LUANA VIRGINIA SOUZA

FUNGAL BIODIVERSITY IN DAIRY INDUSTRY AND ANTIFUNGAL ACTIVITY OF LACTIC ACID BACTERIA IN CHEESE MATRIX

Thesis submitted to the Food Science and Technology Graduate Program of the Universidade Federal de Viçosa in partial fulfillment of the requirements for the degree of *Doctor Scientiae*.

Adviser: Antonio Fernandes de Carvalho

Co-adviser: Rosângela de Freitas

VIÇOSA – MINAS GERAIS
2023

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

T
S729f
2023

Souza, Luana Virginia, 1987-
Fungal biodiversity in dairy industry and antifungal activity of lactic acid bacteria in cheese matrix / Luana Virginia Souza. – Viçosa, MG, 2023.
1 tese eletrônica (150 f.): il. (algumas color.).

Texto em inglês.
Inclui apêndice.
Orientador: Antônio Fernandes de Carvalho.
Tese (doutorado) - Universidade Federal de Viçosa, Departamento de Tecnologia de Alimentos, 2023.
Inclui bibliografia.
DOI: https://doi.org/10.47328/ufvbbt.2023.059
Modo de acesso: World Wide Web.

1. Queijo - Indústria - Microbiologia. 2. Antimicóticos. 3. Fungos - Deterioração. 4. Biodiversidade. 5. Biodiversidade - Conservação. I. Carvalho, Antônio Fernandes de, 1964-. II. Universidade Federal de Viçosa. Departamento de Tecnologia de Alimentos. Programa de Pós-Graduação em Ciência e Tecnologia de Alimentos. III. Título.

CDD 22. ed. 637.3

Bibliotecário(a) responsável: Alice Regina Pinto Pires CRB-6/2523

LUANA VIRGINIA SOUZA

FUNGAL BIODIVERSITY IN DAIRY INDUSTRY AND ANTIFUNGAL ACTIVITY OF LACTIC ACID BACTERIA IN CHEESE MATRIX

Thesis submitted to the Food Science and Technology Graduate Program of the Universidade Federal de Viçosa in partial fulfillment of the requirements for the degree of *Doctor Scientiae*.

APPROVED: February 17, 2023.

Assent:

gov.br Documento assinado digitalmente
LUANA VIRGINIA SOUZA
Data: 28/02/2023 06:47:33-0300
Verifique em https://verificador.iti.br

Luana Virginia Souza
Author

gov.br Documento assinado digitalmente
ANTONIO FERNANDES DE CARVALHO
Data: 27/02/2023 18:12:13-0300
Verifique em https://verificador.iti.br

Antonio Fernandes de Carvalho
Adviser

To my parents, Neuza and Zequinha (In memorian)

To my lover, Vinícius Meloni

ACKNOWLEDGMENTS

First, I would like to thank my mother, Neuza. Who supported me throughout my life and for always believing in me.

To my loved husband Vinícius, who is always by my side in the good and not so good times and who always waited for me while I worked late at the lab.

To my advisor Professor Antonio, for the opportunity to work in the InovaLeite laboratory. Thanks for sharing your knowledge intensely. For the friendship. For personal, professional and philosophical advice. And for always making us inspired by life.

To Coordenação de Aperfeiçoamento de Pessoal de Nível Superior – Brasil (CAPES) on what was financed this study in part by the– Finance Code 001 and to to the Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq), for granting the scholarship DAI (Doutorado Acadêmico de Inovação). To the Fundação de Amparo à Pesquisa do Estado de Minas Gerais (FAPEMIG) for the support.

To DSM Produtos Nutricionais Brasil S.A. for all the support.

To my co-advisor Prof. Evandro, for sharing a lot of knowledge, for the friendship, for all the support in the articles. For the moments of "peteca", "adedanha", snacks and also for many moments involving teaching, research and extension.

To Prof. Caggia and Prof. Randazzo for the opportunity to study at UniCT. For welcoming me so well and making me feel at home. Still, for believing in my potential more than myself.

To all friends of the Inovaleite.Who were with me in times of work and fun too. In particular to Raiane, Sid, Frida, Rafa, Tetê, Virgínia, Camila, Jean, Falqueto, Lari and Andressa, Guilherme, Luana intern. Gusta was my partner at the table and with dull jokes. Bella, my volleyball partner and who was the first person I worked with in the lab. You were fundamental in making this doctorate lighter.

To Raiane (who needs a separate dedication), who, especially in recent years, has grown a beautiful friendship that has given me all of her, in fact, through the 5 languages of love. I have no words to describe my feeling of gratitude for everything you did for me.

To all the people that I met at UniCT for easier the adaption into the new environment, friendship. In particular, my friend Fabrizio, who received me, helped on the several initial "perrengues" when moved to another country and for shared many fun moments.

To Valesca, my best friend for a long time and who has always been by my side during these years. Our connection is so strong that it seems to be from other lives. Thank you for sharing so much with me.

Edna, my life partner. Who I saw grow professionally and I admire in a unique way. Jessica, the first person I met in Viçosa, my sister that life allowed me to choose. Thank you my beautiful ones for so much love. Lara who was with me after a ruptured tendon with all that affection and physical and emotional support. To Renata, to the "friends of wealth", to Dandara for always being with me, even miles away. Amandinha, who is my fut muse. The volleyball, footvolley and Crossfit friends for the relaxing moments.

To the couple, Thamires and Felype for their friendship and for helping me so much in Italy.

To my friends for decades, Lilian, Ruana e Fernando. The fantastic four.

Carol, my little flower and Mister Alex for their friendship over the years and for always believe in my future.

To my parents from the Souza and Diniz Family who contributed a lot to my life development.

My little star Pietro who is up in the sky. Taught me so much about what life is. And how important it is to be grateful for everything.

Finally, to everyone who contributed directly or indirectly to the realization of my PhD project.

And to God for life and for his infinite grace.

“E cada ser em si carrega o dom de ser capaz e ser feliz…”
“Each being in itself carries the gift of being able and being happy…”

Tocando em Frente, Almir Sater

ABSTRACT

SOUZA, Luana Virginia, D.Sc., Universidade Federal de Viçosa, February, 2023. **Fungal biodiversity in dairy industry and antifungal activity of lactic acid bacteria in cheese matrix**. Adviser: Antonio Fernandes de Carvalho. Co-adviser: Rosângela de Freitas.

Spoilage fungi contamination in dairy industries represents an important economic problem due to spoilage of products. In addition, due to the mycotoxins produced by some fungi, it may also represent a public health problem. Considering the current demand for lesser chemical preservatives addition in foods associated to "Clean label" tendency, the application of bioprotective cultures has been widely explored as alternative. Bacteria belonging to the group of lactic acid bacteria (LAB) stand out as possible bioprotective cultures for being widely used in food processing, show antagonist activity against spoilage microorganisms, and some species recognized as GRAS (Generally Recognized As Safe) status. Thus, considering the concern with spoilage fungi in the dairy industry and the ways to prevent these contaminations, this thesis aimed to understand the biodiversity of fungi in the dairy chain and the antifungal activity of some LAB. For that, the construction of this thesis was realized in two steps: (1) 109 filamentous fungi were isolated from spoiled dairy products and dairy production environments and identified through sequencing of the internal transcribed spacer (ITS) region; (2) 52 LAB strains were tested *In vitro* for their antifungal potential against *Aspergillus niger* and *Penicillium chrysogenum* and LAB with the highest antifungal activity were selected for the *in situ* test into cheese matrix inoculated with *A. niger* and *P. chrysogenum.* As result, *Penicillium* and *Cladosporium* were the most frequent genera isolated from spoilage dairy products. *Cladosporium*, *Penicillium*, *Aspergillus,* and *Nigrospora* had high incidence in the processing environment. Four species (*Hypoxylon griseobrunneum*, *Rhinocladiella similis*, *Coniochaeta rosae,* and *Paecilomyces maximus*) were identified for the first time in dairy environments. Three phytopathogenic genera (*Montagnula*, *Clonostachys*, and *Riopa)* and one pathogenic fungi (*R. similis*) were also identified. Regarding the phylogeny, 14 families and most of the fungi belonging to the Ascomycota phylum were observed. For the antifungal activity *in vitro* test, 5 strains of LAB (*W. confusa* (W5, W8), *W. paramesenteroides* (W9), *W. cibaria* (W25), *L. Plantarum* (Q4C3)) had the highest antifungal effect against the target fungi. In general, the five strains had a positive antifungal effect against *A. niger* and *P. Chrysogenum* also in the *in situ* test; however, these strains lose, in different intensities, their antifugal activity in cheese matrix when combined with a starter culture. Thereby, we show with our study that, despite some

LAB didn’t present an antifungal effect when combined with the starter culture, it is important to emphasize that these bacteria may have great antifungal potential when apply in other food matrices which don’t use a starter culture. Finally, they can be tested regarding their other diverse technological applications.

