3.3, M3.6, R3.2, and R3.6, no significant increase in fungal biomass was observed compared to the control ($p < 0.005$) from the seventh day of incubation onward. This inhibitory effect was particularly evident in the interaction with *P. chrysogenum*, as shown in Figure 1 (C), where no visible biomass formation was detected. Regarding *A. niger*, although a small amount of fungal biomass (Figure 1 D) was detected after

12 days of incubation, the dry weight remained substantially lower than that of the control, indicating limited or no significant growth.

It was evident that the antifungal effect of the five *L. plantarum* strains was more pronounced against *P. chrysogenum* than against *A. niger*, as confirmed by the measurement of inhibition halos in the dual agar overlay assay. However, regarding the effect on *A. niger*, greater inhibition was observed under co-inoculation conditions compared to the agar-based assay. This difference may be attributed to microbial metabolism, which occurs under more favorable conditions for growth in liquid media.

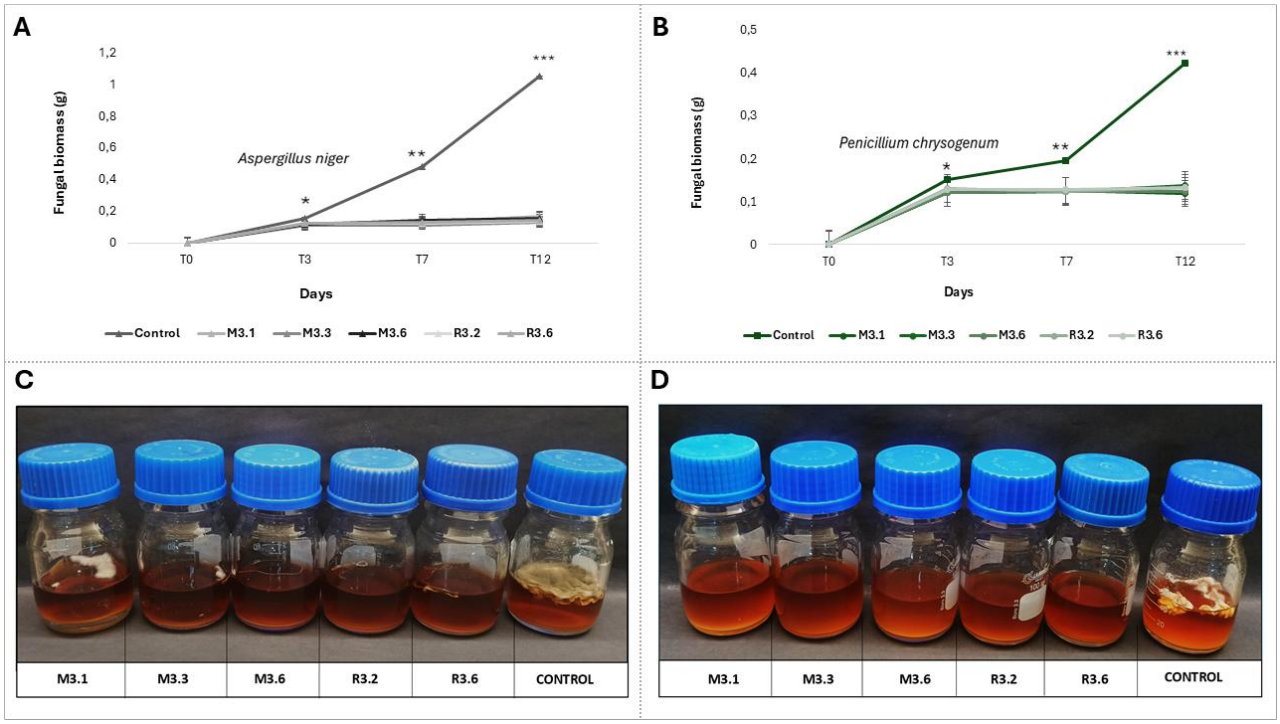


Figure 1. Reduction of fungal biomass (**A, B**) under co-inoculation with *L. plantarum* strains (M3.1, M3.3, M3.6, R3.2, and R3.6) against *A. niger* and *P. chrysogenum*, measured by filtration over different time points for 12 days. Visualization of fungal mycelial growth (**C, D**) after 12 days of co-inoculation with *L. plantarum* strains against *A. niger* and *P. chrysogenum*. Statistical significance at * $p > 0,05$, ** $p < 0,005$, *** $p < 0,0001$.

Under such conditions, a broader range of antifungal compounds can be synthesized, thereby enhancing the inhibition of *A. niger.* The enhanced inhibition in liquid media may also

be linked to the induction of specific secondary metabolites, which are less likely to accumulate or diffuse efficiently on solid surfaces.

Furthermore, the cell viability of the *L. plantarum* strains was maintained throughout the 12-day incubation period, showing no significant differences between strains ($p > 0.05$), and only a slight decline over time, which did not exceed two logarithmic cycles in any case ($p < 0.001$). Such a decline is considered a typical behavior in bacterial growth kinetics, occurring after a certain duration when the cells have transitioned from the stationary phase into the death phase, characterized by a net loss of viable cells due to nutrient depletion and accumulation of toxic metabolites (Madigan et al., 2021). By the end of the experiment, all strains remained above 8 log CFU/mL, indicating that the viability of *L. plantarum* cells was not affected by either *A. niger* or *P. chrysogenum* (Figure 2). In similar studies, Vasundaradevi et al. (2025) and Vasundaradevi et al. (2024) evaluated the antifungal capacity of *L. plantarum* strains against *Fusarium oxysporum* and *Alternaria alternata*, respectively. They reported complete inhibition of fungal biomass formation during co-inoculation assays with the bacterial strains, employing a methodology comparable to that used in the present study. Furthermore, the authors observed notable antifungal activity in the cell-free supernatants (CFS) of the strains, with varying degrees of inhibition. These findings support the hypothesis that the compounds produced by *L. plantarum* possess potent antifungal properties.

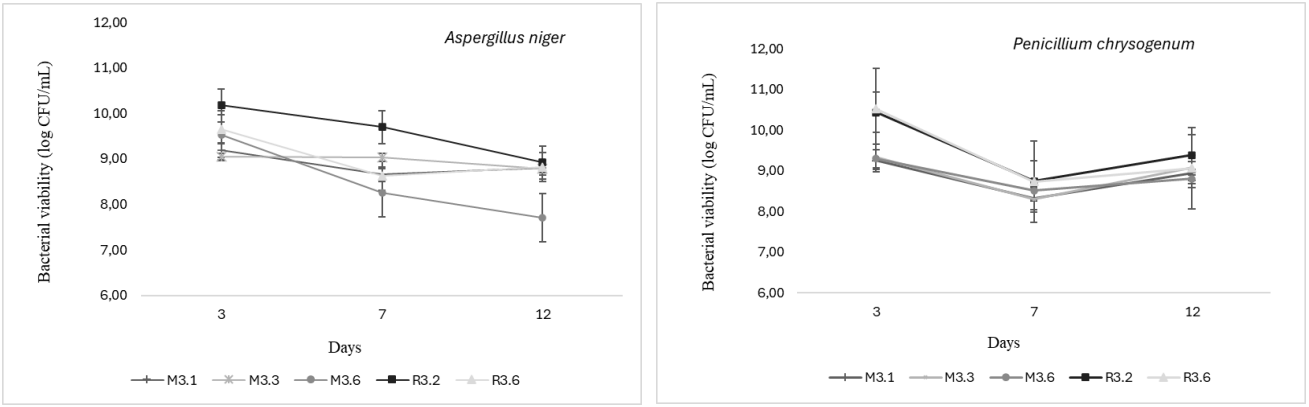


Figure 2. Cell viability of *L. plantarum* strains in co-inoculation with A. niger (A) and P. chrysogenum (B). All strains remained above 8 log CFU/mL. Statistical significance ($p > 0.05$ between strains; $p < 0.001$ over time).

L. plantarum strains have demonstrated a broad spectrum of antifungal activity in various studies against different fungi, including *Aspergillus*, *Penicillium*, *Cladosporium*, *Fusarium*, *Alternaria*, among others (Souza et al., 2023, Cheong et al., 2014; De Simone et al., 2024; Russo et al., 2017). An important consideration emerging from our findings is that, although *L. plantarum* strains exhibit antifungal potential, their efficacy varies not only among fungal species but also among bacterial strains of the same species. While several factors underlying antifungal activity have been previously discussed, these variations may result from strain-specific metabolic profiles and differential production of antifungal compounds. Thus, quantifying and characterizing these metabolites is essential to elucidate the specific mechanisms responsible for the antifungal activity of each strain.

4.3. Fungal Spore Changes by SEM

The morphological changes were observed in spores of *A. niger* and *P. chrysogenum* following co-inoculation with *L. plantarum* strains (M3.1, M3.3, M3.6, R3.2, and R3.6) as shown in Figures 3 and 4. The control spores, which were not subjected to co-inoculation, exhibited well-preserved morphological features. Both *P. chrysogenum* (Figure 3A) and *A. niger* (Figure 4G) displayed intact and regular surface structures, with no evidence of damage such as perforations, fissures, or shrinkage, as clearly observed by SEM. In contrast, spores exposed to *L. plantarum* in co-inoculation exhibited significant structural damage across all treatments and both fungal species. As illustrated in Figure 3 (B–F), *P. chrysogenum* spores showed noticeable surface irregularities, including slight shrinkage and fissuring. Similarly, *A. niger* spores (Figure 4 H–L) presented marked morphological alterations, such surface collapse and fissures leading to a highly irregular appearance.

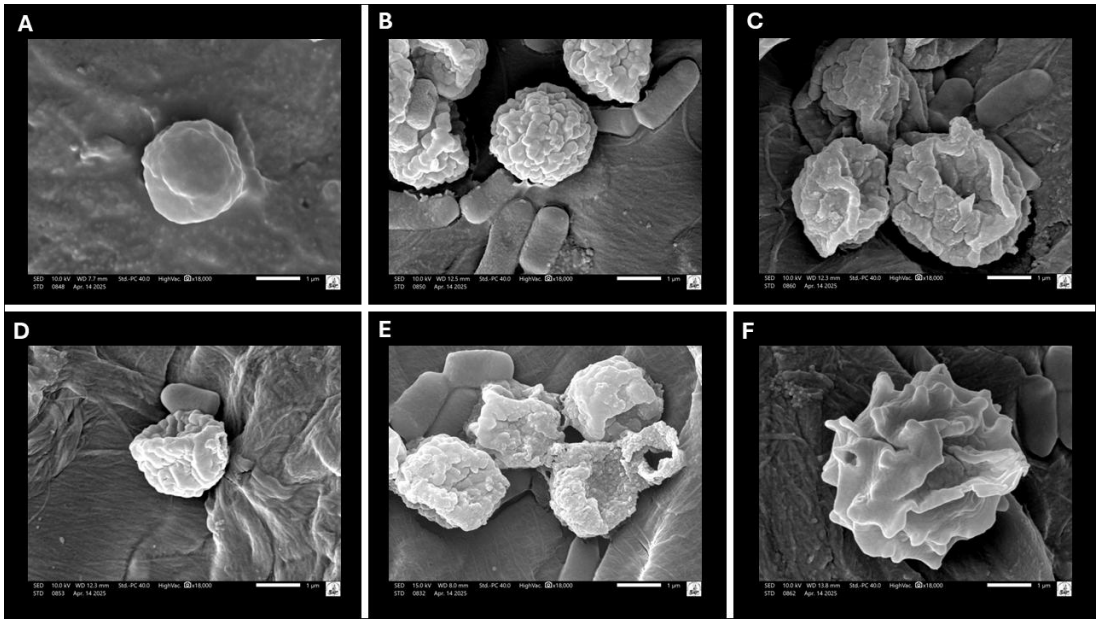


Figure 3. Observation microscope of the morphological structure of the *P.chrysogenum* spore under SEM (A). Observation of changes in *P. chrysogenum* spores in co-inoculation with *L.plantarum* strains M3.1, M3.3, M3.6, R3.2 and R3.6, respectively (B, C, D, E and F).

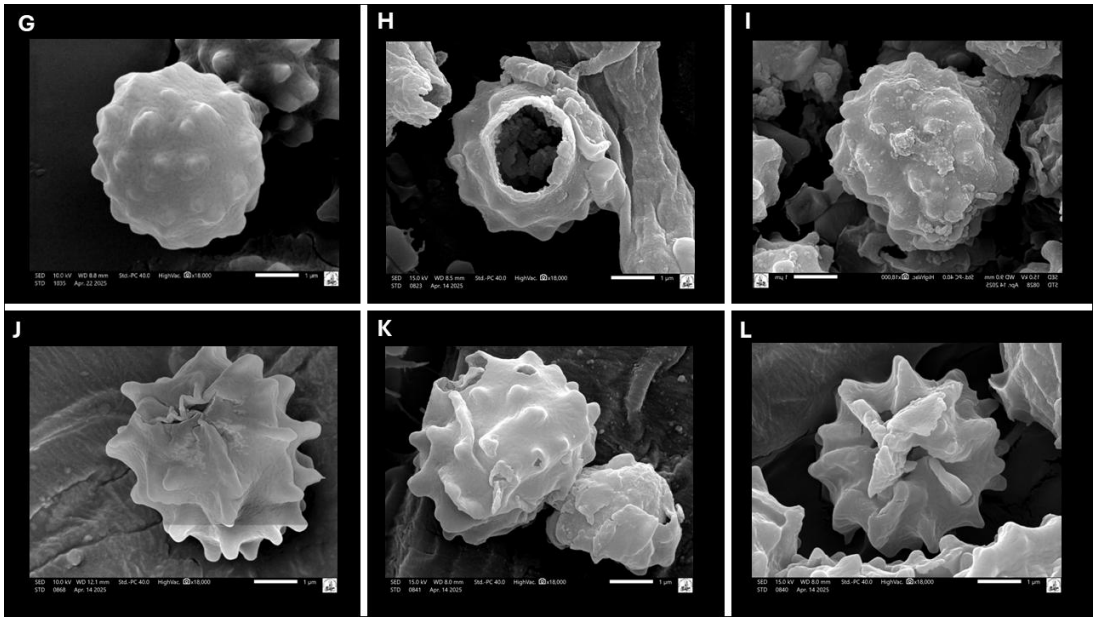


Figure 4. Observation microscope of the morphological structure of the *A.niger* spore under SEM (G). Observation of changes in *A. niger* spores in co-inoculation with *L.plantarum* strains M3.1, M3.3, M3.6, R3.2 and R3.6, respectively (H, I, J, K and L).

Notably, a large perforation was observed in the *A. niger* spore structure (Figure 4H). These findings strongly suggest that co-inoculation with *L. plantarum* strains compromises spore membrane integrity, leading to structural degradation. Our results agree with the results found by Ma et al. (2024) who evaluated the damage caused by *L. plantarum* against *A. niger* by SEM, observing similar structural alterations. The treatment with the antifungal strain caused fissures, shrinkage and overall damage to the spore structure. Several mechanisms may underlie the antifungal activity of *L. plantarum*, primarily related to the direct production of its metabolites. These mechanisms include the induction of oxidative stress in fungal cells, the generation of reactive oxygen species (ROS), modifications of small molecules and protein thiol groups, disruption of the cell wall, DNA degradation, and impairment of fungal membrane integrity through inhibition of ergosterol biosynthesis, which is essential for fungal growth and regulation. Additionally, some interactions may lead to mitochondrial dysfunction and the induction of mycelial apoptosis (Liu et al., 2022; Ma et al., 2024).

4.4. Metabolomic profiling of *L. plantarum* strains

4.4.1. Metabolites produced by *L.plantarum* strains by HPLC

The targeted metabolomic analysis by HPLC revealed that the types and concentrations of organic acids were similar among the five *L. plantarum* strains, particularly with respect to the production of lactic and acetic acids (Table 2). Succinic acid was not produced by strains M3.1 and M3.3 to M3.6, but low concentrations (0,70, 0,91 $mmol.l^{-1}$) were detected in the CFS produced by strains R3.2 to R3.6, respectively. Malic, formic, propionic, and butyric acids were also detected at low concentrations across the CFS produced by all tested strains. Gallic and citric acid were either not detected or present below the detection limit in CFS.

Table 2. Characterization of organic acids produced by *L.plantarum* strains isolated from donkey milk by HPLC

Organic acid (mmol.l^{-1})	**M3.1**	**M3.3**	**M3.6**	**R3.2**	**R3.6**
Lactic	268,11	310,45	273,30	264,14	238,58
Succinic	*ND**	*ND*	*ND*	0,70	0,91
Malic	2,85	3,67	3,89	1,85	1,75
Acetic	68,11	81,63	70,14	73,89	64,36
Propionic	11,16	13,10	11,23	12,92	9,82
Gallic	*ND*	*ND*	*ND*	*ND*	*ND*
Citric	*ND*	*ND*	*ND*	*ND*	*ND*
Butyric	0,48	1,65	0,84	1,50	0,62
Formic	14,86	14,75	12,70	13,08	11,94

*ND Not detected or below detection limits.

The antifungal activity of organic acids is well documented and primarily attributed to the biochemical interactions of undissociated weak acid molecules, which interfere with the proton gradients of the cytoplasmic membrane (Siedler et al., 2019). These molecules can diffuse across the plasma membrane, dissociate in the cytoplasm, and consequently lower the intracellular pH, thereby disrupting the proton motive force (Mieszkin et al., 2017). However, this mechanism depends on the acid's pKa, which must exceed the environmental or matrix pH to ensure efficient membrane permeation and consequent antifungal activity (Liu et al., 2022). Although lactic acid is typically the predominant metabolite produced by *L. plantarum*, studies have shown that its contribution to antifungal activity is limited, and that the inhibitory effect cannot be solely explained solely by pKa-pH dynamics (Guimarães et al., 2018). As

demonstrated by Guimarães et al. (2018), phenyllactic acid (PLA) produced by *L. plantarum* strains exhibited strong antifungal activity against *Aspergillus flavus*, despite only 45% of the compound being in its undissociated form. This effect was more pronounced than that observed for other acids such as hydroxyphenylactic acid (OH-PLA), indole lactic acid (ILA), and lactic acid, which presented higher proportions in their undissociated forms.

Thus, additional mechanisms may contribute to the antifungal activity of *L. plantarum*, indicating also that no single compound is solely responsible. The inhibitory effect is likely the result of a synergistic combination of multiple metabolites, including organic acids, peptides, unsaturated fatty acids, acrolein, diacetyl, reuterin, hydroxy acids, hydrogen peroxide, and other antifungal agents produced by the bacteria. Peng et al. (2023) investigated the antifungal activity of *L. plantarum* against *Penicillium* sp., *Rhizopus delemar*, *A. flavus*, and *A. niger*, and identified several potentially active metabolites. Among them, oleamide, trans-cinnamic acid, and citric acid were the most abundant. Molecular docking analyses predicted that these compounds interact with the active site of Cyp51, a key enzyme involved in fungal membrane biosynthesis, through hydrogen bonding interactions, which may contribute to the disruption of fungal cell membrane integrity.

Additionally, Wang et al. (2024) identified 545 antifungal compounds produced by *L. plantarum*, including 66 organic acids. Among these, trans-cinnamic acid, citric acid, trans-3-indoleacrylic acid, and DL-4-hydroxyphenyllactic acid demonstrated the highest antifungal activity. In addition to organic acids, Muhialdin et al. (2016) evaluated the antifungal potential of peptides produced by *L. plantarum* and identified 20 peptides with inhibitory properties. A novel peptide, FPSHTGMSVPPP, partially inhibited the growth of *Aspergillus flavus*, *Penicillium roqueforti*, and *Eurotium rubrum* by 44%, 60.5%, and 42.9%, respectively.

Therefore, a comprehensive understanding of metabolome is indispensable for unraveling the complex and multifactorial mechanisms underlying antifungal activity. The integration of different analytical approaches, in conjunction with genomic analyses, constitutes a pivotal strategy to advance the elucidation of these biological processes.