Keywords: Dairy products. Antifungal activity. Spoilage fungal. Biopreservation. Biodiversity.

RESUMO

SOUZA, Luana Virginia, D.Sc., Universidade Federal de Viçosa, fevereiro de 2023. **Biodiversidade de fungos filamentosos na indústria de laticínios e atividade antifúngica de bactéria ácido lática em matriz de queijo.** Orientador: Antonio Fernandes de Carvalho. Coorientadora: Rosângela de Freitas.

A contaminação por fungos na indústria de laticínios representa um importante problema econômico devido à deterioração de produtos. Além disso, devido às micotoxinas produzidas por alguns fungos, também pode representar um problema de saúde pública. Considerando a atual demanda por menor adição de conservantes químicos nos alimentos associada à tendência "Clean label", a aplicação de culturas bioprotetoras tem sido amplamente explorada como alternativa. As bactérias pertencentes ao grupo das bactérias ácido láticas (BAL), destacam-se como possíveis culturas bioprotetoras por serem amplamente utilizadas no processamento de alimentos, apresentarem atividade antagonista contra microrganismos patogênicos e deterioradores, sendo algumas espécies reconhecidas como GRAS (Generally Recognized As Safe). Assim, considerando a importância dos fungos deterioradores na indústria de laticínios e as formas de prevenir essas contaminações, esta tese teve como objetivo entender a biodiversidade de fungos na cadeia de laticínios e a atividade antifúngica de estirpes de BAL. Sendo assim, a construção da tese foi realizada em duas etapas: (1) 109 fungos filamentosos foram isolados de produtos lácteos deteriorados e ambientes de produção leiteira e identificados por meio do sequenciamento da região espaçador transcrito interno (ITS); (2) 52 cepas de LAB foram testadas *in vitro* quanto ao seu potencial antifúngico contra *Aspergillus niger* e *Penicillium chrysogenum* e as cepas com maior atividade antifúngica foram selecionadas para o teste *in situ* em matriz de queijo inoculada com *A. niger* e *P. chrysogenum*. Como resultados, *Penicillium* e *Cladosporium* foram os gêneros mais frequentemente isolados de produtos lácteos deteriorados. *Cladosporium*, *Penicillium*, *Aspergillus* e *Nigrospora* tiveram alta incidência no ambiente de processamento. Quatro espécies (*Hypoxylon griseobrunneum*, *Rhinocladiella similis*, *Coniochaeta rosae* e *Paecilomyces maximus*) foram identificadas pela primeira vez em ambientes lacteos. Três gêneros fitopatogênicos (*Montagnula*, *Clonostachys* e *Riopa*) e um fungo patogênico (*R. similis*) também foram identificados. Quanto à filogenia, foram observadas 14 famílias e a maioria dos fungos pertencentes ao filo Ascomycota. Para o teste de atividade antifúngica *in vitro*, 5 cepas de LAB (*W. confusa* (W5, W8), *W. paramesenteroides* (W9), *W. cibaria* (W25), *L. plantarum* (Q4C3)) tiveram o maior efeito antifúngico contra os fungos testados. Em geral,

as cinco cepas tiveram um efeito antifúngico positivo contra *A. niger* e *P. chrysogenum* também no teste *in situ*; no entanto, essas cepas perderam, em diferentes intensidades, sua atividade antifúngica na matriz de queijo quando combinadas com cultura starter. Assim, mostramos com nosso estudo que, apesar de algumas LAB não apresentarem efeito antifúngico quando combinadas com a cultura starter, é importante ressaltar que essas bactérias podem ter grande potencial antifúngico para serem aplicadas em outras matrizes alimentares. E finalmente, também podem ser testadas quanto às suas outras diversas aplicações tecnológicas.

Palavras-chave: Produtos lácteos. Atividade antifúngica. Fungos deterioradores. Biopreservação. Biodiversidade.

RIASSUNTO

SOUZA, Luana Virginia, PhD. Universidade Federal de Viçosa, Febbraio, 2023. **Biodiversità fungina nell'industria lattiero-casearia e studio dell´attività antimicotica dei batteri lattici nel formaggio.**Tutor: Antonio Fernandes de Carvalho. Co-tutora: Rosângela de Freitas.

La contaminazione da funghi nelle industrie lattiero-casearie rappresenta un grave problema economico con perdite di prodotto dovute al deterioramento. Inoltre, a causa delle micotossine prodotte da alcuni funghi può rappresentare un problema di salute pubblica. Considerando l'attuale tendenza a ridurre l'uso di conservanti chimici negli alimenti e la tendenza al "Clean label", l'applicazione di colture bioprotettive è stata ampiamente esplorata come alternativa. I batteri appartenenti al gruppo dei batteri lattici (LAB) sono sempre più utilizzati nel mercato come additivi alimentari poiché molti di loro posseggono lo stato GRAS (Generally Recognized As Safe). Pertanto, poichè risulta necessario focalizzare l´attenzione sulle contaminazioni da funghi nell'industria lattiero-casearia e i modi per prevenirle, questa tesi há avut ló scopo di valutare la biodiversità di funghi isolati nell´ambiente caseario e l'attività antifungina esercitata da alcuni ceppi di batteri lattici, utilizzati come coltura bioprotettiva. Um totale di 109 funghi filamentosi sono stati isolati da vari prodotti lattiero-caseari avariati e dagli ambienti di produzione e successivamente identificati attraverso il sequenziamento della regione “internal transcribed spacer” (ITS). 52 ceppi di LAB sono stati testati *in vitro* per il loro potenziale antimicotico contro *Aspergillus niger* e *Penicillium chrysogenume* e quelli che hanno mostrato attività antimicotica marcata sono stati selezionati per il test *in situ* nella matrice del formaggio, inoculata con *A. niger* e *P. chrysogenum*. I risultati hanno mostrato che *Penicillium* e *Cladosporium* sono stati i generi più frequentemente isolati dai latticini avariati, mentre *Cladosporium*, *Penicillium*, *Aspergillus* e *Nigrospora* hanno avuto un'elevata incidenza nell'ambiente caseario. Inoltre, le specie *Hypoxylon griseobrunneum*, *Rhinocladiella similis*, *Coniochaeta rosae* e *Paecilomyces maximus,* i generi fitopatogeni *Montagnula, Clonostachys* e *Riopa* e il ceppo patogeno *R. Similis* sono stati per la prima volta riscontrati negli ambienti caseari. Per quanto riguarda la filogenesi, sono state osservate 14 famiglie, con la maggior parte dei funghi appartenenti al phylum Ascomycota. Il test di attività antimicotica *in vitro* ha messo in evidenza che i 5 ceppi di LAB (*W. confusa* (W5, W8), *W. paramesenteroides* (W9), *W. cibaria* (W25), *L. plantarum* (Q4C3)) hanno avuto un marcato effetto antimicotico contro i funghi testati. In dettaglio, i sei ceppi hanno mostrato un effetto inibente contro *A. niger* e *P. chrysogenum* nel test *in situ*,

tuttavia, gli stessi hanno perso, a vari livelli, la loro attività antimicotica quando inoculati nella matrice di formaggio, in combinazione con la coltura starter.In definitiva, il presente studio ha dimostrato che, nonostante alcuni ceppi di LAB non abbiano mostrato un effetto antimicotico quando combinati con la coltura starter, gli stessi sono in grado di esercitare un effetto antimicotico marcato se utilizzati in altre matrici alimentari che non prevedono l´utilizzo di colture starter ed essere testati per altre diverse applicazioni tecnologiche.