4.4.2. Untargeted metabolomics of *L. plantarum* strains (HPLC-MS)

Metabolomic analysis by HPLC–MS (in both positive and negative ESI) enabled the identification of total of 35 distinct metabolites produced by the confirmed antifungal *L. plantarum* strains (M3.1, M3.3, M3.6, R3.2, and R3.6). To explore similarities and differences between samples, HCA analysis was performed on the ESI (−) and ESI (+) HPLC-MS data as shown in Figure 5. For ESI (−) HPLC-MS, two major groups were formed, where samples R and samples M were grouped. For ESI (+) HPLC-MS, a tendency of separation between R and M samples also suggests the difference between the metabolism of this group of samples. However, is observed a huge similarity between all samples when compared to ESI (−) LC-MS. An inference regarding the separation of the clusters may be associated with the sampling origin. Although all bacterial strains were isolated from the same source (donkey milk), they were collected at different time points. Additionally, certain phylogenomic characteristics of the strains may have contributed to this divergence. Despite the observed variations in the metabolomic profiles, common traits among the strains are expected to exist, along with distinctive features that are generally unique to each strain.

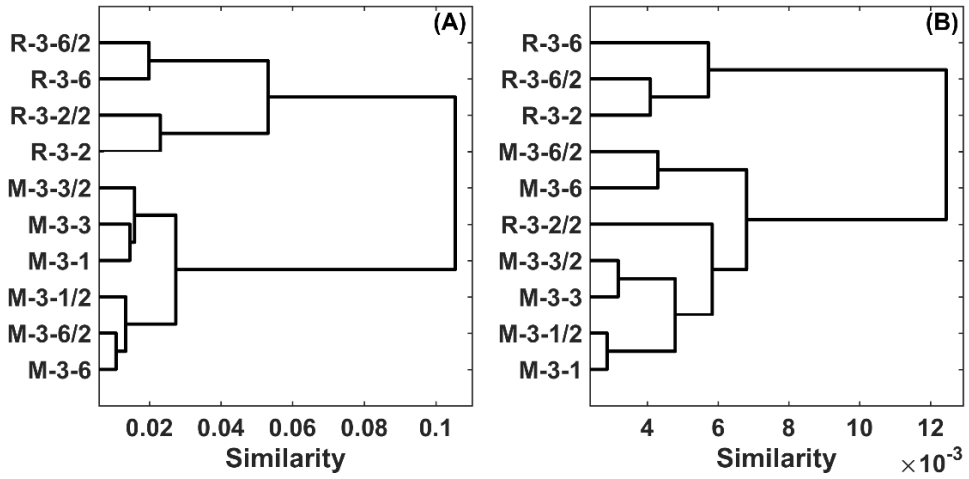


Figure 5. Hierarchical Cluster Analysis (HCA) for: (A) ESI (−) HPLC-MS; (B) ESI (+) HPLC-MS.

In this context, the HCA proved to be a valuable exploratory tool for identifying clustering trends between R and M samples. However, since HCA does not provide information about the distribution of variables within these clusters, additional chemometric methods such as PCA were applied for a more comprehensive interpretation of the data. PCA performs data compression by transforming the dataset into a new vector space defined by orthogonal components, known as principal components (PCs). The scores matrix represents the samples in this new space, while the loadings matrix describes the contribution of each variable. Thus, PCA enables simultaneous visualization of both sample and variable behavior (Granato et al., 2018).

In our findings, the first principal component (PC1) captured most of the data variance, indicating that a single component was sufficient to describe the main clustering tendencies. For the ESI(−) dataset, PC1 accounted for 85.28% of the total variance. Samples from group M showed positive score values, while samples from group R exhibited negative ones (Fig. 6A), confirming the clustering trend observed in the HCA analysis. Evaluation of PC1 loadings (Fig. 6B) revealed that low m/z compounds contributed mainly to group M, whereas compounds with moderate m/z values were more representative of group R. A similar pattern was observed in the ESI(+) dataset, which explained 54.84% of the total variance in PC1. Again, clear clustering between groups M and R was evident, with M samples showing negative scores and R samples positive scores. Moreover, M samples were characterized by compounds with lower m/z values compared to those predominant in R samples.

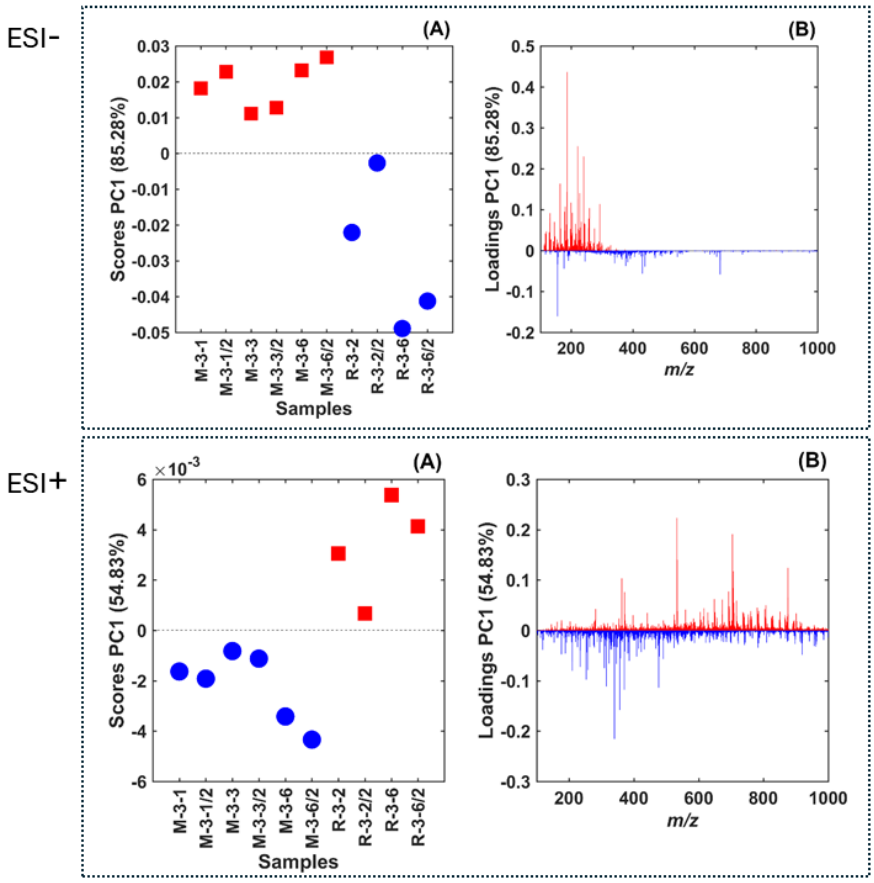


Figure 6. Principal Component Analysis (PCA) for ESI- HPLC-MS and ESI+ HPLC-MS data. (A) Scores; (B) Loadings.

The identified metabolites encompassed various chemical classes, including organic acids, fatty acids, cyclic dipeptides, amino acids, phenolic compounds, and esters (Table 3). These compounds are consistent with the multifactorial antifungal profile of lactic acid bacteria, particularly *L. plantarum*, as previously described in the literature (Li et al., 2022; De Simone et al., 2024).

Table 3. General metabolites produced by *Lactiplantibacillus plantarum* isolated from donkey milk by HPLC-MS

Metabolite	Adduct	*Measured m/z*	error	class
POSITIVE				
Guanosine cyclic monophosphate	$[M+H]^+$	346.055	0.05	Alkaloid
Linoleic acid	$[M+H]^+$	281.247	0.32	Fatty acid
Lysine-valine	$[M+H]^+$	246.181	0.34	Amino acid
1-Hexadecanoyl-sn-glycerol	$[M+H]^+$	331.284	0.48	Monoacylglycerol
Arginine	$[M+H]^+$	175.119	0.48	Amino acid
Triacetin	$[M+Na]^+$	241.068	0.5	Triester
Melibiose	$[M+H-H_2O]^+$	325.113	0.5	Disaccharide
Tyrosine	$[M+H]^+$	182.081	0.5	Amino acid
Indole-3-lactic acid	$[M+H]^+$	206.073	0.5	Monocarboxylic acid
Cyclo(proline-leucine)	$[M+H]^+$	211.144	0.5	Amino acid and peptides
Adenosine 2',3'-cyclic phosphate	$[M+H]^+$	330.060	0.55	Nucleosides
Lactose	$[M+Na]^+$	365.105	0.65	Disaccharide
Guanine	$[M+H]^+$	152.057	0.66	Alkaloid
L-valine	$[M+H]^+$	118.086	0.72	Amino acid
4-hydroxybenzaldehyde	$[M+H]^+$	123.044	0.75	Phenolic acid

2-Amino-1-phenylethanol	$[M+H-H_2O]^+$	120.081	0.80	Alkaloid
1,3-Dicyclohexylurea	$[M+H]^+$	225.196	0.80	Carboxylic acids analogs
Creatine	$[M+H]^+$	132.077	0.81	Amino acid
Aspartic acid	$[M+H]^+$	134.045	1.82	Amino acid
L-α-Palmitin	$[M+NH_4]^+$	348.310	2.80	Fatty acid
Fructoselysine	$[M+H]^+$	309.167	3.20	Carbohydrates
N-Fructosyl isoleucine	$[M+H]^+$	294.156	3.40	Amino acid
Phenylalanine	$[M+H]^+$	166.086	4.9	Amino acid
NEGATIVE				
L-Pyroglutamic acid	$[M-H]^-$	128.035	0.03	Amino acid
Isoleucine	$[M-H]^-$	130.087	0.03	Amino acid
Aconitic acid	$[M-H]^-$	173.009	0.05	Fatty Acid
Latifolicinin c acid	$[M-H]^-$	181.051	0.07	Phenylpropanoid
Inositol	$[M-H]^-$	179.056	0.08	Carbohydrate
Fructose 6-phosphate	$[M-H]^-$	259.022	1.5	Carbohydrate
Adenosine 3'-monophosphate	$[M-H]^-$	346.108	2.9	Carbohydrate
Sucrose	$[M-H]^-$	341.108	2.9	Carbohydrate
N-Fructosyl pyroglutamate	$[M-H]^-$	290.087	3.4	Amino acid derivative
Citrate	$[M-H]^-$	191.026	3.5	Tricarboxylic acid
Malic acid	$[M-H]^-$	133.021	3.5	Dicarboxylic acid

Succinic acid	$[M-H]^-$	117.025	4.0	Dicarboxylic acid

In the comparative analysis of similarities detected by ESI−, all strains (M3.1, M3.3, M3.6, R3.2, and R3.6) produced pyroglutamic acid, isoleucine, leucic acid, glutamic acid, citramalic acid, phenylalanine, inositol, fructose 6-phosphate, tyrosine, and citrate (Table 4). In contrast, 2-hydroxy-3-(4-hydroxyphenyl) propanoic acid was produced by all strains except R3.6. Additionally, aconitic acid and N-fructosyl pyroglutamate were detected only in strains R3.2 and R3.6. Under ESI+, guanosine cyclic monophosphate, linoleic acid, lysine-valine, 1-hexadecanoyl-sn-glycerol, arginine, guanine, L-valine, 1,3-dicyclohexylurea, aspartic acid, N-fructosyl isoleucine, indole-3-lactic acid, and cyclo (proline-leucine) were detected in all strains. Some metabolites, however, were found in one or more strains, for example, 4-hydroxybenzaldehyde in M3.3 and M3.6, and L-α-palmitin exclusively in R3.6.

Table 4. Similarity identified metabolites produced by *Lactiplantibacillus plantarum* isolated from donkey milk by HPLC-MS

Samples	Analyte	*m/z*	Class
POSITIVE			
All	Guanosine cyclic monophosphate	346.055	Alkaloid
All	Linoleic acid	281.248	Fatty acid
All	Lysine-valine	246.181	Amino acid
All	1-Hexadecanoyl-sn-glycerol	331.284	Monoacylglycerol
All	Lactose	365.105	Disaccharide
All	Arginine	175.119	Amino acid

All	Guanine	152.057	Alkaloid
All	L-valine	118.086	Amino acid
All	1,3-Dicyclohexylurea	225.196	Carboxylic acids analogs
All	Aspartic acid	134.045	Amino acid
All	N-Fructosyl isoleucine	294.156	Amino acid
M_3_3 M_3_6	Melibiose	325.113	Carbohydrates
All	Indole-3-lactic acid	206.073	Monocarboxylic acid
All	Cyclo(proline-leucine)	211.144	Amino acid and peptides
M_3_1	Adenosine 2',3'-cyclic phosphate	330.060	Nucleosides
M_3_3 M_3_6	4-hydroxybenzaldehyde	123.044	Phenolic acid
M_3_1 M_3_6 R_3_2 R_3_6	Creatine	132.077	Amino acid
R_3_6	L-α-Palmitin	348.310	Fatty Acid
R_3_2	Fructoselysine	306.166	
NEGATIVE			
All	Pyroglutamic acid	128.034	Amino acid
All	Isoleucine	130.094	Amino acid

All	Leucic acid	131.072	Amino acid derivative
All	Glutamic acid	146.043	Amino acid
All	Citramalic acid	147.027	Carboxylic acid
All	Phenylalanine	164.070	Amino acid
All	Inositol	179.056	Carbohydrate
All	Fructose 6-phosphate	259.021	Carbohydrate
All	Tyrosine	180.066	Amino acid
All	Citrate	191.026	Tricarboxylic acid
M_3_1 M_3_3 M_3_6	2-Hydroxy-3-(4-hydroxyphenyl)propanoic acid	181.050	Carboxylic acid
R_3_2 R_3_6	Norvaline	116.078	Amino acid
R_3_2 R_3_6	Aconitic acid	173.008	Carboxylic acid
R_3_2 R_3_6	N-Fructosyl pyroglutamate	290.087	Carbohydrate

Organic acids, in general, have been extensively studied and are mostly characterized by their antimicrobial activity against a wide range of lactic acid bacteria, as discussed in the previous section. Among the amino acid-derived metabolites identified by HPLC-MS, a noteworthy example is indole-3-lactic acid (ILA), that was produced by all strains in our study, a common metabolite produced by *L. plantarum* with antifungal activity against fungi such as *Aspergillus* and *Penicillium* (Guimarães et al., 2018). In a study conducted by Guimarães,

Venâncio, and Abrunhosa (2018), it was observed that ILA, at concentrations of 8 mg mL^{-1}, was able to inhibit 50% of the fungal growth of *Penicillium nordicum*, while 4 mg mL^{-1} was sufficient to suppress the production of the mycotoxin ochratoxin A (OTA). Additionally, this metabolite originates from successive transamination reactions followed by dehydrogenase activity during tryptophan catabolism carried out by *L. plantarum* and it may also play other important roles, such as regulating the intestinal barrier and alleviating inflammatory conditions when associated with probiotic functions (Wang et al., 2023).

The identification of fatty acids, such as linoleic acid, and lipid-derived metabolites, including monoacylglycerols, further substantiates the antifungal role of the strains under investigation. Unsaturated fatty acids and their metabolites have been extensively reported as bioactive compounds capable of disrupting fungal membrane integrity and inhibiting mycelial proliferation (Guimarães and Venâncio, 2022). Notably, a previous study demonstrated that *L. plantarum* can catalyze the bioconversion of linoleic acid into 10-hydroxy-12-octadecenoic acid (10-HOE) via linoleate hydratase, a metabolite endowed with antifungal activity against fungi such as *Penicillium roquefortii* and *Aspergillus niger* (Chen et al., 2016). The fatty acids identified in our study, including linoleic acid, L-α-palmitin, and aconitic acid, may therefore act as precursors contributing to antifungal activity. Furthermore, Liu et al. (2008) reported that even in its unmodified form, palmitic acid at concentrations of 3,900 µmol/L was able to partially suppress spore germination of *Colletotrichum lagenarium* and *Fusarium oxysporum* f. sp. *lycopersici*, while reducing spore germination of *F. oxysporum* f. sp. *cucumerinum* by 49%.

In our study, we detected the presence of cyclic dipeptides in all strains, such as cyclo (Pro-Leu), as well as several amino acids including lysine-valine, tyrosine, L-valine, N-fructosyl isoleucine, phenylalanine, L-pyroglutamic acid, and isoleucine. Compounds belonging to these classes have previously been isolated from the of bacteria and have demonstrated strong antifungal activity in various experimental models, suggesting an important role in the suppression of multiple fungal species (Ström et al., 2002; Kwak et al., 2014; Vela-Corcia et al., 2024). In a study conducted by Kwak et al. (2014), the antifungal activity of cyclic dipeptides produced by *L. plantarum* against *Ganoderma boninense* was confirmed, including the fractions cis-cyclo(L-Tyr-L-Pro), cis-cyclo(L-Val-L-Pro), cis-

cyclo(L-Ser-L-Pro), cis-cyclo(L-Leu-L-Pro), and cis-cyclo(L-Phe-L-Pro). Similarly to our study, Kanjan and Sakpetch (2023) detected the presence of cyclo (Pro-Leu) produced by *L. plantarum*, which exhibited antifungal activity against fungi of the genera *Aspergillus* and *Penicillium*.

The mechanisms underlying the antifungal activity of cyclic dipeptides involve disruption of membrane integrity, induction of oxidative stress, and impairment of mitochondrial function (Kanjan & Sakpetch, 2023; Vela-Corcia *et al.*, 2024). They also suppress spore germination and biofilm formation, while modulating fungal secondary metabolism by downregulating mycotoxin-related genes such as *aflR* (Ström *et al.*, 2002; Iimura *et al.*, 2017). This activity is further enhanced through synergistic interactions with other metabolites, including lactic acid and 3-phenyllactic acid, forming a cooperative antifungal strategy (Ström *et al.*, 2002; Bojarska *et al.*, 2021).

Accordingly, the results obtained are in full agreement with the literature: the production of antifungal metabolites by *L. plantarum* involves a diverse set of compounds exerting both direct and indirect effects on fungal cells, often acting synergistically. The detection of ILA, fatty acids, cyclic dipeptides, esters, organic acids, and phenolic compounds precisely reflect the patterns reported in previous studies, confirming that the metabolic profile identified in the analyzed strains is consistent with their demonstrated antifungal activity. Moreover, given the multiple mechanisms underlying antifungal activity, the metabolome is highly complex, and bacteria–fungi interactions may influence key molecular targets, potentially modulating the mechanisms involved.

To further strengthen these findings, future studies should include structural confirmation through fragmentation analyses (MS/MS), the use of authentic standards, and bioassay-guided fractionation experiments, which will enable the quantitative association of specific metabolites with the observed antifungal activity, thereby reinforcing the causal relationships suggested in literature. Such comprehensive investigations of the full metabolomic profile and biosynthetic pathways are essential to identify metabolites with genuine antifungal efficacy and to better understand the multifactorial nature of *L. plantarum* antifungal activity.

4.5. *In situ antifungal activity*

All *L. plantarum* strains exhibited strong antifungal activity in a simulated cheese model against *A. niger* and *P. chrysogenum* over a 7-day incubation period at 30°C (Figure 6). In contrast, the fungal controls showed normal growth throughout the incubation period. Using the commercial culture Delvo® Guard, the cheese model remained unaffected for the entire 7-day incubation period (Supplementary material S1). While the antifungal activity demonstrated by the treatments was effective relative to the control for both fungal species tested, incipient growth of *A. niger* was nonetheless detected in cheeses inoculated with strains M3.1 and R3.2. However, no black pigment formation was observed in any of these treatments, indicating that the strains still delayed the diffusion of fungal mycelia during the incubation period. Cheeses treated with strains M3.3, M3.6 and R3.6 completely inhibited fungal growth for both fungi tested.