Parole chiave: Prodotti lattiero-caseari. Attività antimicotica. Funghi. Biocontrollo. Biodiversità.

RÉSUMÉ

SOUZA, Luana Virginia, PhD. Universidade Federal de Viçosa, Février, 2023. **Biodiversité fongique dans L´industrie laitière et activité antifongique des bactéries lactiques dans la matrice du fromage**. Directeur: Antonio Fernandes de Carvalho. Co-Directeur: Rosângela de Freitas.

La contamination par les champignons d'altération dans les industries laitières représente un gros problème économique, avec des pertes de produits dues à l'altération. De plus, du fait des mycotoxines produites par certains champignons, cela peut aussi représenter un problème de santé publique. Compte tenu de la tendance actuelle à réduire l'utilisation de conservateurs chimiques dans les aliments et de la tendance "Clean label", l'application de cultures bioprotectrices a été largement explorée comme alternative. Les bactéries appartenant au groupe des bactéries lactiques (LAB) sont de plus en plus exposées sur le marché en tant qu'additifs alimentaires puisque beaucoup d'entre elles ont le statut GRAS (Generally Recognized As Safe). Ainsi, compte tenu de la préoccupation des champignons d'altération dans l'industrie laitière et des moyens de prévenir ces contaminations, cette thèse visait à comprendre la biodiversité des champignons dans la chaîne laitière et l'activité antifongique de différentes bactéries lactiques (BL) en tant que culture bioprotectrice. Pour cela, la construction de cette thèse a été réalisée en trois étapes. (i) Dans la première étape, 109 champignons filamenteux ont été isolés de divers produits laitiers avariés et environnements de production laitière. Les isolats ont été identifiés par séquençage de la région de l'espaceur transcrit interne (ITS). Dans les produits avariés, *Penicillium* et *Cladosporium* étaient les genres les plus fréquents de champignons filamenteux et également présents dans l'environnement laitier, indiquant qu'ils peuvent représenter une source primaire de contamination. Pour les environnements de production laitière, les genres les plus fréquents étaient *Cladosporium*, *Penicillium*, *Aspergillus* et *Nigrospora*. Quatre espèces (*Hypoxylon griseobrunneum*, *Rhinocladiella similis*, *Coniochaeta rosae* et *Paecilomyces maximus*) ont été identifiées pour la première fois dans des produits laitiers ou des environnements de production laitière. Des genres phytopathogènes ont également été détectés, tels que *Montagnula*, *Clonostachys* et *Riopa*. Une espèce isolée de l'environnement de production laitière est classée comme champignon pathogène, R. similis. Concernant la phylogénie, 14 familles différentes et la plupart des champignons appartenant au phylum Ascomycota ont été observés. A l'étape (ii), 52 souches LAB ont été testées. In vitro pour leur potentiel antifongique against *Aspergillus niger* and *Penicillium chrysogenum*. five souches de LAB *W.*

confusa (W5, W8), de *W. paramesenteroides* (W9), de *W. cibaria* (W25), de *L. plantarum* (Q4C3) ont eu l'effet antifongique le plus élevé contre les champignons cibles testés dans des spores non diluées (10^7/g) et 4 et 16 fois diluées (10^4 et 10^3 spores/g) concentrations. Pour l'étape (iii), ces cultures ont été sélectionnées pour des essais in situ sur une matrice de fromage simulée. En général, les six souches sélectionnées ont eu un effet antifongique positif contre *A. niger* et *P. chrysogenum* inoculés dans la matrice de fromage. La culture bioprotective commerciale a montré une action antifongique combinée ou non avec la culture de départ. Cependant, l'effet antifongique a été perdu lorsque ces LAB ont été combinés avec la culture de départ. Ainsi, nous montrons avec notre étude que, malgré certains LAB n'a pas présenté un effet antifongique lorsqu'il est combiné avec la culture de démarrage, il est important de souligner que ces bactéries peuvent avoir un grand potentiel antifongique lorsqu'elles sont appliquées dans d'autres matrices alimentaires qui n'utilisent pas une culture de démarrage. Enfin, ils peuvent être testés sur leurs autres applications technologiques.

Mots clés : Produits laitiers. Activité antifongique. Champignons détériorants. Biopréservation. Biodiversité.

LIST OF ILLUSTRATIONS

CHAPTER II. REVIEW ARTICLE ONE

CHAPTER III. RESEARCH ARTICLE TWO

CHAPTER IV. RESEARCH ARTICLE THREE

LIST OF TABLES

CHAPTER II. REVIEW ARTICLE ONE

CHAPTER III. RESEARCH ARTICLE TWO

CHAPTER IV. RESEARCH ARTICLE THREE

SUMMARY

CHAPTER I.

GENERAL INTRODUCTION

Context

Filamentous fungi are ubiquitous organisms and because they are present in the environment they can contaminate milk and dairy products, which are a major problem in defining the shelf life, with economic losses and a consumer dissatisfaction impact (Pitt and Hocking, 2009). Dairy products constitute a favorable environment (e.g. acidic pH and intermediate water activity, nutrient composition) for the growth these microorganisms (Garnier, Valence and Mounier, 2017). Products resulting from defects that deteriorating fungi can cause include, visible growth on the product surface, color production, excess musty, bitter flavors and in some cases can produce mycotoxins (Pinches and Apps, 2007; Garnier, Valence and Mounier, 2017). Generally, to avoid spoilage due to filamentous fungi contamination, the industries, normally use chemical preservatives (e.g natamycin, potassium sorbate, sodium benzoate) which are efficient, in addition to good manufacturing practices (GMP).

A new trend has been explored by the food industries called “Clean label”, especially in the post-Covid-19 pandemic scenario. Many consumers began to demand less processed foods, with fewer ingredients and, especially, without any addition of chemical preservatives (Aschemann-Witzel, Varela & Peschel, 2019; Miyah et al., 2022). Considering this strong trend, the research for bioprotective cultures for the market has been increasing estimating even greater growth for the coming years.

The global market for bioprotective cultures projects growth of up to approximately 10% between 2021 and 2026, and since 2018, several cultures have been made available on the market. Several food chains such as dairy, meat, poultry and seafood products are being explored and in various locations such as North and South America, Europe, Asia and Middle East; and Africa (Mordor Intelligence, 2022).

In the dairy industry, the most common technology of food preservation is the fermentation process by LAB strains, in which the main antimicrobial compound is lactic acid to confer protection. In the process of fermentation, other desired compounds such as flavors, are also produced to improve the characteristics of the final product (Bintsis, 2018). These strains can produce several antimicrobial compounds in addition to lactic acids, such as other organic acids (acetic, benzoic, formic, succinic, phenylacetic, indole lactic, and azelaic acids), hydrogen peroxide, acetoin, diacetyl, reuterin, and peptides like bacteriocins (Salas et al., 2019).

Studies have identified autochthonous strains of LAB isolated from artisanal cheese as good candidates with bioprotective potential what allows us the identification of new microbial strains with a wide range of technological applications. Thus, considering the tendency to reduce the use of chemical preservatives, the in-depth study on the potential of bioprotective cultures, identification of their metabolites and especially on the complex microorganism/food matrix interactions has become increasingly inevitable.

Research hypotheses and aims

The hypothesis was to understand the biodiversity of filamentous fungi in the dairy industry and selected LAB strains to provide an antifungal effect in the assay *in vitro* and the simulated cheese-making in the laboratory conditions. To verify this hypothesis, the following steps were defined:

i. To isolate and identify the main filamentous fungi that contaminate dairy products and those present in the environment that may contaminate the products.
ii. To evaluate the antifungal activity of different LAB from a collection of cultures already characterized by 16S rDNA sequencing, and selected the bacteria with the best effect for the assay *In Situ*.

iii. To evaluate the antifungal activity of LAB selected in the *In vitro* assay for the *In situ* tests on cheese obtained through simulated making on laboratory conditions.

Thesis outline

Chapter I gives a general overview of the filamentous fungi and the bioprotective cultures with focus on the antifungal effect, the importance of this work, the research problem, as well as the hypotheses and objectives of the thesis.

Chapter II describes a published review that presents information on the bioprotection mechanisms developed by LAB in foods and describes the main strategies used to identify and characterize bioprotective LAB with potential applications in the food industry.

Chapter III describes the biodiversity of filamentous fungi in the spoilage dairy products and dairy environments from Brazil, considering that have few studies in this country concerning fungi from dairy chain. The understanding of fungal biodiversity in dairy products and environment can support the development of conservation strategies to control food spoilage.

Chapter IV reports the *In vitro* and I*n situ* performance of LAB against two references filamentous fungal. The *In situ* test was realized on a simulated cheese matrix. The findings were important to understand certain interactions between culture starters and non-starters in the cheese matrix.

Chapter V provides the overall experiments, summaries the main findings of this research and provides recommendations for future studies.

Chapter VI brings a survey of the scientific production (Papers and Certificates) carried out during the Ph.D.(March 2019 to December 2022), either through this research or through parallel projects.

References

Aschemann-Witzel, J., Varela, P., & Peschel, A. O. (2019). Consumers' categorization of food ingredients: Do consumers perceive them as 'clean label' producers expect? An exploration with projective mapping. *Food Quality and Preference, 71*, 117–128.

Bintsis, T. (2018). Lactic acid bacteria as starter cultures: An update in their metabolism and genetics. *AIMS microbiology*, *4*(4), 665.

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

Katz, B., & Williams, L. A. (2011). Cleaning up processed foods. *Food Technology (Chicago)*, *65*(12), 33-37.

Miyah, Y., Benjelloun, M., Lairini, S., & Lahrichi, A. (2022). COVID-19 impact on public health, environment, human psychology, global socioeconomy, and education. *The Scientific World Journal*, *2022*.

Mordor Intelligence. (2022). Protective cultures Market – Growth, trends, covid-19, impact, and forecasts 2023-2029, 2022. Acessed 12/12/2022. Available: https://www.mordorintelligence.com/industry-reports/protective-cultures-market

Pitt, J. I., & Hocking, A. D. (2009). *Fungi and food spoilage* (Vol. 519, p. 388). New York: Springer.

CHAPTER II. REVIEW

ARTICLE ONE

Strategies for the Development of Bioprotective Cultures in Food Preservation

SOUZA et al.

Manuscript published in *International Journal of Microbiology* (IF 3.746: 2021), 2022.
DOI: https://doi.org/10.1155/2022/6264170

Chapter presented according to the final format of the journal.

.

Title page

Strategies for the Development of Bioprotective Cultures in Food Preservation

Luana Virgínia SOUZA[a], Evandro MARTINS[a] , Isabella Maria Fernandes Botelho MOREIRA [a], Antônio Fernandes de CARVALHO[a]

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

ORCID Authors:

https://orcid.org/0000-0001-7791-6496 Luana Virgínia Souza

https://orcid.org/0000-0002-5448-8385 EvandroMartins

https://orcid.org/0000-0002-5698-7997 Isabella Maria Fernandes Botelho Moreira

https://orcid.org/0000-0002-3238-936X Antonio Fernandes de Carvalho

* Corresponding author: A. F. Carvalho (antoniofernandes@ufv.br)

Abstract

Consumers worldwide are increasingly demanding food with fewer ingredients, preferably without chemical additives. The trend called “Clean Label” has stimulated the development and commercialization of new types of bioprotective bacterial cultures. These bacteria are not considered new, and several cultures have been available on the market. Additionally, new bioprotective bacteria are being identified to service the clean label trend, extend the shelf life, and, mainly, improve the food safety of food. In this context, the lactic acid bacteria (LAB) have been extensively prospected as a bioprotective culture, as they have a long history in food production and their antimicrobial activity against spoilage and pathogenic microorganisms is well established. However, to make LAB cultures available in the market is not that easy, the strains should be characterized phenotypically and genotypically, and studies of safety and technological application are necessary to validate their bioprotection performance. Thus, this review presents information on the bioprotection mechanisms developed by LAB in foods and describes the main strategies used to identify and characterize bioprotective LAB with potential application in the food industry.

Keywords: Clean label, Antimicrobial, Lactic acid bacteria.

1. Introduction

According to the most recent reports, the 'Clean Label Ingredients' market is projected to grow at a Compound Annual Growth Rate of 6.75% between 2021 and 2026, with the potential to reach up to USD 75.2 billion by 2026 [1]. During the COVID-19 pandemic, people have become more cautious in regards to healthy eating, especially on those who have been infected and the right choice of food can help balance the immune system and optimize its function. This explains the rapid growth of clean label foods in the recent years [2].

The clean label trend has stimulated the food industry into developing new strategies for food production and preservation. Included into this scenario are the bioprotective bacterial cultures, which can answer the consumer's exigencies regarding foods with less ingredients [3, 4, 5].

The first definition of bioprotective bacterial cultures was proposed by Lücke in 1994 [6], who defines them as being microbial cultures added to food for the unique purpose of inhibiting pathogens, extending shelf life and improving their sensory quality. Later, the concept of biopreservation was introduced, and according to this new definition, bioprotection can be achieved through the addition of bioprotective cultures, or their antimicrobial metabolites, to promote extended shelf life and food safety [7, 8]. More recently, Vignolo & Fadda in 2015 unified the previous concepts [9]. Thus, biopreservation has come to be defined as the use of antagonistic microorganisms and/or their metabolites to inhibit undesirable microorganisms to increase the shelf life of food with minimal modifying its sensory properties.

To be considered as a bioprotective culture, the bacterial strain needs to be identified at the genus and species level, to have the GRAS (Generally Recognized as Safe) or QPS (Qualified Presumption of Safety) status; to be stable and remain active under storage conditions and, to inhibit the growth of pathogenic or/and spoilage microorganisms. In

addition, for the commercialization of these cultures, proof of the intended technological effect is required, with the definition of the quantity to be used and effectiveness at safe levels [10, 11].

Some works have highlighted that bioprotective bacteria are mainly classified as *Lactococcus*, *Lactobacillus*, *Lacticaseibacillus, Latilactobacillus, Lactiplantibacillus, Limosilactobacillus, Streptococcus*, *Leuconostoc, Weissella, Pediococcus, Carnobacterium* and *Enterococcus*; with most of these belonging to the lactic acid bacteria group (LAB) (Table 1A). LAB combined with non-LAB bacteria can be found in the market as bioprotective cultures for food application (Table 1B).