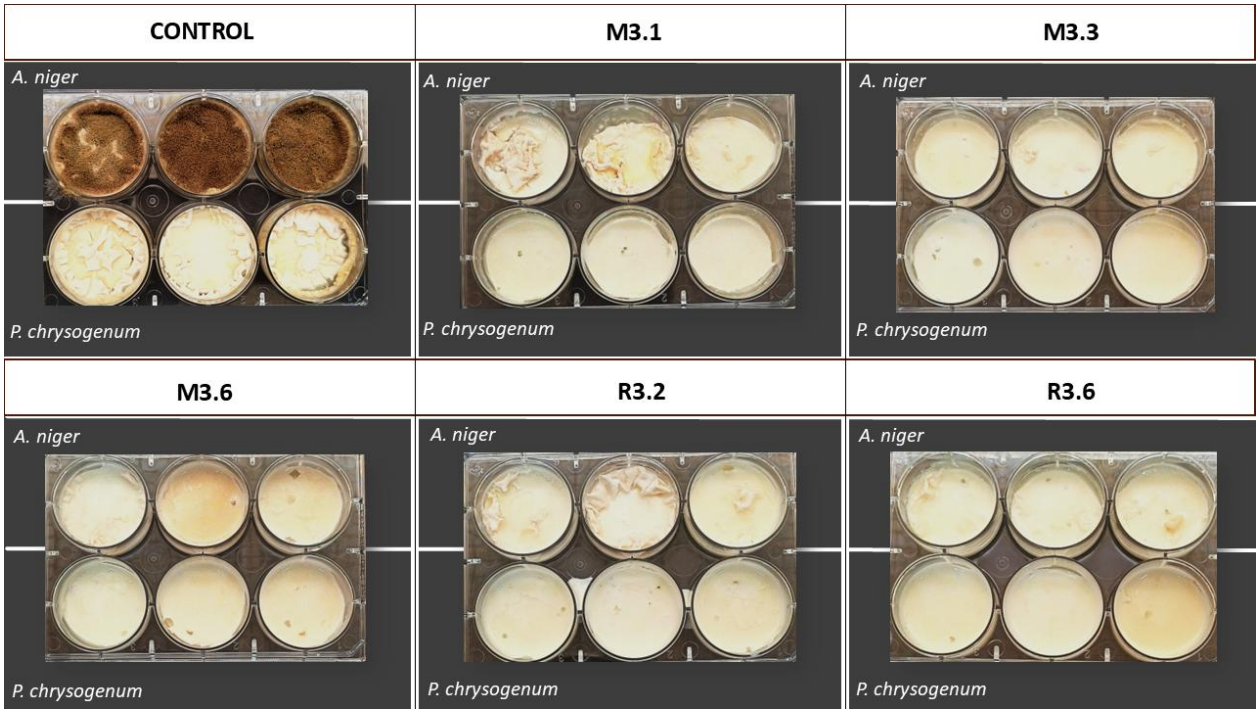


Figure 7. In situ antifungal activity of *L. plantarum* strains (M3.1, M3.3, M3.6, R3.2 and R3.6) against *A. niger* (IOC 207) and *P. chrysogenum* (IOC 132) in matrix under simulated cheese conditions. Each treatment was performed in triplicate for each fungus tested.

Our findings align with those reported by Garnier et al. (2018) and Souza et al. (2023), who demonstrated the antifungal activity of lactic acid bacteria, particularly *L. plantarum*, in a cheese-simulating matrix against *Penicillium commune*, *A. niger*, and *P. chrysogenum*, respectively. Notably, Garnier et al. (2018) observed that *L. plantarum*, when applied as a bioprotective culture, exhibited a more pronounced antifungal effect compared to the use of its fermented products alone. This observation is consistent with our results, which showed that *L. plantarum* cultures displayed significantly greater antifungal efficacy than the corresponding CFS in the cheese model system (Supplementary material S2). In our study, it was observed that none of the CFS were effective against *A. niger* after 7 days of incubation when compared to the control on cheese model. However, a partial inhibitory effect was observed against *P. chrysogenum*, particularly with the CFS derived from *L. plantarum* strains R3.2 and R3.6.

Although the antifungal properties of *L. plantarum*-derived compounds are well established, several factors may influence their effectiveness, including the concentration of the compounds, the amount applied to the dairy product, and potential impacts on processing technology, as well as modifications to texture, taste, and flavor. Conversely, the use of live cultures may foster a more dynamic biochemical interaction with the matrix, particularly in ripened cheeses, where antifungal compounds are gradually produced throughout the ripening process. The efficacy of *L. plantarum* may also be influenced by the composition of the cheese matrix, including fat content, moisture, and pH, which can affect both bacterial growth and the diffusion of antifungal metabolites. Furthermore, antifungal activity may result from the combined action of several metabolites – such as peptides, cyclic dipeptides and organic acid – that can act synergistically to inhibit fungal growth and spore germination.

The antifungal activity of *L. plantarum* strains was also effective in other dairy matrices, including semi-hard cheeses, sour cream, and yogurt, as confirmed by the studies conducted by Leyva Salas et al. (2018) and Wang et al. (2024), respectively. The antifungal effect of *L. plantarum* has been well-documented *in vitro* in several studies (Adithi et al., 2022; Wang et al., 2024; Zha et al., 2024; Souza et al., 2025; Vasundaradevi et al., 2025). In a previous study, we also assessed its antifungal potential through a screening aimed at selecting the most promising bacterial strains (Souza et al, 2025). This screening broadened the evaluation

of antifungal activity and facilitated the subsequent identification of the metabolites involved in this effect, as discussed in the previous section. However, *in situ* applications remain limited and such investigations are essential to confirm the practical applicability of antifungal substances within food matrices, as the mechanisms involved are inherently multifactorial. These mechanisms depend not only on microbial interactions between bacteria and fungi but also on processing technologies, food composition, and complex biochemical interactions among bacteria, fungi, and the food matrix.

5. Conclusion

The present study demonstrates that *L. plantarum* strains exhibit strong antifungal activity against *A. niger* and *P.chrysogenum* in a cheese model, highlighting their potential as bioprotective cultures for dairy products. Live cultures consistently outperformed cell-free supernatants, indicating that continuous metabolite production during storage enhances antifungal efficacy. Structural analyses of fungal spores revealed significant morphological damage, supporting the role of synergistic antifungal metabolites, including peptides, cyclic dipeptides, organic acids, and other bioactive compounds. Metabolomic profiling confirmed a complex and multifactorial mechanism of action, consistent with literature reports on *L. plantarum* antifungal potential.

These findings underscore the practical applicability of *L. plantarum* as a natural biopreservative in situ, capable of inhibiting fungal growth and delaying mycelial diffusion within a real food matrix. The study provides a strong foundation for future investigations aiming to optimize strain selection, dosing, and integration into industrial cheese production, supporting the development of innovative, safer and chemical-free dairy products with extended shelf-life.

CRediT authorship contribution statement

Luana Virgínia Souza: Conceptualization, Methodology, Validation, Formal analysis, Investigation, Writing - Original Draft, Writing - Review & Editing, Visualization. **Andressa Falqueto**: Conceptualization, Formal analysis, Writing - Review & Editing, Visualization.

Valéria Quintana Cavicchioli: Conceptualization, Writing - Review & Editing, Visualization, Supervision. **Almir Custodio Batista Junior**: Methodology, Validation, Formal analysis, Writing - Review & Editing, Visualization. **João Victor Ataíde Oliveira**: Methodology, Validation, Formal analysis, Writing - Review & Editing, Visualization. **Andréa Rodrigues Chaves:** Methodology, Writing - Review & Editing, Visualization, Supervision. **Cíntia Silvia Minafra e Rezende:** Writing - Review & Editing, Visualization, Supervision. **Cinzia Caggia**: Conceptualization, Supervision, Writing - Review & Editing, Visualization. **Cinzia Lucia Randazzo**: Conceptualization, Writing - Review & Editing, Visualization. **Antonio Fernandes de Carvalho**: Conceptualization, Supervision, Writing - Review & Editing, Visualization. **Luís Augusto Nero**: Conceptualization, Resources, Writing - Review & Editing, Visualization, Supervision, Project administration, Funding acquisition. All authors read and approved the final manuscript.

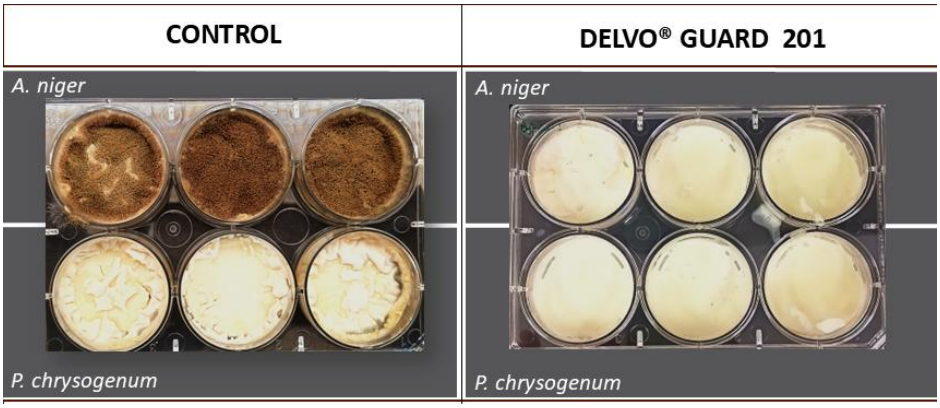
Declaration of competing interest

The authors have no competing interests to declare.

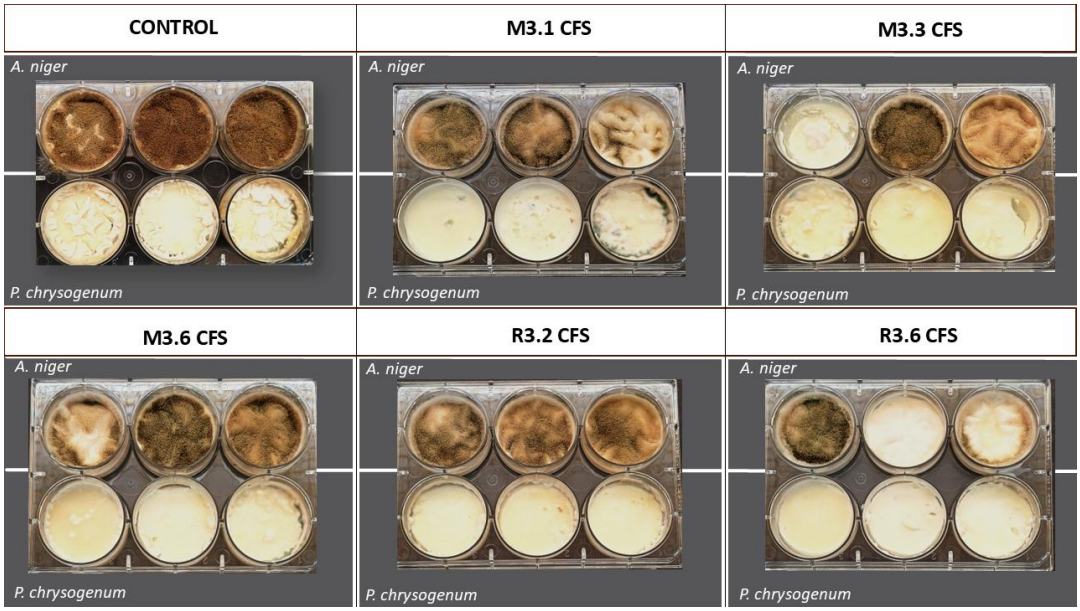
Acknowledgments

The authors are thankful to Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES, Brasília, DF, Brazil, Financial code 001), Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq, Brasília, DF, Brazil), Fundação de Amparo à Pesquisa do Estado de Minas Gerais (FAPEMIG, Belo Horizonte, MG, Brazil), Center for Microscopy and Microanalysis (UFV) and the Chromatography and Mass Spectrometry Laboratory at the Federal University of Goiás (UFG) for their support and laboratory techniques.

Supplementary material



S1. Antifungal effect of the commercial bioprotective culture Delvo® Guard 201 (containing *Lactobacillus rhamnosus* and *Lactobacillus sakei*) by DSM Food Specialties Company against *A. niger* (IOC 207) and *P. chrysogenum* (IOC 132) in a model matrix under simulated cheese conditions after 7 days at 30°C. Each treatment was performed in triplicate for each fungus tested.



S2. Antifungal effect of CFS of *L. plantarum* strains (M3.1, M3.3, M3.6, R3.2 and R3.6) against *A. niger* (IOC 207) and *P. chrysogenum* (IOC 132) in matrix under simulated cheese conditions after 7 days at 30°C. Each treatment was performed in triplicate for each fungus tested

6.References

Adithi, G., Somashekaraiah, R., Divyashree, S., Shruthi, B., & Sreenivasa, M. Y. (2022). Assessment of probiotic and antifungal activity of *Lactiplantibacillus plantarum* MYSAGT3 isolated from locally available herbal juice against mycotoxigenic *Aspergillus* species. *Food Bioscience*, 50, 102118. https://doi.org/10.1016/j.fbio.2022.102118

Bojarska, J., Mieczkowski, A., Ziora, Z. M., Skwarczynski, M., Toth, I., Shalash, A. O., ... & Wolf, W. M. (2021). Cyclic dipeptides: The biological and structural landscape with special focus on the anti-cancer proline-based scaffold. *Biomolecules*, *11*(10), 1515. https://doi.org/10.3390/biom11101515

Chen, Y. Y., Liang, N. Y., Curtis, J. M., & Gänzle, M. G. (2016). Characterization of linoleate 10-hydratase of *Lactobacillus plantarum* and novel antifungal metabolites. *Frontiers in Microbiology*, *7*, 1561. https://doi.org/10.3389/fmicb.2016.01561

Cheong, E. Y., Sandhu, A., Jayabalan, J., Le, T. T. K., Nhiep, N. T., Ho, H. T. M., ... & Turner, M. S. (2014). Isolation of lactic acid bacteria with antifungal activity against the common cheese spoilage mould *Penicillium commune* and their potential as biopreservatives in cheese. *Food Control*, 46, 91-97. https://doi.org/10.1016/j.foodcont.2014.05.011

De Simone, N., López, L., Ciudad, C. S., Scauro, A., Russo, P., Rodríguez, J., ... & Martínez, B. (2024). Antifungal activity of *Lactiplantibacillus plantarum* isolated from fruit and vegetables and detection of novel antifungal VOCs from fungal-LAB co-cultures. *Food Bioscience*, 58, 103824. https://doi.org/10.1016/j.fbio.2024.103824

Digital Science. (2025). Dimensions [Digital Science platform] available from https://www.dimensions.ai (accessed 13 April 2025).

Echegaray, N., Yilmaz, B., Sharma, H., Kumar, M., Pateiro, M., Ozogul, F., & Lorenzo, J. M. (2023). A novel approach to *Lactiplantibacillus plantarum*: From probiotic properties to the omics insights. *Microbiological Research*, 268, 127289. https://doi.org/10.1016/j.micres.2022.127289

Garnier, L., Salas, M. L., Pinon, N., Wiernasz, N., Pawtowski, A., Coton, E., ... & Valence, F. (2018). High-throughput method for antifungal activity screening in a cheese-mimicking model. *Journal of Dairy Science*, 101(6), 4971-4976. https://doi.org/10.3168/jds.2017-13518

Garnier, L., Valence, F., & Mounier, J. (2017). Diversity and control of spoilage fungi in dairy products: An update. *Microorganisms*, 5(3), 42.https://doi.org/10.3390/microorganisms5030042

Graham, A. E., & Ledesma-Amaro, R. (2023). The microbial food revolution. Nature communications, 14(1), 2231. https://doi.org/10.1038/s41467-023-37891-1

Granato, D., Santos, J. S., Escher, G. B., Ferreira, B. L., & Maggio, R. M. (2018). Use of principal component analysis (PCA) and hierarchical cluster analysis (HCA) for multivariate association between bioactive compounds and functional properties in foods: A critical perspective. *Trends in Food Science & Technology*, *72*, 83-90. https://doi.org/10.1016/j.tifs.2017.12.006

Guimarães, A., Venancio, A., & Abrunhosa, L. (2018). Antifungal effect of organic acids from lactic acid bacteria on *Penicillium nordicum*. *Food Additives & Contaminants: Part A*, *35*(9), 1803-1818. https://doi.org/10.1080/19440049.2018.1500718

Guimarães, A., & Venâncio, A. (2022). The potential of fatty acids and their derivatives as antifungal agents: A review. *Toxins*, *14*(3), 188. https://doi.org/10.3390/toxins14030188

Guimarães, A., Santiago, A., Teixeira, J. A., Venâncio, A., & Abrunhosa, L. (2018). Anti-aflatoxigenic effect of organic acids produced by *Lactobacillus plantarum*. *International Journal of Food Microbiology*, 264, 31-38. https://doi.org/10.1016/j.ijfoodmicro.2017.10.025

Kaddouri, A. C., ElOirdi, S., Lakhlifi, T., Miyah, Y., Benjelloun, M., Zakya, M., Belhaj, A. (2025). Isolation and characterization of three *Lactiplantibacillus plantarum* strains with high activity and broad antifungal spectrum. *Natural Resources for Human Health*, 5(2), 196-208.https://doi.org/10.53365/nrfhh/199365

Kanjan, P., & Sakpetch, P. (2023). Effect of antifungal compounds secreted by *Lactiplantibacillus plantarum* 124 against *Aspergillus flavus* and *Penicillium* sp. and its application in Kaeng-Tai-Pla-Haeng to extend the shelf life. International Journal of Food Science & Technology, 58(10), 5376-5387. https://doi.org/10.1111/ijfs.16656

Kwak, M. K., Liu, R., Kim, M. K., Moon, D., Kim, A. H., Song, S. H., & Kang, S. O. (2014). Cyclic dipeptides from lactic acid bacteria inhibit the proliferation of pathogenic fungi. *Journal of Microbiology*, *52*(1), 64-70. https://doi.org/10.1007/s12275-014-3520-7

Leyva Salas, M., Thierry, A., Lemaitre, M., Garric, G., Harel-Oger, M., Chatel, M., ... & Coton, E. (2018). Antifungal activity of lactic acid bacteria combinations in dairy mimicking models and their potential as bioprotective cultures in pilot scale applications. *Frontiers in Microbiology*, 9, 1787. https://doi.org/10.3389/fmicb.2018.01787

Li, Q., Zeng, X., Fu, H., Wang, X., Guo, X., & Wang, M. (2023). *Lactiplantibacillus plantarum*: A comprehensive review of its antifungal and anti-mycotoxic effects. *Trends in Food Science & Technology*, 136, 224-238. https://doi.org/10.1016/j.tifs.2023.04.019

Iimura, K., Furukawa, T., Yamamoto, T., Negishi, L., Suzuki, M., & Sakuda, S. (2017). The mode of action of cyclo (L-Ala-L-Pro) in inhibiting aflatoxin production of *Aspergillus flavus*. Toxins, 9(7), 219. https://doi.org/10.3390/toxins9070219

Liu, S., Ruan, W., Li, J., Xu, H., Wang, J., Gao, Y., & Wang, J. (2008). Biological control of phytopathogenic fungi by fatty acids. *Mycopathologia*, *166*(2), 93-102. https://doi.org/10.1007/s11046-008-9124-1

Liu, A., Xu, R., Zhang, S., Wang, Y., Hu, B., Ao, X., ... & Liu, S. (2022). Antifungal mechanisms and application of lactic acid bacteria in bakery products: a review. *Frontiers in Microbiology*, 13, 924398. https://doi.org/10.3389/fmicb.2022.924398

Ma, M., Li, A., Feng, J., Wang, Z., Jia, Y., Ma, X., & Ning, Y. (2024). Antifungal mechanism of *Lactiplantibacillus plantarum* P10 against *Aspergillus niger* and its in-situ biopreservative application in Chinese steamed bread. *Food Chemistry*, 449, 139181.

https://doi.org/10.1016/j.foodchem.2024.139181

Madigan, M. T., Bender, K. S., Buckley, D. H., Sattley, W. M., & Stahl, D. A. (2021). Brock Biology of Microorganisms (16th ed.). Pearson.