The microorganisms of the LAB group have, as a common characteristic, the ability to produce lactic acid from carbohydrate fermentation and they are commonly used in the production of fermented foods such as yogurts, cheeses, and fermented meats or vegetables [6, 8, 12, 13, 14, 15]. The LAB group is composed of the genera *Lactococcus, Streptococcus, Lactobacillus, Paralactobacillus, Holzapfelia, Amylolactobacillus, Bombilactobacillus, Companilactobacillus, Lapidilactobacillus, Agrilactobacillus, Schleiferilactobacil-lus, Loigolactobacilus, Lacticaseibacillus, Latilactobacillus, Dellaglioa, Liquorilactobacillus, Ligilactobacillus, Lacti-plantibacillus, Furfurilactobacillus, Paucilactobacillus, Limosilactobacillus, Fructilactobacillus, Acetilactobacillus, Apilactobacillus, Levilactobacillus, Secundilactobacillus, Lentilactobacillus, Leuconostoc, Pediococcus, Aerococcus, Carnobacterium, Enterococcus, Oenococcus, Tetragenococcus, Vagococcus*, and *Weissella*; and some species such as *L. lactis*, *L. curvatus* and *L. plantarum* can be considered the GRAS status [8, 16]. The reported bioprotection effects of some LAB cultures may be affected by food factors such as pH, water activity, composition of food matrix, processing type, storage conditions and by microbial factors such as strain type, technological capacity of the strains and genetic expression ; resulting in significant changes in the efficiency of these

bacteria in certain applications [4, 17, 18]. *L. mesenteroides* reduces the population of spoilage microorganisms from 4 to 5 log cycles when inoculated in apples, while a reduction of only 3 log cycles was observed when the same bacterium was added to lettuce [19]. In a study conducted by Mirkovic et al. [20] the LAB *L.lactis*, in addition to demonstrating an antimicrobial effect on *L. monocytogenes* and *S. aureus* in Quark-type cheese, it also demonstrated an effect on filamentous fungi and yeasts from spontaneous growth during 21 days of product storage.

In an antifungal evaluation on the dairy systems model, Salas *et al.* [4] demonstrated that the antifungal activity of LAB was greater in cheese than in yogurt. These studies reinforce the idea that the bioprotection efficiency can change from one food to another, which reinforces the importance for the discovery of other bioprotective cultures that are able to cover all specificities of food industry. By considering that LAB are a viable possibility to replace or reduce the number of preservatives in food, and that few strains have been commercialized until now, research into alternative LAB cultures may help develop new processes and adapt technologies for the "clean label" demand. This review aims to describe the bioprotection mechanisms developed by LAB in foods, and to describe the main strategies used to identify LAB with potential application in the food industry.

Table 1. (A)
Major cultures used as bioprotective in food products

Researches			
Application product	Microorganism	Target microorganisms	References
Marine products			
	Latilactobacillus sakei [1]		[21]
Cold-smoked salmon	*Latilactobacillus curvatus* [2]	*Listeria monocytogenes*	[21]
	Carnobacterium maltaromaticum		[21]
Fish pâté	*Lactococcus lactis* subsp. *lactis*	*Vibrio sp.*	[22]
Gilt head sea bream	*L.sakei* [1]	*Listeria monocytogenes*	[23]
Tuna burgers	*Lacticaseibacillus paracasei* [3]	*Pseudomonas spp.*	[24]
Meat			
Cooked ham	*Lactiplantibacillus plantarum* [4]	*Bacillus cereus*	[25]
Cooked ham	*Pediococcus acidilactici*	*Clostridium sp. like botulinum*	[25]
Fresh sausage	*L. curvatus* [2]	*Listeria monocytogenes*	[26]
Chicken breast	*Enterococcus lactis*	*Listeria monocytogenes*	[27]
Meatballs	*Pediococcus acidilactici*	*Escherichia coli* 0157:H7	[28]
Fermented sausage	*L. curvatus* [2]	*Listeria monocytogenes*	[29]
Speck	*Debaryomyces hansenii*		[30]
Speck	*Saccharomycopsis fibuligera*	*Aspergillus ochraceus/P. nordicum*	[30]
Suckling-lambmeat	*Leuconostoc pseudomesenteroides*	*Listeria monocytogenes*	[31]
Bakery Products			
Pan whole-wheat	*Limosilactobacillus reuteri* [5]	*Aspergillus niger*	[32]
Panettones	*Limosilactobacillus fermentum* [6]	*Molds and yeasts*	[33]
Sourdough bread	*L. paracasei* [3]	*Bacillus spp.*	[34]
Pound cake	*L. reuteri* [5]	*Cladosporium sphaerospermum*	[35]
Pound cake	*Levilactobacillus spicheri* [7]	*Cladosporium sphaerospermum*	[35]
Milk bread rolls	*Lactobacillus citreum*	*Aspergillus niger*	[35]

Fruits and vegetables

Table olive	*L. plantarum* [4]	*Listeria monocytogenes*	[36]
Cabbage	*L. plantarum* [4]	*Listeria monocytogenes*	[37]
Apples	*L. plantarum* [4]	*Escherichia coli / Listeria monocytogenes*	[13]
Apples	*Leuconostoc mesenteroides*	*Listeria monocytogenes*	[19]
Pickles	*L. plantarum* [4]	*Candida albicans*	[38]
Orange	*Weissella paramesenteroides*	*Penicillium digitatum*	[39]
Orange	*Liquorilactobacillus sucicola* [8]	*Penicillium digitatum*	[39]
Grape bunch	*Pediococcus pentosaceus*	*Aspergillus niger/ Aspergillus carbonarius*	[40]
Lettuce	*Leuconostoc mesenteroides*	*Listeria monocytogenes*	[19]

Dairy products

Cheese feta	*Lactobacillus acidophilus*	*Aspergillus niger*	[41]
Sour cream and cheese	*L.plantarum* [4]*/Lactobacillus harbidenses*	*Penicillium commune/Mucor racemosus*	[4]
Sour cream	*L. plantarum* [4]	*Penicillium commune*	[42]
Fermented Milk	*L. harbidenses*	*Yarrowia lipolytica*	[43]
Gorgonzola cheese	*Lactococcus lactis* subsp. *lactis*	*Listeria monocytogenes*	[44]
Fresh cheese	*Lactococcus lactis* subsp. *lactis*	*Bacillus cereus*	[45]
Kasseri cheese	*Streptococcus macedonicus*	*Clostridium tyrobutyricum*	[46]

Table 1. (B)

COMMERCIAL CULTURES

Product/Manufacturer	Application	Target microorganisms	Composition
FreshQ®1/CHR Hansen	Fermented dairy products	Yests and molds	*Lacticaseibacillus rhamnosus*[9] and *L. paracasei*[3]
FreshQ®2/CHR Hansen	Fermented dairy products	Yests and molds	*L. rhamnosus*[9] and *L. paracasei*[3]
FreshQ®4/CHR Hansen	Fermented dairy products	Yests and molds	*L. rhamnosus*[9] and *L. paracasei*[3]
SafePro® B-LC-007/CHR Hansen	Fermented sausage	*Listeria monocytogenes*	*Debaryomyces hansenii,L. sakei*[1],*Pediococcus acidilactici ,Pediococcus pentosaceus,Staphylococcus carnosus,Staphylococcus xylosus*
Viniflora®CH16/CHR Hansen	Wine	Yests and molds	*Oenococcus oeni*
Concerto™/CHR Hansen	Wine	Yests and molds	*Lachancea thermotolerans*
Delvo®Guard 201/ DSM	Soft cheese	Yests and molds	*L. rhamnosus* [9] and *L. sakei*[1]
Delvo®Guard 301/ DSM	Soft cheese	Yests and molds	*L. rhamnosus* [9]
Lyofast LPR A/ SACCO	Cheese hard and semi-hard	Yests and molds	*L. rhamnosus* [9] and *L. plantarum*[4]
Bioprox RP80/ PROXIS	Dairy products	Yests and molds	*L. rhamnosus* [9] and *L. plantarum*[4]
Lyopro ® Tect / Codex-ing	Fermented milk and cheese	Yests and molds	*L. rhamnosus*[9] and *Propionibacterium shermanii*
HOLDBACK® Listeria dairy / Danisco	Soft and smear cheese, dry and semi-dry cured meats, cooked and fresh ground meats	*Listeria monocytogenes*	*L. plantarum*[4]
HOLDBAC® YM-XPM/ Danisco	All cheese types	Yests and molds	*L. paracasei*[3] *and L. plantarum*[4]
M-CULTURE® Safe 1100/ Meatcracks	Raw sausages	*Listeria monocytogenes*	*Staphylococcus carnosus, L. curvatus* [2] *and Staphylococcus xylosus*
Protek/ BIOCHEM	Fresh and soft cheese	Yests and molds	*L. rhamnosus* [9]