Magnusson, J., Ström, K., Roos, S., Sjögren, J., & Schnürer, J. (2003). Broad and complex antifungal activity among environmental isolates of lactic acid bacteria. *FEMS microbiology letters*, *219*(1), 129-135. https://doi.org/10.1016/S0378-1097(02)01207-7

Mieszkin, S., Hymery, N., Debaets, S., Coton, E., Le Blay, G., Valence, F., & Mounier, J. (2017). Action mechanisms involved in the bioprotective effect of *Lactobacillus harbinensis* K. V9. 3.1. Np against *Yarrowia lipolytica* in fermented milk. *International Journal of Food Microbiology*, 248, 47-55. https://doi.org/10.1016/j.ijfoodmicro.2017.02.013

Ming, T., Han, J., Li, Y., Lu, C., Qiu, D., Li, Y., ... & Su, X. (2018). A metabolomics and proteomics study of the *Lactobacillus plantarum* in the grass carp fermentation. *BMC Microbiology*, 18, 1-13. https://doi.org/10.1186/s12866-018-1354-x

Muhialdin, B. J., Hassan, Z., Bakar, F. A., & Saari, N. (2016). Identification of antifungal peptides produced by *Lactobacillus plantarum* IS10 grown in the MRS broth. *Food Control*, 59, 27-30. https://doi.org/10.1016/j.foodcont.2015.05.022

Murtagh, F., & Contreras, P. (2012). Algorithms for hierarchical clustering: an overview. *Wiley interdisciplinary reviews: data mining and knowledge discovery*, *2*(1), 86-97. https://doi.org/10.1002/widm.53

Peng, Q., Yang, J., Wang, Q., Suo, H., Hamdy, A. M., & Song, J. (2023). Antifungal effect of metabolites from a new strain *Lactiplantibacillus plantarum* LPP703 isolated from naturally fermented Yak Yogurt. *Foods*, 12(1), 181. https://doi.org/10.3390/foods12010181

Pitt J, Hocking A (2022) Fungi and Food Spoilage. https://doi.org/10.1007/ 978-3-030-85640-3

Prabawati, E. K., Turner, M. S., & Bansal, N. (2023). *Lactiplantibacillus plantarum* as an adjunct culture exhibits antifungal activity in shredded Cheddar cheese. *Food Control*, 144, 109330. https://doi.org/10.1016/j.foodcont.2022.109330

Rouxel, M., Barthe, M., Marchand, P., Juin, C., Mondamert, L., Berges, T., Blanc, P., Verdon, J., Berjeaud, JM & Aucher, W. (2020). Characterization of antifungal compounds produced by lactobacilli in cheese-mimicking matrix: Comparison between active and inactive strains. *International Journal of Food Microbiology*, 333, 108798. https://doi.org/10.1016/j.ijfoodmicro.2020.108798

RStudio Team (2019). RStudio: Integrated Development for R. RStudio, Inc., Boston, MA URL. http://www.rstudio.com/

Roy, U., Batish, V. K., Grover, S., & Neelakantan, S. (1996). Production of antifungal substance by *Lactococcus lactis* subsp. *lactis* CHD-28.3. *International Journal of Food Microbiology*, 32(1-2), 27-34. https://doi.org/10.1016/0168-1605(96)01101-4

Russo, P., Arena, M. P., Fiocco, D., Capozzi, V., Drider, D., & Spano, G. (2017). *Lactobacillus plantarum* with broad antifungal activity: A promising approach to increase safety and shelf-life of cereal-based products. *International Journal of Food Microbiology*, 247, 48-54. https://doi.org/10.1016/j.ijfoodmicro.2016.04.027

Sadiq, F. A., Yan, B., Tian, F., Zhao, J., Zhang, H., & Chen, W. (2019). Lactic acid bacteria as antifungal and anti-mycotoxigenic agents: a comprehensive review. *Comprehensive Reviews in Food Science and Food Safety*, 18(5), 1403-1436. https://doi.org/10.1111/1541-4337.12481

Siedler, S., Balti, R., & Neves, A. R. (2019). Bioprotective mechanisms of lactic acid bacteria against fungal spoilage of food. *Current Opinion in Biotechnology*, 56, 138-146. https://doi.org/10.1016/j.copbio.2018.11.015

Souza, L. V., da Silva, R. R., Falqueto, A., Fusieger, A., Martins, E., Caggia, C., ... & de Carvalho, A. F. (2023). Evaluation of antifungal activity of lactic acid bacteria against fungi in simulated cheese matrix. *LWT*, 182, 114773. https://doi.org/10.1016/j.lwt.2023.114773

Souza, L. V, Rodrigues, R. R, Fusieger, A., Ribeiro, L. O. T., Nicosia, F. D., Cavicchioli, V. Q., ... & Nero, L. A. (2025). Selection of Lactic Acid Bacteria from Dairy Matrices with Antifungal Activity to be Used as Biopreservative Adjunct Cultures. *Food Biotechnology*, 39(2), 185-207. https://doi.org/10.1080/08905436.2025.2501215

Souza, L. V., da SILVA, R. R., Cavicchioli, V. Q., de Melo TAVARES, R., Randazzo, C. L., Caggia, C., ... & Nero, L. A. (2025). The duality of filamentous fungi: Beneficial uses and risks in the food industry. *Food Research International*, *220*, 117191. https://doi.org/10.1016/j.foodres.2025.117191

Ström, K., Sjögren, J., Broberg, A., & Schnürer, J. (2002). *Lactobacillus plantarum* MiLAB 393 produces the antifungal cyclic dipeptides cyclo (L-Phe-L-Pro) and cyclo (L-Phe-trans-4-OH-L-Pro) and 3-phenyllactic acid. *Applied and environmental microbiology*, *68*(9), 4322-4327. https://doi.org/10.1128/AEM.68.9.4322-4327.2002

Vanitha, P. R., Somashekaraiah, R., Divyashree, S., Pan, I., & Sreenivasa, M. Y. (2023). Antifungal activity of probiotic strain *Lactiplantibacillus plantarum* MYSN7 against *Trichophyton tonsurans*. *Frontiers in Microbiology*, 14, 1192449. https://doi.org/10.3389/fmicb.2023.1192449

Vasundaradevi, R., Sarvajith, M., & Somashekaraiah, R. (2024). Antagonistic properties of *Lactiplantibacillus plantarum* MYSVB1 against *Alternaria alternata*: a putative probiotic strain isolated from the banyan tree fruit. *Frontiers in Microbiology* 15: 1322758. https://doi.org/10.3389/fmicb.2024.1322758

Vasundaradevi, R., Sarvajith, M., Divyashree, S., Deepa, N., Achar, P. N., & Sreenivasa, M. Y. (2025). Tropical fruit-derived *Lactiplantibacillus* as potential probiotic and antifungal agents against *Fusarium oxysporum*. *Scientific Reports*, 15(1), 2144. https://doi.org/10.1038/s41598-025-85190-0

Vela-Corcia, D., Hierrezuelo, J., Pérez-Lorente, A. I., Stincone, P., Pakkir Shah, A. K., Grélard, A., ... & Romero, D. (2024). Cyclo (Pro-Tyr) elicits conserved cellular damage in fungi by

targeting the [H+] ATPase Pma1 in plasma membrane domains. *Communications biology*, 7(1), 1253. https://doi.org/10.1038/s42003-024-06947-3

Wang, A., Guan, C., Wang, T., Mu, G., & Tuo, Y. (2023). Indole-3-lactic acid, a tryptophan metabolite of *Lactiplantibacillus plantarum* DPUL-S164, improved intestinal barrier damage by activating AhR and Nrf2 signaling pathways. *Journal of Agricultural and Food Chemistry*, *71*(48), 18792-18801. https://doi.org/10.1021/acs.jafc.3c06183

Wang, M., Carver, J. J., Phelan, V. V., Sanchez, L. M., Garg, N., Peng, Y., ... & Bandeira, N. (2016). Sharing and community curation of mass spectrometry data with Global Natural Products Social Molecular Networking. *Nature biotechnology*, 34(8), 828-837. https://doi.org/10.1038/nbt.3597

Wang, Q., Zhang, F., Zhang, Y., Zhang, Y., Wang, H., Song, J., & Suo, H. (2024). A novel strain *Lactiplantibacillus plantarum* LPP95 isolated from Chinese pickles: Antifungal effect, mechanism, and potential application in yogurt. *Food Bioscience*, 58, 103640. https://doi.org/10.1016/j.fbio.2024.103640

Wu, X., Liu, Y., Guo, Z., Ji, N., Sun, Q., Liu, T., & Li, Y. (2025). Antifungal activities of *Pediococcus pentosaceus* LWQ1 and *Lactiplantibacillus plantarum* LWQ17 isolated from sourdough and their efficacies on preventing spoilage of Chinese steamed bread. *Food Control*, 168, 110940. https://doi.org/10.1016/j.foodcont.2024.110940

Yilmaz, B., Bangar, S. P., Echegaray, N., Suri, S., Tomasevic, I., Manuel Lorenzo, J., ... & Ozogul, F. (2022). The impacts of *Lactiplantibacillus plantarum* on the functional properties of fermented foods: A review of current knowledge. *Microorganisms*, 10(4), 826. https://doi.org/10.3390/microorganisms10040826

Zha, M., Li, J., Zhu, S., Huang, W., Sun, Z., Xia, Y., & Chen, Y. (2024). Selection and analysis of the characteristics of *Lactiplantibacillus plantarum* with antifungal activity. *LWT*, 202, 116277. https://doi.org/10.1016/j.lwt.2024.116277

Zheng, J., Wittouck, S., Salvetti, E., Franz, C. M., Harris, H. M., Mattarelli, P., ... & Lebeer, S.

(2020). A taxonomic note on the genus *Lactobacillus*: Description of 23 novel genera, emended description of the genus *Lactobacillus* Beijerinck 1901, and union of *Lactobacillaceae* and *Leuconostocaceae*. *International Journal of Systematic and Evolutionary Microbiology*, 70(4), 2782-2858. https://doi.org/10.1099/ijsem.0.004107

CHAPTER 5

5.FOURTH PAPER

FOURTH PAPER - Comparative genomics of antifungal *Lactiplantibacillus plantarum* strains: Insights into technological, safety, virulence and flavor production in fermented milk

Luana Virgínia Souza et al.,

Manuscript to be submitted in Food Microbiology *2026*

Comparative genomics of antifungal *Lactiplantibacillus plantarum* strains: Insights into technological, safety, virulence and flavor production in fermented milk

Luana Virgínia Souza[a,b], Rafaela da Silva Rodrigues[a], Fabrizio Cincotta[d], Ricardo Seiti Yamatogi[a], Luigi Liotta[d], Cinzia Lucia Randazzo[b], Cinzia Caggia[b], Antonio Fernandes de Carvalho [b,c], Luís Augusto Nero [a,b]

[a] InsPOA - Laboratório de Inspeção de Produtos de Origem Animal, Departamento de Veterinária, Universidade Federal de Viçosa, 36570 900, Viçosa, MG, Brazil

[b] Di3A - Dipartimento di Agricoltura, Alimentazione e Ambiente, Università degli Studi di Catania, 95123, Catania, CT, Italy

[c] InovaLeite - Laboratório de Pesquisa em Leites e Derivados, Departamento de Tecnologia de Alimentos, Universidade Federal de Viçosa, 36570 900, Viçosa, MG, Brazil

[d]Dipartimento di Scienze Veterinarie, Università degli Studi di Messina, 98122, Messina, ME, Italy

*Corresponding author: Luís Augusto Nero. InsPOA - Laboratório de Inspeção de Produtos de Origem Animal, Departamento de Veterinária, Universidade Federal de Viçosa, 36570 900, Viçosa, MG, Brazil.

E-mail address: nero@ufv.br (L. A. Nero)

Abstract

Lactiplantibacillus plantarum has attracted considerable attention due to its technological potential in fermented foods, including antimicrobial activity and the production of flavor and aroma compounds. However, despite being extensively studied, each strain exhibits distinct metabolic and functional profiles, highlighting the importance of genomic characterization to guide their effective application in food systems. In this study, the genomes of five *L. plantarum* strains with proven antifungal activity were analyzed to assess their safety, presence of virulence factors, and technological potential. Genome sizes ranged from 3.20 to 3.27 Mb, with GC contents of approximately 44.5%, and no virulence or antimicrobial resistance genes were detected, except for intrinsic vancomycin resistance. Comprehensive genome mining identified bacteriocin areas of interest (AOIs) corresponding to plantaricin and sactipeptide clusters, as well as terpene, RiPP-like, and type III polyketide synthase (T3PKS) biosynthetic gene clusters. Additionally, a wide range of carbohydrate-active enzymes (CAZymes) belonging to GH, GT, CE, and AA families were detected, supporting the strains' versatile carbohydrate metabolism. To evaluate their functional expression in a dairy matrix, volatile metabolites produced during milk fermentation were analyzed by GC–MS. Significant strain-dependent differences were observed in ketones, alcohols, esters, and organic acids. Strain M3.1 produced higher levels of alcohols and esters, associated with fruity and aromatic notes, whereas M3.3 showed increased ketones and acetic acid, related to buttery, creamy, and acidic attributes. Overall, the integration of genomic and metabolomic analyses highlights the safety, metabolic versatility, and technological potential of these strains as promising adjunct cultures for flavor development and biopreservation in dairy fermentations.

Keywords: *Lactiplantibacillus plantarum*; aromatic compounds, safety; mining genome, technological.

1. Introduction

Lactic acid bacteria (LAB) are attracting growing interest in the food science and biotechnology sectors due to their multifaceted functional and technological potential. These microorganisms serve not only as agents of biopreservation, by inhibiting or reducing contamination from pathogenic and spoilage microorganisms, including bacteria and filamentous fungi, but also can be a key contributors to human health through probiotic actions and to food processing through desirable technological traits (Adesulu-Dahunsi et al., 2018; Ağagündüz et al., 2022; De Simone et al., 2024). Among LAB species, *Lactiplantibacillus plantarum* (formerly *Lactobacillus plantarum*) stands out for its broad repertoire of functional and technological features, making it an excellent candidate for applications in fermented foods, and in particular in dairy systems (Costa et al., 2024, Cai et al., 2024).

In human health contexts, *L. plantarum* has been widely studied for its probiotic potential and its ability to influence lipid metabolism, among other benefits. For example, supplementation with *L. plantarum* has demonstrated reductions in total cholesterol, LDL-cholesterol and triglycerides in human and animal models (Zuo et al., 2025).

In addition to probiotic and health-promoting functions, *L. plantarum* exhibits antimicrobial capabilities. Notably, many strains harbour bacteriocin genes encoding plantaricins, ribosomally-synthesised antimicrobial peptides that can inhibit important foodborne pathogens such as *Listeria monocytogenes* and *Escherichia coli,* among others (Kawahara et al., 2022; Ruiz et al., 2023). More broadly, genomic surveys of *L. plantarum* have revealed that many strains carry multiple bacteriocin biosynthetic gene clusters (BGCs) including plantaricin loci, underscoring the wide antimicrobial potential of this species (Choi et al., 2021).

Besides producing bacteriocins, *L. plantarum* contributes to microbial antagonism and preservation through metabolic acidification and the secretion of a diverse range of organic acids, such as lactic, acetic, formic, phenyl-lactic, butyric, citric, succinic, among others, which lower the pH and inhibit undesirable microorganisms (Souza et al., 2022). Notably, the accumulation of these organic acids has been linked to antifungal activity and the suppression of filamentous fungi in fermented matrices (Huang et al., 2024). Moreover, *L. plantarum* is

increasingly recognised for its antifungal activity. Recent studies show that this species can produce a range of compounds, acting alone or in combination,that inhibit moulds and yeasts, extending its usefulness beyond suppressing bacteria to supporting fungal biopreservation (De Simone et al., 2024, Souza et al., 2026).

L. plantarum has valuable technological traits for the food industry, including flavour and aroma production, coagulation activity, proteolytic and lipolytic functions, and versatile carbohydrate metabolism. These capabilities allow it to be used as a starter, adjunct, or protective culture in fermented foods (Tian et al., 2025). These traits making genomic characterization crucial that helps identify biosynthetic gene clusters, carbohydrate-active enzymes, and stress-resistance systems, enabling targeted exploitation of specific strains (Isaac et al., 2024).

Given the combination of antimicrobial, antifungal, functional, flavour/aroma and carbohydrate-metabolism traits, *L. plantarum* appears particularly well suited for application in fermented dairy systems, not only to ensure bioprotection, but also to contribute to the sensory and functional quality of the final product.Within this context, this study aims to expand the genomic and functional understanding of five *L. plantarum* strains with known antifungal activity by assessing their safety, biosynthetic potential, and metabolite production in fermented milk. By integrating genomic and metabolomic analyses, the work provides insights to guide the selection of strains for bioprotective/adjunct applications in dairy fermentations, linking strain characteristics also to their technological potential.

2. Materials and methods

2.1. Bacterial strains and culture condition

Five *L. plantarum* strains (M3.1, M3.3, M3.6, R3.2, and R3.6) from the Food Agricultural Microbiology Laboratory (Department of Agriculture, Food and Environment, University of Catania, Sicily, Italy) were used in this study. These isolates had been previously identified and characterized according to Souza et al. (2025) and are well recognized for their antifungal activity, as reported by Souza et al. (2026). For whole genome sequencing and

inoculation in fermented milk, cultures from the stock were grown in MRS broth (Kasvi, São José dos Pinhais, PR, Brazil) and incubated at 37 °C for 24 h.

2.2. DNA extraction, whole genome sequencing and assembly

The *L. plantarum* strains were first activated at 37 °C for 24 h, subsequently centrifuged (6,500 × g, 5 min), and the resulting pellets were subjected to DNA extraction using the MagMAX™ CORE kit (Thermo Fisher Scientific), following the manufacturer's instructions with slight modifications. For each strain, 350 µL MagMAX™ CORE Lysis Solution, 350 µL MagMAX™ CORE Binding Solution, 10 µL Proteinase K (20 mg/mL), and 20 µL Magnetic Beads were added, and samples were vortexed until clarification. Beads were washed twice (Wash Solutions 1 and 2), the supernatant was discarded, and beads were air-dried. DNA was then eluted with 50 µL MagMAX™ CORE Elution Buffer and homogenized. Sequencing libraries were prepared using the Nextera® XT DNA Library Preparation Kit (Illumina, San Diego, CA, USA). Whole-genome sequencing was performed on the Illumina MiSeq® platform (2 × 150 bp paired-end reads) using the V3-600 cycle configuration. The sequenced genomic data were analyzed and assembled using the open-source, online bioinformatics platform Galaxy (version 5.3.0; https://usegalaxy.org/). The following tools were used: FastQC (Galaxy Version 0.74+galaxy1) for read quality control; Trimmomatic (Galaxy Version 0.39+galaxy2) for trimming; Unicycler pipeline for bacterial genome assembly (Galaxy Version 0.5.1+galaxy0); checkM assign taxonomic labels to sequencing reads (Galaxy Version 2.1.3+galaxy2) and Prokka (Galaxy Version 1.14.6+galaxy1) for gene annotation. The draft genome sequences were deposited in GenBank.

2.3. Functional annotation, identification and phylogenomic analysis

Functional annotation and metabolic pathway prediction were performed using the Rapid Annotation using Subsystem Technology (RAST) server (https://rast.nmpdr.org/rast.cgi) and BlastKOALA (KEGG Orthology and Links Annotation) (https://www.kegg.jp/blastkoala/). Taxonomic identification was performed using the Type Strain Genome Server (Meier-Kolthoff et al., 2019). Whole-genome comparisons of *L. plantarum* strains M3.1, M3.3, M3.6, R3.2, and R3.6 were performed against the reference genome *L. plantarum* subsp. *plantarum*

ATCC 14917 using the BLAST Ring Image Generator (BRIG, version 3.0). Genomes were aligned with BLASTn, and concentric colored rings were generated to depict homologous regions relative to the reference. Shading intensity within each ring represented different nucleotide identity thresholds (70–95%), enabling visualization of conserved and variable genomic regions among the strains. Orthologous cluster identification algorithm and visualization Identifying orthologous clusters was performed using OrthoVenn3 (https://orthovenn3.bioinfotoolkits.net/home) to compare the evolutionary relationships between strains.

The phylogenomic analysis included the five strains of *L. plantarum* together with the type strain *L. plantarum* subsp. *plantarum* ATCC 14917 (GCF_000143745.1). To root the tree, *Lactiplantibacillus paraplantarum* DSM 10667^{T} (GCF_001435655.1) was used as outgroup. Initially, the GFF files generated by Prokka annotation were used for pangenome analysis with Roary v3.13.0, employing MAFFT for multiple sequence alignment (Page et al. 2015). Maximum-likelihood inference was conducted in IQ-TREE v2.3.4, based on the core genome alignment, with 1,000 ultrafast bootstrap replicates (Nguyen et al. 2015). The best-fit substitution model (GTR+I+G4) was determined using ModelTest-NG v0.1.7 (Darriba et al. 2020). The final tree was visualized and edited in the Interactive Tree of Life (iTOL) v7 (Letunic and Bork 2024).

2.4. Safety and Virulence Gene Annotation

The draft genome sequences of *L. plantarum* were screened for antimicrobial resistance gene to verify the safety of the strains using Resfinder (Camacho et al., 2009; Bortolaia et al., 2020) and Comprehensive Antibiotic Resistance Database – CARD (https://card.mcmaster.ca/analyze/rgi). Additionally, the genomes were evaluated for their potential pathogenicity using the PathogenFinder web server (http://cge.cbs.dtu.dk/services/PathogenFinder/). The identification and annotation of prophage sequences in bacterial genomes and plasmids was also evaluated using the PHAge Search Tool Enhanced Release – Phaster (https://phaster.ca/) (Zhou et al., 2011; Arndt et al., 2016).

2.5. Technological Traits Gene

The tools used for the technological analyses were: BAGEL4 and antiSMASH 7.0, which were employed to predict the identification of putative gene clusters responsible for the synthesis of bacteriocin clusters and secondary metabolites, respectively (van Heel et al., 2018; Blin et al., 2023). Additional functional analyses were performed using the CAZy database (https://www.cazy.org/), which classifies families of structurally related catalytic and carbohydrate-binding modules present in enzymes involved in the degradation, modification, and synthesis of glycosidic bonds (Drula et al., 2022). Carbohydrate-active enzymes (CAZymes) were identified through the dbCAN server (https://bcb.unl.edu/dbCAN2/), using HMMER searches against the dbCAN and dbCAN_sub databases, the latter being a comprehensive resource of CAZyme subfamilies specifically developed for substrate annotation (Zhang et al., 2018*b*).

2.6. Screening of volatile aroma compounds

The capacity of *L. plantarum* strains to produce volatile organic compounds (VOCs) was assessed and for this purpose, a fermented milk was prepared as described below, allowing the evaluation of metabolic differences among strains and the complexity of their metabolism in the milk matrix.

2.6.1. Preparation of Fermented Milk

Pasteurized milk (100 mL; 90 °C, 5 min) supplemented with 20% (w/v) skim milk powder (Nestlé) served as the fermentation substrate. The five *L. plantarum* strains (M3.1, M3.3, M3.6, R3.2 and R3.6) were inoculated at approximately 10^3 CFU/mL. The homogenized milk was aliquoted into triplicate tubes and incubated at 30 °C for 48 h to allow fermentation.

2.6.2. Volatile Organic compounds (VOCs) in Fermented Milk Analyzed by GC-MS

VOCs of unfermented and fermented milk samples were analyzed by headspace solid-phase microextraction coupled with gas chromatography–mass spectrometry (HS-SPME–GC–MS). Eighteen mL of each sample were placed in a 40 mL glass vial and equilibrated for 30

minutes at 35 °C. Then, a triphasic DVB/CAR/PDMS (divinylbenzene/carboxen/polydimethylsiloxane) fiber was exposed to the headspace for 30 min and subsequently desorbed for 3 min in the GC injector maintained at 260 °C.

GC-MS analyses were performed using a Shimadzu GC-2010 Plus system coupled to a TQMS 8040 triple-quadrupole mass spectrometer (Shimadzu, Milan, Italy), equipped with a Vf-WAX-ms capillary column (60 m × 0.25 mm i.d., 0.25 µm film thickness). Separation was performed under the following chromatographic conditions: injector temperature 260 °C in splitless mode; oven program starting at 45 °C (held for 5 min), ramped to 80 °C at 5 °C/min, then to 150 °C at 2 °C/min, then to 240 °C at 10 °C/min and held for 15 min; carrier gas helium at a constant flow of 1 mL/min; transfer line temperature at 250 °C; mass acquisition range 40-300 m/z, scan speed 1250 amu/s; Electron Ionization (EI) at 70 eV.

Compound identification was based on comparison of the obtained mass spectra with those in the NIST 2024 (NIST/EPA/NIH, Wiley, USA) and FFNSC 3.0 libraries, and by linear retention indices (LRI) calculated according to the van den Dool and Kratz equation, literature data, and injection of authentic standards when available. The relative abundance of each volatile compound was expressed as absolute peak area from three replicate determinations.

3. Results and Discussion

3.1. Genome features of L. plantarum strains

The whole genome sequencing of the strains M3.1, M3.3, M3.6, R3.2 and R3.6 demonstrate some genomic characteristics in common with some differences in comparison as shown in table 1. The GC content was 44.5% for strains M3.1, M3.3, and R3.2, and 44.4% for strains M3.6 and R3.6. The number of contigs ranged from 46 to 171, with the highest values observed for strains M3.3 and M3.6 (142 and 171, respectively).

Table 1. Genome characteristics of *L.plantarum* strains

Key atributes	M3.1	M3.3	M3.6	R3.2	R3.6
% GC	44.5	44.5	44.4	44.5	44.4
Seq Length (bp)	3,269,683	3,250,201	3,227,078	3,267,144	3,205,294
N50	346307	59255	43473	346306	148800
L50	4	16	21	4	7
Number of Contigs (PEGs)	46	142	171	49	61
Number of Subsystems	215	216	213	216	197
Number of Coding Sequences	3208	3232	3241	3205	3157
Number of RNAs	63	61	59	62	63
Genbank Acession No.	xx	xx	xx	xx	xx

The total genome size varied between 3,205,294 and 3,269,683 bp, while the number of coding sequences (CDS) ranged from 3,205 to 3,241. Despite minor variations among the sequenced *L. plantarum* strains, the genomic features obtained are consistent with those reported in the National Center for Biotechnology Information (NCBI) database to date, including annotated genomes and excluding atypical genomes and metagenome-assembled genomes (MAGs). The database currently lists approximately 1,697 *L. plantarum* genomes, with an average genome size of 3.2 to 3.4 Mb and a GC content of 44.4 to 44.5%, although most genomes show a GC content of 44.5% (NCBI, 2026).

The comparison of the genomes, as illustrated by the BRIG-generated figure, revealed that all strains share a conserved genomic backbone with *Lactiplantibacillus plantarum* subsp. *plantarum* ATCC 14917, while also displaying strain-specific variable regions (Figure 1).

Among them, M3.1 and R3.2 showed the highest similarity to the reference genome, whereas M3.3, M3.6, and R3.6 contained more divergent genomic segments.

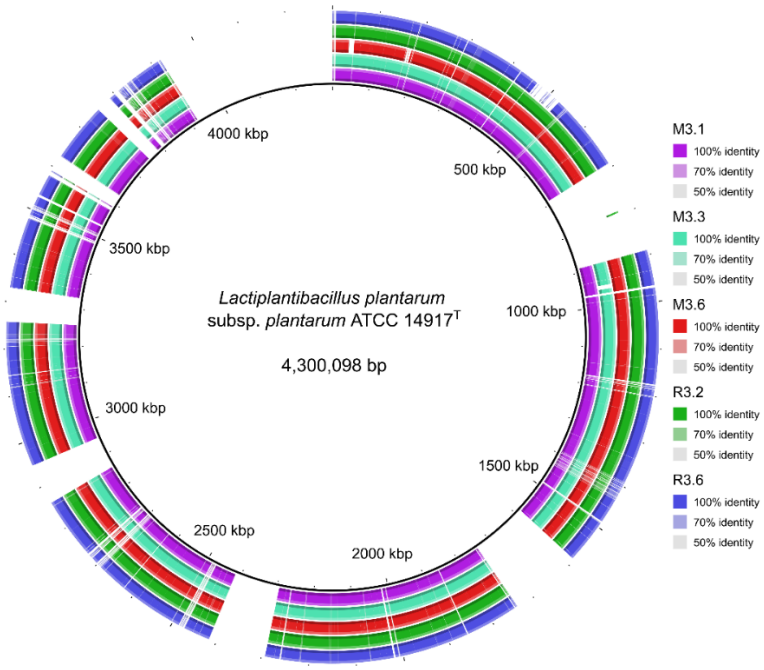


Figure 1. Genome comparison of strains M3.1, M3.3, M3.6, R3.2, and R3.6 against the genome *Lactiplantibacillus plantarum* subsp. *plantarum* ATCC 14917 using the BLAST Ring Image Generator (BRIG). Colored rings depict the aligned regions of each strain relative to the reference, with shading corresponding to different nucleotide identity thresholds.

The rooted phylogenetic tree, constructed using *Lactiplantibacillus paraplantarum* DSM 10667 as an outgroup, revealed that the five strains clustered within the same clade as the type strain *Lactiplantibacillus plantarum* subsp. *plantarum* ATCC 14917, confirming their identity as belonging to this species (Figure 2). Within this clade, the five strains formed a cluster supported by a bootstrap value of 100%, indicating a recent common ancestry. A subclade comprising strains M3.3 and R3.6 showed moderate support (68%), whereas another grouping containing strains R3.2 and M3.1, along with the position of M3.6, exhibited low support (25%), suggesting limited resolution of the internal relationships among the strains.

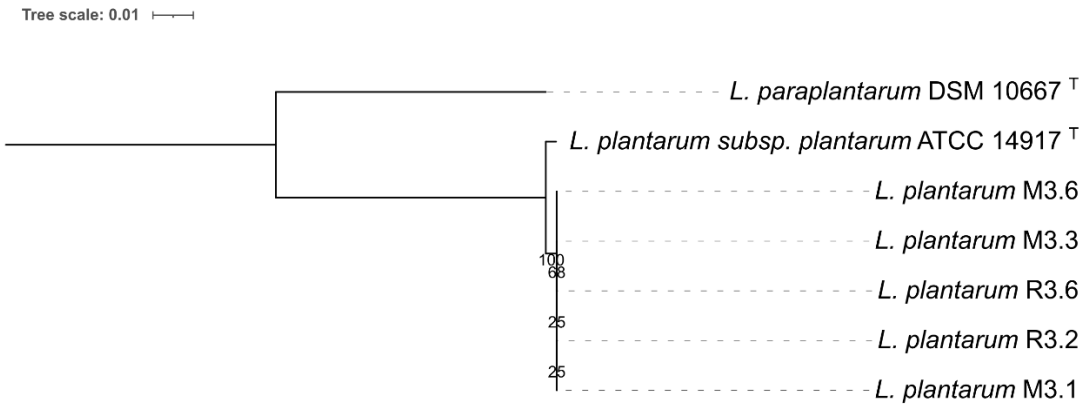


Figure 2. Maximum-likelihood phylogenomic tree based on the core genome alignment of five *Lactiplantibacillus plantarum* strains, the type strain *L. plantarum* subsp. *plantarum* ATCC 14917, and *L. paraplantarum* DSM 10667T as outgroup. Bootstrap values (1,000 replicates) are showed.

Corroborating the phylogenetic tree identification, a taxonomic comparison was also performed using the TYGS server. In addition to providing high accuracy in species identification, the server conducted comparisons with the reference strain *L. plantarum* DSM 20174. As shown in Table 2, digital DNA–DNA hybridization (dDDH) values above 70%, and even exceeding 90% as observed in our study, indicate a strong level of confidence in species identification. Furthermore, the small difference in GC content supports that the isolates are indeed *L. plantarum* strains. A G+C content difference greater than 1% is generally considered to indicate a potentially unreliable identification result when computed from genome sequences; therefore, the values obtained in our study, which are closer to 0%, confirm the accurate identification of the species.

Table 2. Genome-based taxonomic comparisons between *L. plantarum* strains on TYGS

Key atributes	M3.1	M3.3	M3.6	R3.2	R3.6
dDDH (d_0, in %)	89.7	89.2	89.2	89.7	87.8

dDDH (d_4, in %)	92.3	92.3	92.3	92.3	92.2
dDDH (d_6, in %)	92.7	92.3	92.2	92.7	91.2
Diff. G+C Percent	0.04	0.04	0.06	0.04	0.07

The orthology analysis performed with OrthoVenn3 enabled the comparative assessment of the predicted proteomes of the *L. plantarum* strains, providing insights into their genomic conservation and diversity. OrthoVenn3 groups protein-coding genes based on sequence similarity, allowing the identification of core and accessory gene clusters among the analyzed genomes. The analysis revealed a total of 2.888 orthologous clusters shared among all strains, representing the core genome of *L. plantarum* (Figure 3).

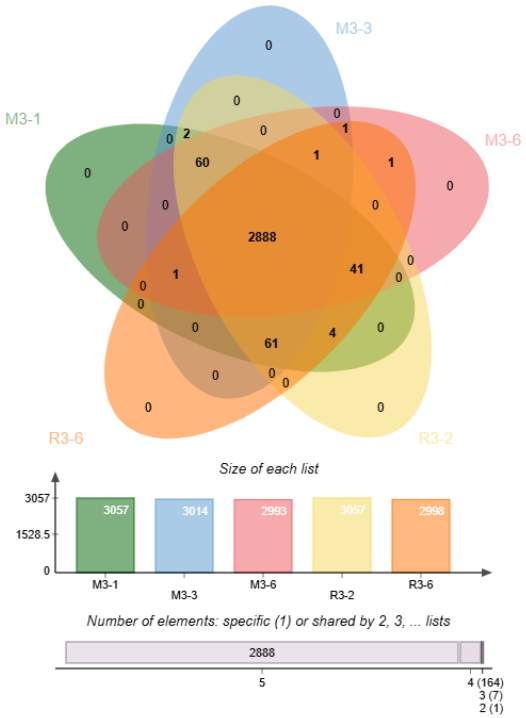


Figure 3. Comparative orthology analysis of *L. plantarum* strains performed using OrthoVenn3. The Vem diagram shows the distribution of orthologous gene clusters among the analyzed genomes.

This large proportion of conserved genes supports the high genomic stability characteristic of the species. In contrast, 8 or 7 clusters were found to be unique to individual strains, suggesting potential functional specialization. These unique genes may be associated with adaptation to specific ecological conditions, stress responses, or technological properties relevant to fermentation environments. Overall, the orthology-based comparison reinforces the view that *L. plantarum* possesses a highly conserved core genome complemented by a flexible accessory genome, which contributes to its remarkable ecological adaptability and metabolic versatility. These results provide a foundation for further comparative and functional analyses aiming to elucidate the genetic basis of phenotypic variation among strains.

3.2. Safety and Virulence Gene Annotation

In the platforms evaluated for the verification of acquired genes and/or chromosomal mutations mediating antimicrobial resistance, using ResFinder against several antibiotic classes, including aminoglycosides, aminocyclitols, quinolones, β-lactams, fosfomycin, glycopeptides, lincosamides, streptogramins, macrolides, tetracyclines, and ionophores, no resistance genes were detected. According to the Comprehensive Antibiotic Resistance Database (CARD) platform, which employs the Resistance Gene Identifier (RGI) to predict resistomes from protein, genome, or genome assembly data based on homology and SNP models, all genomes exhibited cluster gene for only to the *vanY* gene within the *vanB* (Vancomycin) under the RGI "strict" detection criterion, in "protein homolog" mode, showing a matching region of 31.93% and a reference sequence length coverage of 95.90%.

The intrinsic resistance to vancomycin observed in the *L. plantarum* is consistent with previous reports describing this characteristic as a natural and species-specific trait rather than an acquired resistance mechanism (Shao et al., 2015, Lu et al., 2025). This intrinsic resistance is associated with the structure of the peptidoglycan precursors in the bacterial cell wall, in which the terminal D-alanyl–D-alanine (D-Ala–D-Ala) dipeptide is replaced by D-alanyl–D-lactate (D-Ala–D-Lac). This substitution significantly reduces the affinity of vancomycin for its target, thereby rendering the bacterium naturally resistant to the antibiotic (Zhang et al., 2018 *a*). As a result, this resistance is considered intrinsic, non-transferable, and not of clinical concern according to the guidelines of the European Food Safety Authority (EFSA, 2018).

Therefore, the vancomycin resistance detected in the present *L. plantarum* strains likely reflects this inherent characteristic rather than the presence of acquired resistance genes.

According to the PathogenFinder2 platform, none of the isolates were predicted to possess pathogenic potential toward humans. The analysis did not identify any protein signatures or genomic features associated with pathogenicity, indicating that all isolates are likely non-pathogenic according to the model's prediction.

3.3. Technological Traits Gene

3.3.1. Bacteriocin gene predictions

Analysis of all *L. plantarum* genomes revealed the presence of bacteriocin gene clusters, as predicted by BAGEL. The prediction identified two areas of interest (AOI) in the *L. plantarum* genomes, corresponding to sactipeptides and plantaricins (Supplementary material S1 and S2). Regarding the plantaricin AOI, the genes detected within these clusters were associated with various functions, including biosynthesis, modification, immunity, transport, core peptide processing, regulation, and leader cleavage/protease activity (Figure 4). These genes were detected in all *L. plantarum* genomes analyzed (M3.1, M3.3, M3.6, R3.2, and R3.6). In contrast, the sactipeptide AOI was absent only in the genome of strain M3.6.

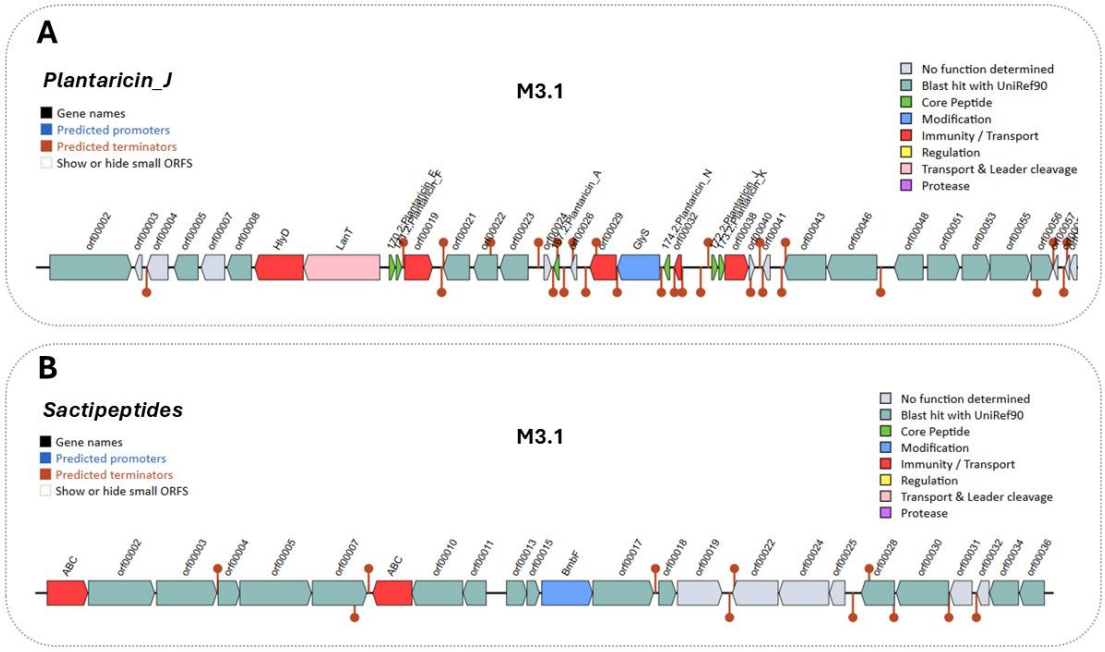


Figure 4. Graphical illustration of the bacteriocin gene clusters detected in the genome of *L. plantarum* M3.1 by BAGEL, highlighting the structure and organization of biosynthetic, transport, and regulatory genes. (A) Plantaricin_J gene cluster and (B) Sactipeptides gene cluster.