[1] Previous *Lactobacillus sakei* [2] Previous *Lactobacillus curvatus* [3] Previous *Lactobacillus paracasei* [4] Previous *Lactobacillus plantarum* [5] Previous *Lactobacillus reuteri* [6] Previous *Lactobacillus fermentum* [7] Previous *Lactobacillus spicheri* [8] Previous *Lactobacillus sucicola* [9] Previous *Lactobacillus rhamnosus*

2. Identified bioprotection mechanisms of LAB

The major preservative effects on food by LAB is associated with the rapid acidification of raw material due to the production and accumulation of mainly lactic acid. As it is a weak organic acid, with a pKa close to 3.0 and a pH greater than 3 in food, the antimicrobial activity of lactic acid is related to; the denaturation of membrane proteins, blocking transmembrane transport, proton gradient interference, enzyme inhibition, and reactive oxygen species (ROS) production, which disturbs the cell metabolism resulting in growth inhibition [43, 47, 48, 49]. However, higher antagonistic activity can be expected in food with high acidity (pH < 3.0), since the non-dissociated form of acid prevails at these pH conditions. The non-dissociated form of lactic acid is apolar and can cross through the cytoplasmatic membrane of the target microorganism, reaching the cytosol [50]. Once inside the cytoplasm, whose pH is close to neutral (pH > pKa), the lactic acid dissociates form and the release of hydrogen ions promotes the acidification of the cytoplasm. As a general consequence, the internal proteins are denatured and the enzymatic activities are interrupted, leading to the death of the microorganism (Fig. 1) [51].

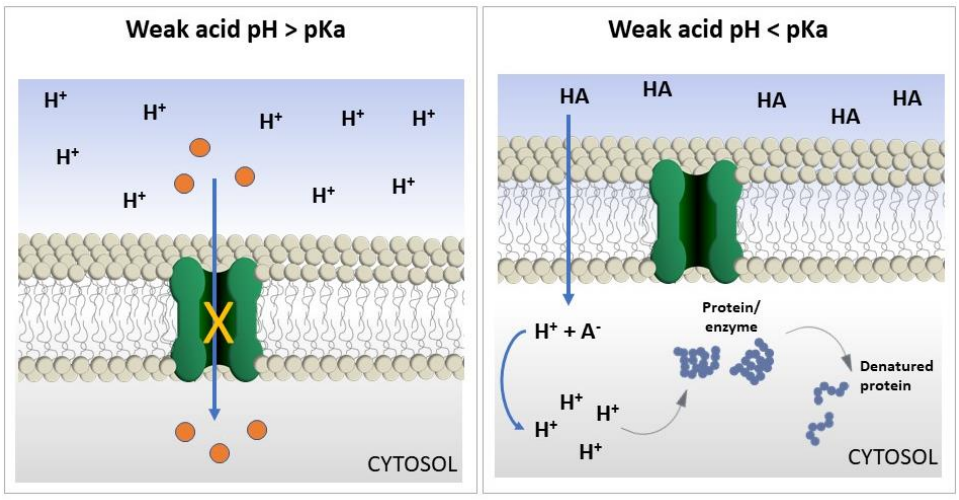


Figure 1. Mechanisms of weak acid.

In addition to lactic acid production, the bioprotective cultures are responsible for producing various other compounds, including; other types of organic acids like acetic, benzoic, formic, succinic, phenyllactic, indole latic and azelaic acids, hydrogen peroxide, acetoin, diacetyl, reuterin and peptides with antimicrobial activity such as bacteriocins (Fig. 2). The action mechanisms of the organic acids produced by LAB are very close to the one described for lactic acid, because they are weak acids [50, 51]. However, some studies suggest that acetic acid, for example, can also inactivate other microorganisms by synergy or through another type of mechanism not yet elucidated [52].

Due to a low molecular weight and absence of charge, hydrogen peroxide crosses the membrane of the target microorganism and reaches the cytoplasm where it is reduced and decomposed to the hydroxyl radical [53]. This radical is highly reactive with organic substances and can promote irreversible damage to enzymes and nucleic acids [54].

In the case of acetoin or diacetyl, both forms can coexist through oxi-reduction reactions, however, studies suggest that these compounds can interact with the arginine amino acid, compromising the structure of some proteins; despite the antagonistic action mechanism of diacetyl not being well established. In relation to diacetyl, another possible mechanism is that this compound can be able to link to DNA molecules, promoting its unfolding [55].

Up until twenty years ago, the exact mechanism of action for reuterin was undefined. This is because reuterin has an aldehyde compound in its molecular composition that is highly reactive and forms several other compounds in an aqueous solution. Thus, studies have characterized that this compound can induce oxidative stress in cells, through the modification of thiol groups in proteins or in small molecules [17, 56].

Regarding bacteriocins by definition are synthesized ribosomal antimicrobial peptides produced by bacteria and which have activity against another bacteria. Their activity can be between same species as narrow spectrum, or among other genera as broad spectrum. As a

defense measure, bacteriocin-producing organisms are immune to their own bacteriocins [57, 58].

The bacteriocins studied can be classified and defined into classes: Class I - small modified peptides; Class II - unmodified peptides and Class III - large peptides > 10 kDa, that are based on their biosynthesis, mechanism, size, and type of molecule (Fig. 2) [59, 60]. Also according to a significant increase in the characterization of new bacteriocins, there is a difficulty in performing the classification. Considering this, new class proposals will emerge [61]. Also, other types of bacteriocins, their mechanisms and classifications possible are well documented in a review presented by Lozo, Topisirovic and Kojic [62]. According to the regulatory agency of the FDA (Food and Drug Administration) and the European Union, nisin is allowed for commercialization and others preparations containing pediocin PA-1 also can use in the food industry as food preservative [63, 64]. However, the application of nisin in food should be improved as it can interact with the food matrix (adsorption to salts, fat, and protein surfaces) and lose antimicrobial activity [65].

It is also important to note that although nisin is a biologically synthesized antimicrobial compound, perhaps it cannot be claimed as a clean label. This is because it is a food additive included and approved for use in foods (Code E234 for Europe) [146]. So, can generate confusion for a consumer, that is, when seeing the food additive claim in the list of ingredients, the consumer is induced to think that it is not something clean label.