Within the plantaricin AOI, the identified bacteriocin gene clusters included those encoding Plantaricin A, Plantaricin E, Plantaricin F, Plantaricin J, Plantaricin K, and Plantaricin N. Our findings are consistent with those of Aziz et al. (2025), who also reported the presence of the same bacteriocin genes. In addition, the authors described other proteins related to bacteriocin secretion, such as ABC transporters (e.g., *PlnG* and its accessory protein *PlnH*). Other regulatory components, including the histidine kinase (*PlnC*) and the response regulator (*PlnD*), were also observed, suggesting that the bacteriocin biosynthetic unit is well coordinated. Similarly, in our study, all *L. plantarum* genomes analyzed contained these regulatory elements, with *PlnC* and *PlnD* genes identified within the same genomic operons. This supports the hypothesis that the bacteriocin production system in these strains is complete and functionally.

Regarding the sactipeptide AOI, based on the current gene annotations of *L. plantarum* genomes, the analyzed region contains several genes related to Fe–S cluster assembly and recycling, ABC transporters, and cofactor metabolism (e.g., probable molybdenum cofactor). This finding is consistent with a genomic environment capable of supporting radical SAM–dependent enzymatic activity potentially involved in sactipeptide production (Chen et al., 2021). However, no gene annotation of a radical SAM enzyme of the "sactisynthase" type (such as the conserved Cys motif or SPASM/twitch domain) nor a short RiPP precursor gene (leader–core peptide structure) was detected. These two components, a radical SAM/sactisynthase enzyme with auxiliary Fe–S motifs and a small precursor peptide encoding the substrate, are essential and nearly invariant features of functional sactipeptide biosynthetic gene clusters (Grove et al., 2017, Chen et al., 2021). Therefore, given the available data, this region cannot be confidently classified as a functional sactipeptide biosynthetic locus. It is more accurate to state that it possesses supportive genetic elements (Fe–S cluster biogenesis genes, ABC exporters) but lacks the key biochemical and genetic signatures (rSAM enzyme and precursor peptide) required for confirmed sactipeptide biosynthesis.

3.3.2. Biosynthetic gene cluster (BGC) predictions

Regarding the analyses performed using antiSMASH, biosynthetic gene cluster (BGC) regions for terpene precursors, terpenes, Type III polyketide synthase (T3PKS), ribosomally synthesized and post-translationally modified peptides (RiPP-like), and cyclic-lactone inducers were detected in all genomes of the *L. plantarum* strains (Table 3) (Figure 5). Similarly, Li et al. (2024b), while studying the genomes of *L. plantarum* strains and their metabolites, also identified BGCs corresponding to T3PKS, terpene, and cyclic-lactone autoinducer metabolism, which were consistent with those found in our study. Considering that the strains evaluated have been previously investigated for their antifungal activity, as reported by Souza et al. (2026), it is plausible to infer that some of these BGCs may be associated with the production of secondary metabolites exhibiting antifungal properties. The antifungal phenotype observed in the studied *L. plantarum* strains may have a genomic basis in several predicted biosynthetic gene clusters detected by BAGEL/antiSMASH. RiPP-like loci are plausible sources of ribosomally-synthesized peptide antimicrobials (Li et al., 2024).

Isaac et al. (2024) reported the presence of four BGCs, like Terpene, T3PKS, Cyclic-lactone autoinducer, and RiPP-like, suggesting their involvement in the production of antimicrobial compounds, as demonstrated by activity against nine methicillin-resistant *Staphylococcus aureus* (MRSA) strains and three of 13 *Klebsiella pneumoniae* clinical isolates. Accordingly, the presence of each of these BGCs in the genome of *L. plantarum* supports the strain's capacity to produce antimicrobial compounds.

Table 3. Secondary metabolite clusters identified by antiSMASH in *L.plantarum s*trains.

Strain	Total Clusters	Types identified	Genomic regions	Size(pb)
M3.1	5	Terpene precursor	1.1	20,927
		Cyclic-lactone-autoinducer	2.1	20,706
		RiPP-like	5.1	12,151
		T3PKS	9.1	30,830
		Terpene	11.1	20,882
M3.3	5	Cyclic-lactone-autoinducer	5.1	16,418
		RiPP-like	7.1	12,151
		Terpene	20.1	20,882
		Terpene precursor	39.1	12,002
		T3PKS	55.1	16,442
M3.6	5	T3PKS	3.2	30,844
		Terpene	6.1	20,882
		RiPP-like	8.1	12,151
		Terpene precursor	17.1	20,927
		Cyclic-lactone-autoinducer	20.1	20,706
R3.2	5	Terpene precursor	1.1	20,927
		RiPP-like	3.1	12,151
		Cyclic-lactone-autoinducer	5.1	20,597
		T3PKS	7.1	30,844
		Terpene	10.1	20,882
R3.6		RiPP-like	1.1	12,151
		Cyclic-lactone-autoinducer	6.1	20,706

Figure 5. Graphical illustration of the secondary metabolites clusters detected in the genome of *L. plantarum* M3.1 by ANTISMASH. The regions describes clusters: Region 1.1 (Terpene-precursor), 2.1 (Cyclic-lactone-autoinducer), 5.1 (RiPP-like), 9.1 (T3PKS), 11.1 (Terpene).

Furthermore, biosynthetic gene clusters (BGCs) are increasingly recognised not only for their role in metabolite synthesis relevant to the food industry but also for their contribution to the probiotic potential of strains, especially those of *L. plantarum*. For instance, genomic analyses have revealed that certain *L. plantarum* strains harbour clusters encoding ribosomally-synthesised and post-translationally modified peptides (RiPPs) such as sactipeptides and

bacteriocins, which enhance their functional attributes in food- and health-related applications (Liu et al., 2023b, Lu et al., 2024). At the same time, in vitro and in silico studies demonstrate that many *L. plantarum* strains possess antioxidant, anti-inflammatory and adhesion-targeting activities consistent with probiotic functionality (El-Dein et al., 2025).

Regarding the biochemical functions of BGCs, T3PKSs are responsible for catalyzing the iterative condensation of malonyl-CoA (and occasionally acetyl-CoA) to produce polyketide chains that undergo cyclization and modification to form diverse aromatic or lactone-type metabolites (Austin et al., 2004). In LAB, genome-wide analyses have revealed the presence of T3PKS gene clusters, indicating a potential for specialized metabolite biosynthesis beyond primary metabolism (Isaac et al., 2024, Li et al., 2024b). These metabolites, particularly those derived from metabolic pathways such as pentose and glucuronate interconversions and the biosynthesis of alkaloids from terpenoid and polyketide routes, may contribute to flavor development through volatile aromatic compounds or exert antimicrobial and antifungal activities (Liu et al., 2023). Therefore, elucidating the genomic context and expression of T3PKS clusters is crucial for optimizing LAB strains in dairy fermentation, enabling both flavor innovation and bioactive compound production.

Terpene or precursors BGCs derive from the methylerythritol phosphate pathway (MET OR DOXP) or mevalonate pathways, which generate isopentenyl pyrophosphate (IPP) and dimethylallyl pyrophosphate (DMAPP) precursors for the terpenes and terpenoids **(**Helfrich et al., 2019). These volatile compounds contribute to flavor formation and may also enhance the antimicrobial and antioxidant properties of fermented dairy products.

RiPP clusters comprise a gene encoding a precursor peptide plus tailoring enzymes (e.g., cyclases, dehydratases, oxidases) that install diverse post-translational modifications to yield mature bioactive peptides such as bacteriocins (Truman, 2016). In LAB, RiPP-like clusters are by far the most abundant BGC class and are strongly associated with antagonistic activities (antimicrobial/antifungal) and potentially probiotic traits.

Thus, genome mining of *L. plantarum* reveals biosynthetic clusters such as T3PKS, terpene, and RiPP that redirect primary metabolites toward compounds influencing aroma,

flavour, and bioactivity. Recognizing the presence and regulation of these clusters allows prediction of each strain's metabolic versatility, competitiveness in fermentation, and biopreservation or probiotic potential. Therefore, genomic characterization is essential for guiding strain selection and fully exploiting the biosynthetic capacity of *L. plantarum* in dairy applications.

3.3.3. Carbohydrate-active enzymes (CAZymes)

Regarding the analysis of CAZymes, including glycosyltransferases (GTs), glycoside hydrolases (GHs), carbohydrate esterases (CEs), carbohydrate-binding modules (CBMs), and auxiliary activities (AAs), a total of 120 genes were identified in the genomes of *L. plantarum* strains M3.1, M3.3, R3.2 and R3.6, distributed across 61 GH, 39 GT, 1 AA, 15 CBM, and 4 CE families (Figure 6). For strains M3.6, 121 genes were identified, comprising 61 GH, 40 GT, 1 AA, 15 CBM, and 4 CE families. These results are consistent with previously reported data for *L. plantarum* (Lu et al., 2025; Zhao et al., 2025; Iarusso et al., 2025). For instance, Lu et al. (2025) reported approximately 100 carbohydrate-active enzymes in the genome of *L. plantarum* GUANKE, including 63 GH, 31 GT, and 3 AA, although the authors did not report CE or CBM families. Similarly, Zhao et al. (2025) identified 173 carbohydrate-active enzymes in *L. plantarum* Z45, including 46 GT, 59 GH, 21 AA, 17 CBM, 28 CE, and 2 polysaccharide lyases (PL).

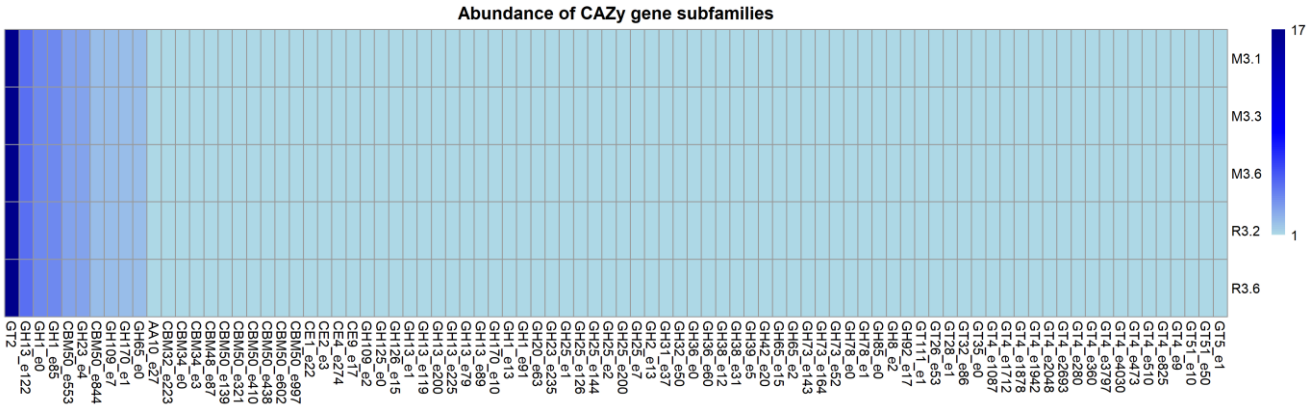


Figure 6. Heatmap showing variation in CAZyme family abundance among *L. plantarum* strains M3.1, M3.3, M3.6, R3.2, and R3.6.

The broad diversity of CAZymes identified in the analyzed *L. plantarum* strains indicates a highly complete and versatile carbohydrate metabolism (Iarusso et al., 2025). In *L. plantarum*, CAZymes belonging to the GH, GT, CE, CBM, and AA families are linked to the efficient degradation of complex carbohydrates (polysaccharides and oligosaccharides) and the subsequent production of fermentation-derived metabolites with recognized immunomodulatory and bioactive functions (Iarusso et al., 2025, Borjihan et al., 2025).

GH13 and GH1 families are known to play crucial roles in the metabolism of complex carbohydrates and were identified in the genomes of *L. plantarum* strains (Iarusso et al., 2025). Lin et al. discussed the main functions of CAZymes, which participate in various processes related to polysaccharide synthesis and degradation. For instance, GTs may be involved in cellulose and chitin synthesis, while GHs such as GH32 and GH43 contribute to the breakdown of fructans and hemicelluloses. Specific CBMs can bind to cellulose, lactose, or β-glucans, and some CBMs, along with AA10, are associated with chitin cleavage, a common component of fungal cell walls (Yao et al., 2023). CBM50 modules, known to bind chitin and peptidoglycan, may act as anchoring domains and potentially contribute to fungal cell wall disruption, whereas AA10 enzymes can oxidatively cleave chitin, weakening the fungal structure.

Additionally, as reported by Liang et al. (2023), CAZymes belonging to GH and CE families have also been associated with the production of flavor compounds through the fermentation and degradation of complex carbohydrates. The authors identified numerous carbohydrate-degrading enzymes, including GH1, GH13, GH32, GH25, GH65, GT2, GT4, GT8, GT51, and CE11. Therefore, integrating CAZyme analysis with BGCs and bacteriocin production predictions can provide insights into the functional mechanisms of the bacterial strains of interest and facilitate the identification of novel, previously uncharacterized features.

In general, our findings similarly suggest that *L. plantarum* strains possess the ability to utilize a wide range of complex carbohydrates as energy sources. Several of these genes, particularly those from GH families, which were predominant in the analyzed genomes, contribute to carbohydrate degradation processes. Considering that the dairy environment is rich and diverse, maintaining such metabolic versatility may be advantageous for the production

of bioactive, functional, or technological compounds by *L. plantarum* strains in the food industry.

3.4. Volatile Organic compounds (VOCs) in Fermented Milk Analyzed by GC-MS

The VOCs produced during milk fermentation varied significantly depending on the LAB strains ($P < 0.05$) (Table 4). These compounds, including ketones, alcohols, esters, and organic acids, are important contributors to the sensory characteristics of fermented dairy products. Similar strain-specific differences in volatile compounds production have also been observed in fermented dairy systems, where distinct *L. plantarum* strains generated markedly different aromatic fingerprints due to variations in carbohydrate metabolism, redox balance, and amino acid catabolism, according to the findings of Tian et al. (2025).

Table 4. Volatile organic compounds (Absolute peak area/1,000) of milk samples fermented with different LAB strains.

	LRI	**Control**	**M3.1**	**M3.3**	**M3.6**	**R3.2**	**R3.6**
Ketones							
Acetone	822	1903^{d}	2671^{c}	6256^{a}	3267^{b}	1873^{d}	3443^{b}
2-Butanone	908	951a	633^{b}	942^{a}	302^{c}	194^{d}	323^{c}
Acetoin	1295	75^{d}	169^{c}	494^{b}	176^{c}	1228^{a}	60^{d}
2-Nonanone	1385	642^{b}	1164^{a}	348^{c}	20^{d}	283^{c}	1379^{a}
Total		***3571^{c}***	***4637^{b}***	***8040^{a}***	***3765^{c}***	***3578^{c}***	***5205^{b}***
Alchohols							
Isopropyl Alcohol	929	-	306^{b}	218^{c}	328^{b}	425^{a}	382^{a}
Ethanol	936	-	114791^{a}	36274^{b}	12193^{d}	24828^{c}	13473^{d}
1-Butanol	1161	214^{d}	4726^{a}	394^{c}	-	1379^{b}	425^{c}
3-methyl-3-Buten-1-ol	1260	-	1234^{a}	684^{c}	1104^{a}	915^{b}	1269^{a}
2-Furanmethanol	1663	$-^{b}$	137^{a}	$-^{b}$	$-^{b}$	$-^{b}$	$-^{b}$
Total		***214^{e}***	***121194^{a}***	***37570^{b}***	***13625^{d}***	***27547^{c}***	***15548^{d}***

Esters							
Ethyl acetate	894	1210^{d}	3947^{a}	3304^{b}	3508^{a}	2591^{c}	3173^{b}
Ethyl butyrate	1039	-^{e}	2910^{a}	391^{d}	1237^{c}	1346^{c}	1606^{b}
Isobutyl butyrate	1150	-^{e}	231^{c}	233^{c}	454^{b}	663^{a}	64^{d}
Ethyl hexanoate	1222	305^{b}	790^{a}	248^{c}	314^{b}	348^{b}	808^{a}
Hexyl acetate	1264	283^{d}	725^{a}	389^{c}	355^{c}	331^{c}	603^{b}
Hexyl formate	1351	777^{c}	1326^{a}	1433^{a}	851^{b}	661^{c}	952^{b}
Total		***2575^{e}***	***9929^{a}***	***5998^{d}***	***6720^{c}***	***5941^{d}***	***7205^{b}***
Acids							
Acetic acid	1461	-^{c}	712^{b}	18757^{a}	-^{c}	-^{c}	-^{c}
Butanoic acid	1631	-^{d}	988^{a}	-^{d}	-^{d}	233^{b}	109^{c}
Isovaleric acid	1671	-^{c}	748^{a}	-^{c}	-^{c}	-^{c}	93^{b}
Total		***-^{d}***	***2448^{b}***	***18757^{a}***	***-^{d}***	***233^{c}***	***202^{c}***

LRI: Linear retention index; Different letters in the same row indicate statistically significant differences at $P<0.05$ among samples; –: Not detected.

Among the ketones detected in our findings, acetone, 2-butanone, acetoin, and 2-nonanone were present in most fermented samples. The highest total ketone concentration was observed in milk fermented with strain M3.3, mainly driven by the elevated production of acetone, which accounted for the majority of the ketone fraction in this sample. Ketones such as acetoin and diacetyl derivatives are well-known aroma compounds contributing buttery, creamy, and dairy-like notes in fermented milk products (Dan et al., 2019). Their formation is generally associated with pyruvate metabolism via the α-acetolactate pathway, where pyruvate is converted into aroma-active intermediates through the activity of α-acetolactate synthase and related enzymes. Variations in the expression of genes involved in this metabolic pathway, including alsS and redox-related enzymes such as NADH oxidase, may explain the substantial strain-dependent differences in ketone production observed in *L. plantarum* strains (Tian et al., 2025). Furthermore, the detection of T3PKS biosynthetic gene clusters (BGCs) and Terpene clusters, as discussed in the previous section, supports the notion that the all observed VOC

profiles can derive these genome-encoded pathways, which appear to be fully active and functionally expressed.

Alcohols were the most abundant VOCs in all samples, particularly in milk fermented with strain M3.1, which exhibited markedly higher ethanol concentrations. Ethanol formation during LAB fermentation may result from acetaldehyde reduction or heterofermentative carbohydrate metabolism, reflecting differences in redox balance and NADH regeneration pathways (Gänzle, 2015). Such strain-dependent variation in *L. plantarum* is often associated with differences in central carbon metabolism and metabolic adaptation to the fermentation environment. The elevated ethanol production observed in M3.1, may also be related to its genomic repertoire of carbohydrate-active enzymes (CAZymes), characterized by the predominance of glycoside hydrolase families (GH1, GH2, GH3, GH13, and GH43). These enzymes can facilitate the hydrolysis of complex carbohydrates present in the milk matrix, increasing the availability of fermentable sugars that can enter central carbon metabolism and potentially enhance glycolytic flux and ethanol formation (Wardman et al., 2022).

Although alcohols generally present relatively high sensory thresholds, they contribute to the aromatic complexity of fermented foods and serve as important precursors for ester formation. In particular, ethanol can react with organic acids to generate ethyl esters, key contributors to fruity aromas in fermented products (Liu et al., 2024). Thus, the elevated ethanol levels detected in M3.1 may partially explain the higher ester production also observed in this sample, suggesting a coordinated metabolic relationship between carbohydrate utilization, alcohol formation, and ester biosynthesis influenced by strain-specific genomic traits.

Organic acids were primarily detected in samples M3.1 and M3.3. Strain M3.3 produced notably higher levels of acetic acid, whereas M3.1 generated greater concentrations of butanoic and isovaleric acids. These compounds play a key role in both the sensory profile and microbiological stability of fermented dairy products. Acetic acid, for instance, contributes pungent, vinegar-like notes The production of organic acids may also be partially linked to the CAZyme repertoire detected in the genomes of these strains. In particular, the presence of glycoside hydrolase families such as GH1, GH2, and GH3 may enhance the hydrolysis of

carbohydrate substrates, increasing the availability of fermentable sugars that feed central carbon metabolism and promote the formation of organic acids. Additionally, carbohydrate esterases (CEs) may release acetyl groups from carbohydrate esters, providing precursors that can contribute to acetic acid formation during fermentation and is widely recognized for its antimicrobial activity.

Short-chain fatty acids such as butanoic and isovaleric acids are commonly formed through amino acid catabolism, particularly from the degradation of branched-chain amino acids (leucine, isoleucine, and valine). Previous metabolomic screening described by Souza et al., (2026) detected these amino acids in all evaluated strains, supporting their role as metabolic precursors for the formation of these short-chain acids. Such metabolic pathways generate compounds associated with strong cheesy or pungent aromas that are characteristic of many fermented dairy products. This highlights the importance of VOCs analysis for defining and proportioning enhanced sensory characteristics for fermented dairy products.

Overall, the VOC profiles suggest that strain selection strongly shapes the sensory characteristics of fermented milk. In general, the strains exhibited distinct aromatic metabolisms, indicating that they may be used individually or in co-cultures to modulate the sensory profile of fermented dairy products. Strain M3.1 appears more suitable for products with fruity notes, whereas M3.3 tends to generate a more pronounced acidic and intense profile. In contrast, M3.6 and R3.6 displayed more intermediate profiles and may contribute to aromatic complexity without dominating the overall sensory perception. Nevertheless, the definitive selection of the most suitable strain for fermented dairy production should be confirmed through future sensory analyses and validation in pilot-scale applications.

Importantly, beyond their role in flavor formation, these strains also exhibit previously characterized antifungal activity and distinct genomic traits associated with antimicrobial peptide production and carbohydrate metabolism. The convergence of aroma-forming capacity, antifungal potential, and genomic features linked to bacteriocin synthesis and carbohydrate utilization underscores the promising potential of these strains as multifunctional adjuncts or

starter cultures. Such combined properties may contribute to the development of fermented dairy products with enhanced sensory quality and improved natural preservation.

4. Conclusion

Our findings demonstrated that the five *L. plantarum* strains evaluated present a strong safety profile and considerable technological potential for dairy fermentations. Genomic analysis revealed biosynthetic gene clusters associated with antimicrobial compounds, including plantaricin-related regions, as well as a diverse repertoire of carbohydrate-active enzymes, supporting their metabolic versatility. The metabolomic analysis of fermented milk confirmed clear strain-dependent differences in volatile compound production, with M3.1 showing enhanced alcohol and ester formation associated with fruity aromatic notes, whereas M3.3 produced higher levels of ketones and acetic acid related to creamy and acidic attributes. Overall, the integration of genomic and metabolomic approaches highlights the potential of these antifungal *L. plantarum* strains as multifunctional adjunct cultures capable of contributing simultaneously to flavor development and natural biopreservation in fermented dairy products. These findings provide a foundation for the development of innovative fermented dairy products combining improved sensory quality with natural microbial stability.

CRediT authorship contribution statement

Luana Virgínia Souza: Conceptualization, Methodology, Validation, Formal analysis, Investigation, Writing - Original Draft, Writing - Review & Editing, Visualization. **Rafaela da Silva Rodrigues**: Conceptualization, Formal analysis, Writing - Review & Editing, Visualization. **Fabrizio Cincotta**: Writing - Review & Editing, Formal analysis, Visualization. **Ricardo Seiti Yamatogi**: Conceptualization, Supervision, Writing - Review & Editing, Visualization. **Luigi Liotta**: Writing - Review & Editing, Visualization. **Cinzia Lucia Randazzo**: Conceptualization, Supervision, Writing - Review & Editing, Visualization. **Cinzia Caggia**: Conceptualization, Writing - Review & Editing, Visualization. **Antonio Fernandes de Carvalho**: Resources, Conceptualization, Supervision, Writing - Review & Editing, Visualization. **Luís Augusto Nero**: Conceptualization, Resources, Writing - Review & Editing,

Visualization, Supervision, Project administration, Funding acquisition. All authors read and approved the final manuscript.

Declaration of competing interest

The authors have no competing interests to declare.

Acknowledgments

The authors are thankful to Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES, Brasília, DF, Brazil, Financial code 001), Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq, Brasília, DF, Brazil), Fundação de Amparo à Pesquisa do Estado de Minas Gerais (FAPEMIG, Belo Horizonte, MG, Brazil).

5.References

Adesulu-Dahunsi, A. T., Jeyaram, K., & Sanni, A. I. (2018). Probiotic and technological properties of exopolysaccharide producing lactic acid bacteria isolated from cereal-based nigerian fermented food products. *Food Control*, *92*, 225-231. https://doi.org/10.1016/j.foodcont.2018.04.062

Ağagündüz, D., Şahin, T. Ö., Ayten, Ş., Yılmaz, B., Güneşliol, B. E., Russo, P., ... & Özogul, F. (2022). Lactic acid bacteria as pro-technological, bioprotective and health-promoting cultures in the dairy food industry. *Food Bioscience*, *47*, 101617. https://doi.org/10.1016/j.fbio.2022.101617

Arndt, D., Grant, J. R., Marcu, A., Sajed, T., Pon, A., Liang, Y., & Wishart, D. S. (2016). PHASTER: a better, faster version of the PHAST phage search tool. *Nucleic acids research*, *44*(W1), W16-W21.

Austin, M. B., Izumikawa, M., Bowman, M. E., Udwary, D. W., Ferrer, J. L., Moore, B. S., & Noel, J. P. (2004). Crystal structure of a bacterial type III polyketide synthase and enzymatic control of reactive polyketide intermediates. *Journal of Biological Chemistry*, *279*(43), 45162-45174. https://doi.org/10.1074/jbc.M406567200

Aziz, T., Shabbir, M. A., Sarwar, A., Khan, A. A., Zhao, L., Yang, Z., ... & Al-Joufi, F. A. (2025). Exploring the multifaceted probiotic potential of *Lactiplantibacillus plantarum* NMGL2, investigating its antimicrobial resistance profiles and bacteriocin production. *Journal of Microbiological Methods*, *236*, 107178. https://doi.org/10.1016/j.mimet.2025.107178

Blin, K., Shaw, S., Augustijn, H. E., Reitz, Z. L., Biermann, F., Alanjary, M., ... & Weber, T. (2023). antiSMASH 7.0: new and improved predictions for detection, regulation, chemical structures and visualisation. *Nucleic acids research*, *51*(W1), W46-W50. https://doi.org/10.1093/nar/gkad344

Borjihan, Q., Yang, Z., Liu, K., Yang, C., Zhang, X., & Yao, G. (2025). Comparative genomics and metabolite analysis of *Lactiplantibacillus plantarum* strains in dairy and vegetable fermentation environments. *Food Bioscience*, *65*, 106058.

https://doi.org/10.1016/j.fbio.2025.106058

Bortolaia, V., Kaas, R. S., Ruppe, E., Roberts, M. C., Schwarz, S., Cattoir, V., ... & Aarestrup, F. M. (2020). ResFinder 4.0 for predictions of phenotypes from genotypes. *Journal of Antimicrobial Chemotherapy*, *75*(12), 3491-3500.

Cai, Z., Guo, Y., Zheng, Q., Liu, Z., Zhong, G., Zeng, L., ... & Wu, Z. (2024). Screening of a potential probiotic *Lactiplantibacillus plantarum* NUC08 and its synergistic effects with yogurt starter. *Journal of Dairy Science*, *107*(5), 2760-2773. https://doi.org/10.3168/jds.2023-24113

Camacho, C., Coulouris, G., Avagyan, V., Ma, N., Papadopoulos, J., Bealer, K., & Madden, T. L. (2009). BLAST+: architecture and applications. *BMC bioinformatics*, *10*(1), 421.

Chen, Y., Wang, J., Li, G., Yang, Y., & Ding, W. (2021). Current advancements in sactipeptide natural products. *Frontiers in Chemistry*, *9*, 595991. https://doi.org/10.3389/fchem.2021.595991

Choi, S., Baek, M. G., Chung, M. J., Lim, S., & Yi, H. (2021). Distribution of bacteriocin genes in the lineages of *Lactiplantibacillus plantarum. Scientific Report. 11, 20063*. https://doi.org/10.1038/s41598-021-99683-1

Costa, C. N. M., de Oliveira, J. M. C., da Silva Alves, A. M., de Lima, K. Y. G., Campos, M. I. F., do Egito, A. S., ... & de Oliveira, M. E. G. (2024). Effect of *Lactiplantibacillus plantarum* CNPC003 and milk pasteurization on artisanal goat coalho cheese characteristics. *International Dairy Journal*, *155*, 105972. https://doi.org/10.1016/j.idairyj.2024.105972

Dan, T., Ren, W., Liu, Y., Tian, J., Chen, H., Li, T., & Liu, W. (2019). Volatile flavor compounds profile and fermentation characteristics of milk fermented by *Lactobacillus delbrueckii* subsp. *bulgaricus*. *Frontiers in Microbiology*, 10, 2183. https://doi.org/10.3389/fmicb.2019.02183

De Simone, N., López, L., Ciudad, C. S., Scauro, A., Russo, P., Rodríguez, J., ... & Martínez, B. (2024). Antifungal activity of *Lactiplantibacillus plantarum* isolated from fruit and

vegetables and detection of novel antifungal VOCs from fungal-LAB co-cultures. *Food Bioscience*, *58*, 103824. https://doi.org/10.1016/j.fbio.2024.103824

Drula, E., Garron, M. L., Dogan, S., Lombard, V., Henrissat, B., & Terrapon, N. (2022). The carbohydrate-active enzyme database: functions and literature. *Nucleic acids research*, *50*(D1), D571-D577. https://doi.org/10.1093/nar/gkab1045

EFSA Panel on Additives and Products or Substances used in Animal Feed (FEEDAP). (2018). *Guidance on the assessment of bacterial susceptibility to antimicrobials of human and veterinary importance.* EFSA Journal, 16(6), 5315.

El-Dein, A. N., Alshehri, W. A., Khalel, A. F., & Awad, H. M. (2025). Comparative in silico analyses between *Lactiplantibacillus plantarum* and *Bifidobacterium longum* concerning probiotic properties, anti-lipidemic, and anti-diabetic in vitro activities. *BMC microbiology*, *25*(1), 356. https://doi.org/10.1186/s12866-025-04062-9

Gänzle, M. G. (2015). Lactic metabolism revisited: metabolism of lactic acid bacteria in food fermentations and food spoilage. Current Opinion in Food Science, 2, 106-117. https://doi.org/10.1016/j.cofs.2015.03.001

Grove, T. L., Himes, P. M., Hwang, S., Yumerefendi, H., Bonanno, J. B., Kuhlman, B., ... & Bowers, A. A. (2017). Structural insights into thioether bond formation in the biosynthesis of sactipeptides. *Journal of the American Chemical Society*, *139*(34), 11734-11744. https://doi.org/10.1021/jacs.7b01283

Helfrich, E. J., Lin, G. M., Voigt, C. A., & Clardy, J. (2019). Bacterial terpene biosynthesis: challenges and opportunities for pathway engineering. *Beilstein journal of organic chemistry*, *15*(1), 2889-2906. https://doi.org/10.3762/bjoc.15.283

Huang, J., Yang, G., Chen, K., Du, M., Zalán, Z., Hegyi, F., & Kan, J. (2024). Anti-fungal effects of lactic acid bacteria from pickles on the growth and sterigmatocystin production of *Aspergillus versicolor*. *International Journal of Food Microbiology*, *422*, 110809. https://doi.org/10.1016/j.ijfoodmicro.2024.110809

Iarusso, I., Mahony, J., Pannella, G., Lombardi, S. J., Gagliardi, R., Coppola, F., ... & Tremonte, P. (2025). Diversity of *Lactiplantibacillus plantarum* in Wild Fermented Food Niches. *Foods*, *14*(10), 1765. https://doi.org/10.3390/foods14101765

Isaac, S. L., Abdul Malek, A. Z., Hazif, N. S., Roslan, F. S., Mohd Hashim, A., Song, A. A. L., ... & Wan Nur Ismah, W. A. K. (2024). Genome mining of *Lactiplantibacillus plantarum* PA21: insights into its antimicrobial potential. *BMC genomics*, *25*(1), 571. https://doi.org/10.1186/s12864-024-10451-7

Kawahara, A., Zendo, T., & Matsusaki, H. (2022). Identification and characterization of bacteriocin biosynthetic gene clusters found in multiple bacteriocins producing *Lactiplantibacillus plantarum* PUK6. *Journal of bioscience and bioengineering*, *133*(5), 444-451. https://doi.org/10.1016/j.jbiosc.2022.01.008

Letunic, I., & Bork, P. (2024). Interactive Tree of Life (iTOL) v6: recent updates to the phylogenetic tree display and annotation tool. Nucleic acids research, 52(W1), W78-W82. https://doi.org/10.1093/nar/gkae268

Li, H., Ding, W., & Zhang, Q. (2024). Discovery and engineering of ribosomally synthesized and post-translationally modified peptide (RiPP) natural products. *RSC Chemical Biology*, *5*(2), 90-108. https://doi.org/10.1039/D3CB00172E

Li, I. C., Lee, Y. L., Li, T. J., Tsai, Y. S., Chen, Y. L., & Chen, C. C. (2024b). Whole-Genome Sequencing of Three *Lactiplantibacillus plantarum* Strains Reveals Potential Metabolites for Boosting Host Immunity Safely. *Journal of Microbiology and Biotechnology*, *34*(10), 2079.

Liang, T., Jiang, T., Liang, Z., Zhang, N., Dong, B., Wu, Q., & Gu, B. (2023). Carbohydrate-active enzyme profiles of *Lactiplantibacillus plantarum* strain 84-3 contribute to flavor formation in fermented dairy and vegetable products. *Food Chemistry: X*, *20*, 101036. https://doi.org/10.1016/j.fochx.2023.101036

Lin, M., Jama, S. M., Cheng, Z., Zong, Y., Su, Y., Liu, W., & Liu, L. (2025). Exploring CAZymes Differences in *Pediococcus acidilactici* Strain OM681363 and *Lacticaseibacillus*

paracasei Strain ON606241 Based on Whole-Genome Sequencing. *Fermentation*, *11*(2), 64. https://doi.org/10.3390/fermentation11020064

Liu, B., Zhong, X., Liu, Z., Guan, X., Wang, Q., Qi, R., ... & Huang, J. (2023b). Probiotic potential and safety assessment of *Lactiplantibacillus plantarum* cqf-43 and whole-genome sequence analysis. *International Journal of Molecular Sciences*, *24*(24), 17570.

Liu, W., Luo, X., Qiu, S., Huang, W., Su, Y., & Li, L. (2023). Determining the changes in metabolites of Dendrobium officinale juice fermented with starter cultures containing *Saccharomycopsis fibuligera* FBKL2. 8DCJS1 and *Lactobacillus paracasei* FBKL1. 3028 through untargeted metabolomics. *BMC microbiology*, *23*(1), 67. https://doi.org/10.1186/s12866-023-02807-y

Liu, Y., Du, S., Sun, L., Li, Y., Liu, M., Sun, P., ... & Wang, Z. (2024). Volatile metabolomics and metagenomics reveal the effects of lactic acid bacteria on alfalfa silage quality, microbial communities, and volatile organic compounds. *Communications Biology*, *7*(1), 1565. https://doi.org/10.1038/s42003-024-07083-8

Lu, S., Yue, K., He, S., Huang, Y., Ren, Z., & Xu, J. (2025). Safety Assessment of *Lactiplantibacillus plantarum GUANKE* Based on Whole-Genome Sequencing, Phenotypic, and Anti-Inflammatory Capacity Analysis. *Microorganisms*, *13*(4), 873. https://doi.org/10.3390/microorganisms13040873

Lu, Y. H., Liang, W. S., Wang, R., Liang, Q. C., Zeng, X. A., & Huang, Y. Y. (2024). Assessment of the safety and probiotic properties of *Lactiplantibacillus plantarum* HYY-DB9 based on comprehensive genomic and phenotypic analysis. *LWT*, *203*, 116386. https://doi.org/10.1016/j.lwt.2024.116386

Meier-Kolthoff, J. P., & Göker, M. (2019). TYGS is an automated high-throughput platform for state-of-the-art genome-based taxonomy. *Nature communications*, *10*(1), 2182. https://doi.org/10.1038/s41467-019-10210-3

National Center for Biotechnology Information (NCBI) Datasets. Genome assemblies for *Lactiplantibacillus plantarum* (taxon 1590) [Internet]. National Library of Medicine (US); accessed 2026 Mar 15. Retrieved from: https://www.ncbi.nlm.nih.gov/datasets/genome/?taxon=1590

Nguyen, L. T., Schmidt, H. A., Von Haeseler, A., & Minh, B. Q. (2015). IQ-TREE: a fast and effective stochastic algorithm for estimating maximum-likelihood phylogenies. Molecular biology and evolution, 32(1), 268-274. https://doi.org/10.1093/molbev/msu300

Page, A. J., Cummins, C. A., Hunt, M., Wong, V. K., Reuter, S., Holden, M. T., ... & Parkhill, J. (2015). Roary: rapid large-scale prokaryote pan genome analysis. Bioinformatics, 31(22), 3691-3693. https://doi.org/10.1093/bioinformatics/btv421

Ruiz, M. J., García, M. D., Canalejo, L. M. M., Krüger, A., Padola, N. L., & Etcheverría, A. I. (2023). Antimicrobial activity of *Lactiplantibacillus plantarum* against shiga toxin-producing *Escherichia coli*. *Journal of Applied Microbiology*, *134*(9), lxad202. https://doi.org/10.1093/jambio/lxad202

Shao, Y., Zhang, W., Guo, H., Pan, L., Zhang, H., & Sun, T. (2015). Comparative studies on antibiotic resistance in *Lactobacillus casei* and *Lactobacillus plantarum*. *Food Control*, *50*, 250-258. https://doi.org/10.1016/j.foodcont.2014.09.003

Souza, L. V., Falqueto, A., Cavicchioli, V. Q., Junior, A. C. B., Oliveira, J. V. A., Chaves, A. R., ... & Nero, L. A. (2026). Metabolomic profiling of antifungal *Lactiplantibacillus plantarum* strains and their inhibition of *Aspergillus niger* and *Penicillium chrysogenum* in a cheese matrix. *International Journal of Food Microbiology*, 111658. https://doi.org/10.1016/j.ijfoodmicro.2026.111658

Souza, L.V., Rodrigues, R.R., Fusieger, A., Ribeiro, L.O.T., Nicosia, F.D., Cavicchioli, V. Q., Russo, N., Caggia, C., Randazzo, C.L., De Carvalho, A.F., Nero, L.A. (2025). Selection of lactic acid bacteria from dairy matrices with antifungal activity to be used as biopreservative adjunct cultures. *Food Biotechnology*. 39 (2), 185–207. https:// doi.org/10.1080/08905436.2025.2501215.