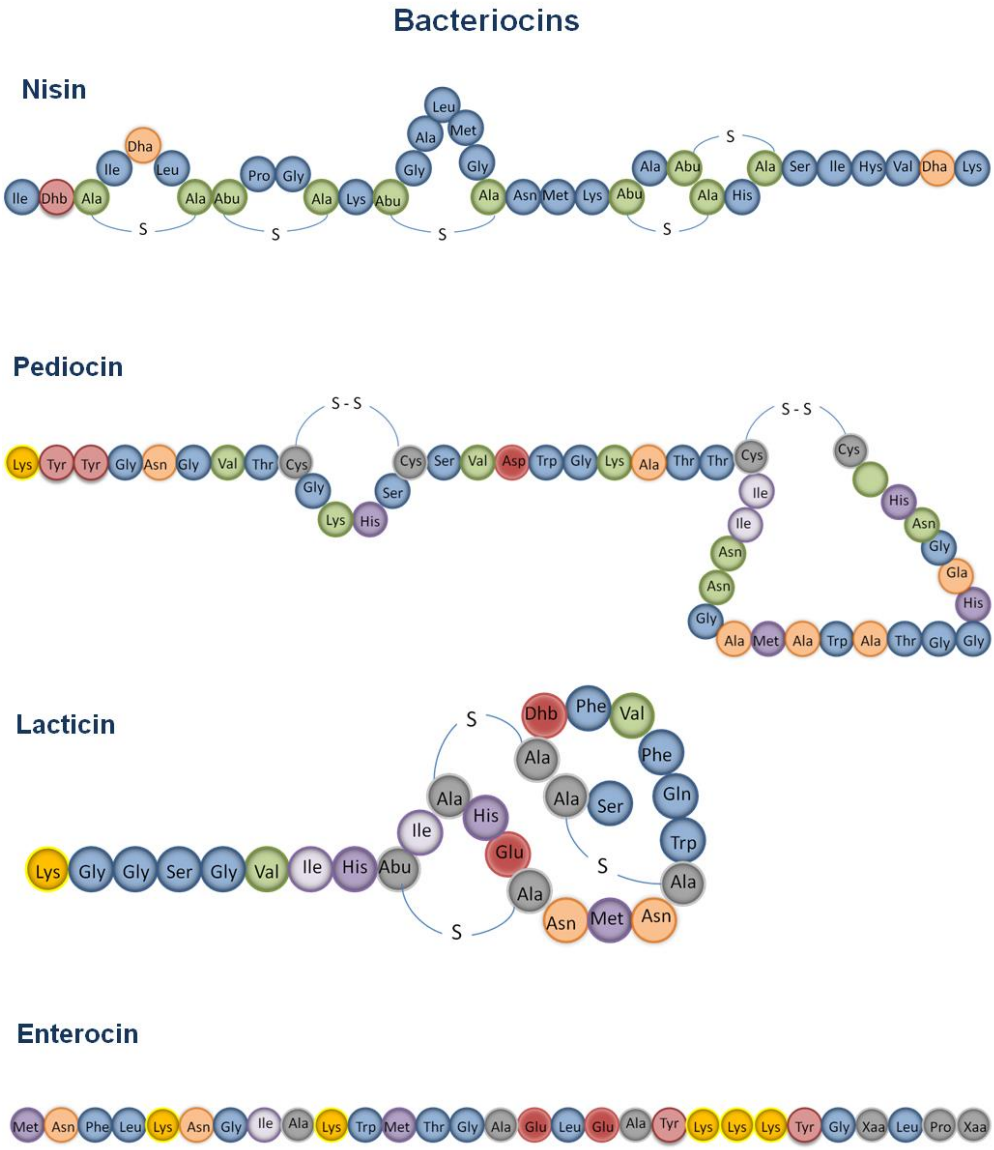


Figure 2. Bacteriocin molecules.

Bacteriocins show several action mechanisms against microorganisms, and these are different from antibiotics. As already well documented in another review by Cotter, Ross and Hill [66] and Lozo et al. [62], some bacteriocins, especially those that act on Gram-positive bacteria, work by targeting the cell wall. It is understood that some Class I bacteriocins link to lipid II of the cytoplasmatic membrane, preventing peptidoglycan synthesis. Despite blocking peptidoglycan synthesis, nisin can also insert into the cell membrane, forming pores. Some class II bacteriocins, such as lactococcin A, bind to the pore-forming receptor mannose

phosphotransferase system (Man-PTS), and thus, eventually form pores. Bacteriocins, especially those that act on Gram-negative bacteria, have a particular mechanism of action which is based on the interference of protein synthesis, DNA and RNA; the action inhibits the production of DNA gyrase and RNA polymerase [66]. Indeed, the mechanism of action on Gram-negative bacteria is expected to be similar. The main point to be questioned is the contact of bacteriocin with the membrane due to the presence of an outer layer of lipopolysaccharide (LPS) in these bacteria. Several bacteriocins have been shown to be effective on gram-negative, particularly when they are in combination with compounds that wash out the outer layer of bacteria. The combined use of bacteriocins with ethylenediaminetetraacetic acid (EDTA), for example, is one of the most common strategies for sensitizing Gram-negative bacteria. EDTA acts by promoting the release of the LPS layer and synergistically potentiates the antimicrobial activity of bacteriocins, as reviewed by Prudêncio, Santos and Vanetti [67]. Recent studies report the use of bacteriocins with other compounds, as verified by Soltani et al. [61] that verified the synergistic effect of using Pediocin PA-1 bacteriocins combined with citric acid and/or lactic acid and which inhibited gram negative strains such as *Aeromonas hydrophila* and *Klebsiella pneumoniae*. In another study by Wang et al. [68] verified the synergistic effect of pediocin PA-1 with lactic acid which also inhibited the Gram-negative *A. hydrophila*.

In addition to inhibiting the growth of bacterial cells, some compounds produced by LAB are also related to the inhibition of fungal toxin production. According to Guimarães *et al.* [18], *L. plantarum* UM55 was able to inhibit the growth and aflatoxin production in five species of *Aspergillus.* According to the same authors, the absence of aflatoxin production is not associated to low fungal growth, but related to the production of phenylactic acid (PLA), hydroxyphenylactic acid (OH-PLA) and indolatic acid (ILA) by *L. plantarum* UM55. In addition to repressing fungal toxin production, compounds released by LAB also inhibit the

growth of against some types of fungi. Zhao *et al.* [69] verified that compounds produced by *L. plantarum* were able to inhibit *Aspergillus niger, Aspergillus oryzae, Trichoderma longibrachiatum, Aspergillus flavus* and *Fusarium graminearum.* In another study performed by Guimarães *et al.* [70], compounds produced by *L. plantarum* and *Lactobacillus buchneri* were able to prevent the growth of *Penicillium nordicum* as well ochratoxin production.

Although several studies demonstrate that LAB cultures have antagonistic activity on fungi and prevent toxin production in foods, the exact mechanism of action remains unclear for now [71, 72].

3. Steps for identifying a potential bioprotective LAB

For the application LAB as a bioprotective culture in the food industry, a series of characteristics need to be assessed. These include; genetic stability; efficiency at low concentrations against a wide spectrum of pathogens and spoilage microorganisms in different food matrices; low nutritional requirements; survival in harsh environments, including food processing conditions; and not be pathogenic or toxic to humans [7, 73].

The development of new bioprotective LAB cultures with the potential to be commercialized and applied in the food industry requires several research steps which will be detailed in the following sections.

3.1. Isolation and identification of LAB strains

The LAB strains are distributed in a wide variety of ecosystems, including fresh and fermented foods, the gastrointestinal tract and mucosa of humans and animals, feces, water, pasture, leaf surfaces, rocks, the surface of equipment, and utensils used in food manufacture [13, 74, 75, 76, 77, 78]. Many studies suggest that LAB with antagonistic activity against pathogens and spoilage microorganisms can be isolated from fresh food, like milk, fruits, vegetables, fish, and even some meat products [13, 22, 26, 40, 62, 79].

Properly following the basic laboratory procedures for the isolation of a bacteria is essential to obtain success in the isolation and identification of a strain with desired characteristics and thus avoid problems such as loss of viability and changes in its antimicrobial activity. More information on these basic procedures can be found in a microbiological methods manual such as the one described by Da Silva *et al* [80].

In general, all bacteria have specific biochemical and physiological growth requirements and, therefore, the formulated culture media must contain the required nutrients to support the microorganism growth. In regards to LAB, a considerable number of representative bacteria are fastidious, and require a rich and complex medium with different carbon sources [81, 82, 83, 84]. LAB cultivation media generally has several sources of nitrogen (such as peptone, and yeast extract), minerals (such as Mn^{2+} and Mg^{2+}), and buffering agents (such as sodium acetate). Monosaccharides are the main sources of energy and carbon required by bacteria, but other substrates can also perform these functions [85]. The Man Rogosa and Sharpe (MRS) medium is the most well-known and the oldest medium for LAB isolation, and was developed in 1960 for the selective cultivation of *Lactobacillus* species [86]. In 1975, Terzaghi and Sandine [87] developed the M17 medium for the cultivation of the *Streptococcus* bacteria genus. Since then, other specific media, such as the MSE was developed by Mayeux *et al.* [88], and MRS has had its composition revised and modified to support the growth of specific LAB [89, 90]. Likewise, Saccaro *et al.* [90] enumerated *L. rhamnosus* and *L. acidophilus* on MRS supplemented with vancomycin and orclindamycin, while Vinderola & Reinheimer [89] enumerated *L. acidophilus* in MRS modified with trehalose.