Souza, L. V., Martins, E., Moreira, I. M. F. B., & De Carvalho, A. F. (2022). Strategies for the development of bioprotective cultures in food preservation. *International journal of microbiology*, *2022*(1), 6264170. https://doi.org/10.1155/2022/6264170

Tian, H., Hang, S., Huang, N., Yu, H., Chen, C., & Ge, C. (2025). Strain-level variation in diacetyl production by *Lactiplantibacillus plantarum* reveals genetic drivers of flavor in fermented dairy. *Journal of Dairy Science*. https://doi.org/10.3168/jds.2025-27128

Truman, A. W. (2016). Cyclisation mechanisms in the biosynthesis of ribosomally synthesised and post-translationally modified peptides. *Beilstein journal of organic chemistry*, *12*(1), 1250-1268. https://doi.org/10.3762/bjoc.12.120

van Heel, A. J., de Jong, A., Song, C., Viel, J. H., Kok, J., & Kuipers, O. P. (2018). BAGEL4: a user-friendly web server to thoroughly mine RiPPs and bacteriocins. *Nucleic acids research*, *46*(W1), W278-W281. https://doi.org/10.1093/nar/gky383

Wardman, J. F., Bains, R. K., Rahfeld, P., & Withers, S. G. (2022). Carbohydrate-active enzymes (CAZymes) in the gut microbiome. *Nature Reviews Microbiology*, *20*(9), 542-556. https://doi.org/10.1038/s41579-022-00712-1

Yao, R. A., Reyre, J. L., Tamburrini, K. C., Haon, M., Tranquet, O., Nalubothula, A., ... & Berrin, J. G. (2023). The Ustilago maydis AA10 LPMO is active on fungal cell wall chitin. *Applied and Environmental Microbiology*, *89*(10), e00573-23. https://doi.org/10.1128/aem.00573-23

Zhang, D., Zhang, J., Kalimuthu, S., Liu, J., Song, Z. M., He, B. B., ... & Li, Y. X. (2023). A systematically biosynthetic investigation of lactic acid bacteria reveals diverse antagonistic bacteriocins that potentially shape the human microbiome. *Microbiome*, *11*(1), 91. https://doi.org/10.1186/s40168-023-01540-y

Zhang, H., Yohe, T., Huang, L., Entwistle, S., Wu, P., Yang, Z., ... & Yin, Y. (2018*a*). dbCAN2: a meta server for automated carbohydrate-active enzyme annotation. *Nucleic acids research*, *46*(W1), W95-W101.

Zhang, S., Oh, J. H., Alexander, L. M., Özçam, M., & van Pijkeren, J. P. (2018*b*). d-Alanyl-d-alanine ligase as a broad-host-range counterselection marker in vancomycin-resistant lactic acid bacteria. *Journal of bacteriology*, *200*(13), 10-1128. https://doi.org/10.1128/jb.00607-17

Zhao, M., Zhang, Y., Wu, Y., Liang, S., & Li, G. (2025). *Lactiplantibacillus plantarum* Z45 from Sour Soup Improves Flavor and Safety of Fermented Corn: Insights from Genomic and Metabolomic Approaches. *Foods*, *14*(21), 3803. https://doi.org/10.3390/foods14213803

Zhou, Y., Liang, Y., Lynch, K. H., Dennis, J. J., & Wishart, D. S. (2011). PHAST: a fast phage search tool. *Nucleic acids research*, *39*(suppl_2), W347-W352.

Zuo, J., Huang, D., Liu, J., Wang, Z., Ren, Y., Su, Y., & Ma, Y. (2025). Effect of Probiotics Containing *Lactobacillus plantarum* on Blood Lipids: Systematic Review, Meta-Analysis, and Network Pharmacological Analysis. *Foods*, *14*(19), 3300. https://doi.org/10.3390/foods14193300

CHAPTER 6

6.CONCLUSION AND OUTPUTS

Conclusion

The results obtained so far highlight the technological relevance and bioprotective potential of *L. plantarum* strains for application in the food industry, particularly as natural agents to enhance the safety, quality, and shelf life of fermented dairy products. The initial literature review emphasized the dual nature of filamentous fungi within the food chain—serving as valuable biotechnological resources while simultaneously representing a major challenge due to their role in spoilage and mycotoxin production. In this scenario, the use of lactic acid bacteria with antifungal properties emerges as a promising and sustainable bioprotective strategy, aligning with the growing consumer and industrial demand for clean-label and additive-free preservation approaches.

The screening of lactic acid bacteria from Sicilian dairy products revealed that *L. plantarum* strains possess desirable technological and safety traits, including diacetyl production, proteolytic and aminopeptidase activities, and the absence of hemolytic or virulence factors. Several isolates also demonstrated strong antifungal activity against *A. niger* and *P. chrysogenum*, confirming their inhibitory capacity against common spoilage fungi and their potential as safe, multifunctional starter or adjunct cultures. Further investigation of five selected *L. plantarum* strains (M3.1, M3.3, M3.6, R3.2, and R3.6) demonstrated consistent antifungal efficacy in both in vitro assays and cheese model systems. Microscopic analyses confirmed structural disruption of fungal spores, while metabolomic profiling identified a wide array of organic acids, amino acids, cyclic dipeptides, and phenolic compounds associated with antifungal effects and possible flavor-enhancing functions. These findings reinforce the dual functionality of *L. plantarum* strains as both bioprotective and aroma-contributing cultures.

The genomic and metabolomic analyses were essential to deepen this understanding by identifying the genetic determinants responsible for antifungal activity, metabolic diversity, and technological performance. Comparative genomics, pangenome analysis, and orthology-based clustering clarified strain-specific adaptations, while volatile compound profiling was important to understand their role in aroma formation during fermentation. In summary, these results demonstrated that *L. plantarum* strains possess a highly versatile genetic and functional profile, making them promising candidates for use as bioprotective and adjunct cultures in dairy fermentations. Their combined ability to suppress spoilage fungi and enhance sensory properties supports their potential for commercial application in the production of high-quality, naturally preserved dairy products with extended shelf life.

Outputs

Published papers

1. Bactericidal and antibiofilm activity of lactic acid bacteria-derived cell free extracts against dairy-associated spoilage and pathogenic bacteria

Manuscript published in *Frontiers in Microbiology*

DOI: https://doi.org/10.3389/fmicb.2026.1783760

Abstract

This study aimed to evaluate the antimicrobial and antibiofilm potential of 15 LAB strains using five types of LAB-derived preparations: cell-free supernatants (CFS), sonicated-inactivated cells (IC), their combination (ICS), and their neutralized variants (CFS N, ICS N) to identify the most effective strain-extract combinations for potential application as natural biocontrol agents in dairy systems. Antimicrobial activity was assessed through agar diffusion assays, growth and biofilm inhibition, and determination of minimum inhibitory (MIC) and bactericidal (MBC) concentrations. The extracts were further characterized by pH and organic acid profiles using high-performance liquid chromatography (HPLC), and their mechanisms of action were investigated through cellular leakage assays, time–kill kinetics, and scanning electron microscopy (SEM). Several LAB-derived extracts exhibited strong antagonistic and antibiofilm activity against both spoilage and pathogenic bacteria. Non-neutralized CFS and ICS showed pronounced bactericidal activity, confirming the central role of organic acids in microbial inhibition. Z-score ranking and leakage assays identified *Lactiplantibacillus plantarum* Q4C3, *Lactococcus lactis* subsp. *lactis* biovar *diacetylactis* SBR4, *Weissella cibaria* W21, and *Weissella viridescens* W23 as the most effective strains. Time–kill assays demonstrated rapid microbial reductions (>3 log CFU/mL) within 4 h by CFS, whereas ICS required longer exposure. SEM analysis revealed severe membrane disruption in CFS-treated cells and the presence of LAB-derived debris surrounding ICS-exposed cells. These findings demonstrate that acidic LAB-derived extracts, particularly CFS, efficiently disrupt microbial cells and support their use as safe and effective natural biocontrol agents for improving the microbial safety and quality of dairy products.

2. Bactericidal and bacteriostatic effects of sodium polyphosphate emulsifying salts on selected targets in processed cheese

Manuscript published in *Food Control*

DOI: https://doi.org/10.1016/j.foodcont.2022.109580

Abstract

Processed cheeses (PC) are products with extended shelf-lives, they have in their composition such additives as emulsifying salts and preservatives. Emulsifying salts are used due their desirable emulsification properties, however, little is known about their inhibitory activity against microorganisms that can survive and multiply in these products; jeopardizing cheese quality or even posing as hazards to consumers. The aim is to determine how mixtures of emulsifying salts (ESSP: emulsifying salt composed of short polyP; BSLP: bacteriostatic salt composed of long polyP; and trisodium citrate) can influence the microbial populations in PC during 90 days of storage. A total of 14 treatments (T1 - T14) were evaluated against eight target strains (*Bacillus cereus* INV 10(3); *Bacillus subtilis* ATCC 19659; *Bacillus thuringiensis* CFBP 3476; *Clostridium perfringens* ATCC 13124; *Enterococcus faecalis* FAIR-E 179; *Listeria monocytogenes* Scott A; *Pseudomonas fluorescens* 07A; and *Staphylococcus aureus* ATCC 6538) inoculated into PC. A treatment composed of emulsifying salts without antimicrobial effect (ESSP 1.5%) was considered as the positive growth control, and another treatment composed of antimicrobial additives (nisin and potassium sorbate) was considered as the negative growth control. Bacterial growth was analyzed using ANOVA and Tukey ($p < 0.05$), based on selected treatment grouping. Most treatments resulted in some level of bactericidal or bacteriostatic effect against the target microorganisms. Bactericidal activity was evident against *Bacillus* spp., and bacteriostatic effect was clear against *C. perfringens, E. faecalis, L. monocytogenes* and *S. aureus* ($p < 0.05$) during the storage period. T13, composed of 1.5% BSLP, presented results comparable to those observed for the negative control for bacterial inhibition in PC samples during 90 days of storage. T13 was able to reduce the *B. cereus* INV 10(3) and *B. thuringiensis* CFBP 3476 population more than the negative control ($p < 0.001$), as well as present better results for the

inhibition of *C. perfringens* ATCC 13124 and *L. monocytogenes* Scott A by the end of storage. The performance of the negative control was better than T13 only against *S. aureus* - ATCC 6538 ($p < 0.001$). Taken together, the results contribute to expanding the understanding of emulsifying salt application in PC, with a focus on microbiological safety and shelf-life.

3.Inhibitory activity of an emulsifying salt polyphosphate (JOHA HBS®) used in processed cheese: An in vitro analysis of its antibacterial potential

Manuscript published in *Lebensmittel-Wissenschaft und-Technologie*
DOI: https://doi.org/10.1016/j.lwt.2022.113777

Abstract

In the present study, the inhibitory activity of a commercial polyphosphate (JOHA® HBS) was tested *in vitro* against 21 bacterial strains. Firstly, the antimicrobial activity of JOHA® HBS, at different concentrations (from 0.5 to 2.0%) was evaluated in different agar and broth media (namely, Brain Heart Infusion: BHI; Nutrient: NT; Plate Count: PCA; Trypticase Soy: TSA/B), using streak assay (agar) and culture enumeration (broth). Furthermore, the bacterial inhibition of JOHA® HBS (at different concentrations, from 0.2% to 3.0%) was evaluated both on NT agar and broth medium, by streak assay, agar-spot method, and by spot-on-the-lawn and well-diffusion method. Finally, the JOHA® HBS antimicrobial activity was tested on NT agar at pH 6.3. Results of the streak assay on NT agar showed that 11 out the 21 tested strains were highly inhibited at 0.5% of JOHA® HBS. Interestingly, at adjusted pH levels, JOHA® HBS concentrations of 1.0% (w/v) were able to inhibit the same targets, confirming the antimicrobial effect of JOHA® HBS. This study reveals that NT medium and agar-based tests are required to properly test the inhibitory activity of polyphosphates. Furthermore, results confirmed the antimicrobial activity of JOHA® HBS, at low concentrations, against target bacteria of interest to the dairy industry.

Chapter book

1.Fusieger, A., Camargo, A. D., Rodrigues, R. S., Souza, L. V., de Carvalho, A. F., & Nero, L. A. (2024). *Milk and dairy products* (Cap. 4). In P. M. L. Germano & M. I. S. Germano (Orgs.), *Higiene e vigilância sanitária de alimentos* (7ª ed., p. 476). Editora Manole. ISBN 978-8520465165.

Conference abstracts

1. FUSIEGER, A.; SOUZA, L.V.; RODRIGUES, R.S.; SILVA, R.R.; SILVA, S.R.J.; MARTINS, E.; MACHADO, S.G.; CAGGIA, C.; RANDAZZO, C.L.; CARVALHO, A.F. (2023). "Diversity of filamentous fungi in dairy spoiled products and dairy processing environments from the Zona da Mata region" - *International Symposium ABRAPA 2023*. March 15, 2023. University of São Paulo, SP, Brazil. (Honorable Mention)

2. SOUZA, L.V., RIBEIRO, L.O.T., RODRIGUES, R.S., RANDAZZO, C.L., CAGGIA, C., CARVALHO, A.F., NERO, L.A. (2023). "Assessment of technological properties of lactic acid bacteria isolated from food matrices in the region of Sicily – Italy". *32º Congresso Brasileiro de Microbiologia*. Outubro 2023. Foz do Iguaçu-PR.

3. SOUZA, L.V, DA SILVA, R.R, FUSIEGER, A., RODRIGUES, R.S., MARTINS, E., RANDAZZO, C.L., CAGGIA, C., NERO, L.A., CARVALHO, A.F. (2023) "Antifungal Potential of lactic acid bacteria in a simulated mini cheese". *32º Congresso Brasileiro de Microbiologia*. Outubro 2023. Foz do Iguaçu-PR.

4.SOUZA, LUANA VIRGÍNIA; RIBEIRO, L. O. T.; VENTURA, N. K. O.; RANDAZZO, C. L.; CAGGIA, C.; CARVAHO, A. F.; NERO, L. A. Evaluation of technological traits and antibiotic resistance of lactic acid bacteria isolated from food matrices in Sicily- Italy. (2024) *28th International ICFMH Conference FOOD MICRO 2024*, 2024, Burgos.

5.Luana Virgínia Souza , Luís Otavio Toledo Ribeiro, Fabrizio Domenico Nicosia, Rafaela da Silva Rodrigues , Cinzia Lucia Randazzo, Cinzia Caggia, Antônio Fernandes de Carvalho , Luís Augusto Nero. "Antifungal and technological atributes of lactic acid bactéria isolated from

daury matrices in Sicily – Italy". (2024) - *IAFP Latino 2024 – Latin American Symposium on Food Safety*. November 2024.

6. Júlya Beatriz Martins da Silva Lopes, Maria Eduarda Fernandes Mazzini Villa, Luana Virgínia Souza, Francine Oliveira Souza Duarte, Cíntia Silva Minafra, Valéria Quintana Cavicchioli. " Public Health Risks in Informal Fresh Cheese Commerce at Goiânia Street Markets" (2024). - *IAFP Latino 2024 – Latin American Symposium on Food Safety*. November 2024.

Online Website publication

1.SOUZA, LUANA VIRGÍNIA; MARTINS, E.; NERO, L. A.; NUNES, I. A.; MINAFRA, C.; CAVICCHIOLI, V. Q. *Campylobacter: a silent threat in milk and dairy products.* Milkpoint - Columns, p.1, June 17, 2024. (https://www.milkpoint.com.br/colunas/cpa-ufg/campylobacter-ameaca-silenciosa-em-leite-e-derivados-237051/)

2. SOUZA, LUANA VIRGÍNIA; RANDAZZO, C. L.; CAGGIA, C.; CARVALHO, A. F.; NERO, L. A.; MINAFRA, C.; CAVICCHIOLI, V. Q. *Spoilage fungi in milk and dairy products.* Milkpoint - Columns, p.1, February 27, 2024. (https://www.milkpoint.com.br/colunas/cpa-ufg/fungos-deterioradores-em-leite-e-derivados-236278/)

External Projects

1. InovaRe - Research and application of innovative tools to improve resilience in ruminants – Università degli Studi di Messina – Messina – Coordinator Luigi Liotta. (2025)

2. Quality, safety, and identity of artisanal cheeses produced in Minas Gerais, Brazil – Universidade Federal de Viçosa – Coordinator Luís Augusto Nero – FAPEMIG Agency. (2024)

3.Internationalization Project – Universidade Federal de Viçosa e Università di Catania. Title: Identity of Artisanal Cheese from Minas Gerais: Hygienic Quality, Biological and Chemical Safety, and Composition of the Bacterial and Fungal Microbiota in Different Production Microregions of Minas Gerais. (2024)

4.Research Project in partnership with the Universidade Federal de Goias (UFG). Title: Dairy in Focus: Popularizing Knowledge in Science, Technology, and Innovation in Milk and Dairy Products. Coordinator: Valéria Quintana Cavichiolli (2023).

Course Taught

Dulce de Leche Manufacturing Technology at the "Academic Week of the School of Veterinary Medicine and Animal Science". Organizing Institution: Academic Center of the School of Veterinary Medicine and Animal Science of the Universidade Federal de Goiás - Brasil (2024).

Membership in student course completion project

1. Participation in the evaluation of the final work of Lanussy Luanna Queiroz Silva entitled “Diagnosis of Milk Producer Profiles” (Veterinary Medicine –Universidade Federal de Goiás - UFG) (2024)

2. Participation in the evaluation of the final work of Lanussy Campos da Silva Dantas entitled “Importance of outbreak monitoring and public health risks” (Veterinary Medicine – Universidade Federal de Goiás - UFG) (2024)

3. Participation in the evaluation of the final work of Sidney Rodrigues de Jesus Silva entitled “Evaluation of different fungal DNA extraction techniques and identification of contaminating microbiota from UHT cream” (Food Engineering –Universidade Federal de Viçosa - UFV) (2024)

4. Participation in the evaluation of the final work of Helen Lisboa Resende Rodrigues entitled “Animal welfare in pre-slaughter handling of cattle” (Veterinary Medicine –Universidade Federal de Goiás - UFG) (2024)

5. Participation in the evaluation of the final work of Francismar de Camargo Anchieta Trevisoli entitled Family farmers producing artisanal fresh cheese in the Quirinópolis region, Goiás: socioeconomic, productive and quality aspects for prospecting regional development (PhD in Agribusiness - Universidade Federal de Goiás - UFG) (2024).

Future Perspectives

The growing demand for safe, sustainable, and clean label food products continues to drive the search for innovative and natural preservation strategies. In this context, the use of bioprotective cultures, particularly *Lactiplantibacillus plantarum*, represents a promising approach to enhance both the safety and sensory quality of dairy and fermented foods. However, despite the advances achieved in this study, several aspects warrant further investigation.

Future research should focus on validating the antifungal efficacy of selected strains under industrial scale conditions and across a range of food matrices. The complexity of real production environments, including interactions with native microbiota and processing methods, may influence the performance of these cultures and should be carefully assessed.

Advances in omics technologies, including metabolomics and genomics, open new opportunities to better understand the mechanisms underlying antifungal activity and metabolite production. Integrating multi-omics approaches may enable the identification of key biomarkers and regulatory pathways, supporting the rational selection and optimization of strains with enhanced functionality. Furthermore, the exploration of synergistic effects between different bioprotective cultures or with other preservation strategies could lead to more robust and effective solutions.

Finally, the integration of multifunctional microbial cultures into food systems represents a key step toward more sustainable and natural preservation strategies. Continued research in this field will contribute to bridging the gap between laboratory findings and industrial applications, supporting the development of new generation of fermented foods aligned with clean label trends and food safety demands.