Regarding LAB with bioprotective capacity, studies carried out using only standard media, such as MRS, M17 and MSE, with few modifications or supplementation were sufficient for the isolation of new strains [91, 92]. After isolation of new strains, verify the

biochemical characteristics is the important point for select the specific bacteria. The biochemical tests are based on the biochemical characteristics of the bacteria, these include the production of enzymes like catalase and oxidase; capacity to ferment specific carbohydrates such as lactose, mannitol, maltose, fructose, glucose, xylose, and esculin; or the production of specific compounds such as diacetyl and acetoin. The biochemical tests aim to eliminate the colonies with biochemical characteristics that are different from LAB [93]. Despite biochemical tests being used in several research for reducing the number of colonies that need be genetically sequenced, these tests have been considered questionable, since LAB can assume different biochemical behaviors due to the constant genetic evolution of the strains in the environment [94]. In addition to genetic evolution, is know that most of the characteristics assumed by LAB are associated with the presence of plasmids in them and that they are important in carrying genes responsible for modifying and/or maintaining the characteristics of LAB in its ecological niche [95].

LAB screening is finished with the genome sequencing of all microorganisms that have gone through all the previous steps and, in this case, the bacterium is identified to the genus and species level [96]. Genome sequencing can be carried out using individual genes or the entire genome. In both cases, the data obtained by sequencing is compared with sequences deposited in the NCBI database (National Center for Biotechnological Information), allowing for the identification of the isolated microorganisms.

3.2. Characterization of potential LAB bioprotection activity

Apart from being applied to LAB identification, genomic sequencing data can also be used to select potential bioprotective strains. Nowadays, at NCBI, more than 34,000 complete bacterial genomes are available, in addition to genes of interest [97]. Thus, through genome analysis, genes, operons or gene groups of interest can be searched, these include those related

to the synthesis of antimicrobial compounds; therefore, this ability can considerably favor the selection of a new bioprotective strain.

In a study carried out by Fusieger *et al.* [98], a strain of *Lactococcus lactis* subsp. *lactis* bv. diacetylactis had the complete gene cluster for nisin synthesis and export, similar to that of nisin Z. Urso *et al.* [99] demonstrated through sequencing and expression analyses the ability of a *L. sakei* strain to produce sakacin P. In another study carried out by Qi *et al.* [100], three complete gene clusters involved in the synthesis and secretion of homologous paracin bacteriocins, and 7 clusters of new bacteriocins were identified in a strain of *L. paracasei*.

Although genomic research yields clues, genetic analysis does not rule out the need for *in vitro* or *in situ* (inside the food matrix) analysis. This is because a bacterium may have a gene related to the production of bacteriocins, organic acids or any other antimicrobial compound but not express them under certain culture conditions.

Some studies demonstrate that LAB strains with bioprotection potential in food may have particular characteristics, such as the production of specific acids, these include: succinic acid, vanillic acid, hydroxyisocaproic acid and others with antifungal activity - phenylpropanoic acid, hydroxyphenyllactic acid, propanoic acid, DL-pyroglutamic acid, 5-oxoproline, pidolic acid and hydrocinnamic acid [101].

For characterization of the metabolite profile produced by LAB, chromatography is the most used [102]. When compound identification is also linked to separation analysis, different types of techniques can be employed, including liquid chromatography (LC), gas chromatography (GC), capillary electrophoresis (CE) and supercritical gas chromatography (SFC) [103]. As an example, for the characterization of the organic acid profile, techniques such as GC-MS, which is a gas chromatography coupled with mass spectrometry, can be explored. Bacteriocins can be characterized using HPLC – RP which is a reversed-phase chromatography (RP-HPLC) technique, which separates components of a mixture by the

difference in hydrophobicity (Table 2). Thus, these compounds can be detected, quantified and fractionated.

For the chromatographic methods used in metabolite separation procedures, the most common systems in use are those based on the reverse phase (RP) and the hydrophilic interaction chromatograph (HILIC). RP-based methods are used to separate average and non-polar metabolites, and the HILIC system for polar metabolites that cannot be retained in RP. In detection methods, systems coupled to mass spectroscopy (MS) is widely used; however, the efficiency of compound detection may depend on the matrix complexity and the analyte [101, 104].

Table 2.
Application of techniques for identification of compounds from bioprotective cultures.

Technique employed	Some detected metabolite	Reference
HPLC	Lactic, citric, acetic, succinic PLA, OH-PLA acids	[101, 105] [106]
HPLC – RP	Benzoic Bacteriocin	[102] [107]
ESI- MS/MS	Short polycyclic lactates	[102]
GC-MS	6-octadecenoic acid methyl ester, hexadecanoic acid methyl ester, phenol, 2-4 bis (1,1 dimethylethyl)	[108]
GC-FID	Stearic, palmitic, oleic, mystiric caproic, caprylic acids	[101]
HS-GC-MS	7-methyl-Z-tetradecen-1-ol acetate, 9-Hexadecenoic acid, 9-Octadecenamide	[109]

Sharaf *et al.* [109] used the Headspace GC–MS technique for the characterization of several compounds produced by *Lactobacillus helveticus* and *L. plantarum.* Tian *et al.* [104] also used this same technique to identify volatile compounds, mainly diacetyl and acetoin produced by *L. plantarum.* Also, through an innovative and recent technique, biochromatography coupled with reversed-phase high-performance liquid chromatography (RP-HPLC), Pei *et al.*, [107] managed to identify a new type of bacteriocin found in the cell suspension of *L. plantarum*.

3.3. Safety aspects of bioprotective cultures

In order to have new bioprotective cultures on the market, it is important to highlight the risks and to assess the safety aspects related to the strains. Despite some LAB showing beneficial effects on consumer health and have the GRAS status, certain strains can produce harmful substances, such as the biogenic amines, synthesize enzymes that degrade human's tissues, such as hemolysins, gelatinases and cytolysins; or disseminate and/or transfer antibiotic resistance genes to pathogens [110].

Therefore, before a bacterium can be approved as a bioprotective culture, it is necessary to prove that the strain is safe for the host [10, 11]. As already mentioned, bacteria of the genus *Lactobacillus* have been historically used as bioprotective agents and probiotics, followed by *Streptococcus*, *Leuconostoc* and *Pediococcus*; and are considered safe due to their history of use [111, 112, 113, 114]. However, even in isolated cases involving patients with underlying medical conditions such as underweight neonates, adults and babies in intensive care units and postoperative patients, these LAB genera have already been associated with systemic infections [111, 115, 116, 117, 118, 119, 120].

Concerning these aspects, strain safety should be demonstrated through *in vitro* and *in vivo* testing. One of them includes the expression of virulence or antibiotic resistance genes, such as *Enterococcus* spp., which can carry virulence genes and express them when applied to the food matrix. One of the major concerns regarding microorganisms with antibiotic resistance genes is the horizontal transfer of these genes to other lactic acid cultures and/or other pathogenic bacteria [121, 122, 123]. Another is the research on virulence factors that include production of biogenic amines, toxins, toxic metabolites, enzymes such as hemolysins and gelatinase [111, 124]. Regarding the production of biogenic amines, these compounds have organic, heterocyclic and aromatic bases. They are molecules that are generated primarily by the decarboxylation of their corresponding precursor amino acid [125,

126]. The amines have the potential to cause health risks to consumers by increasing blood pressure, causing food poisoning and can also react with nitrite to form carcinogenic nitrosamines [127]. When microorganisms have high proteolytic activity, the chances of biogenic amine formation increase, due to the availability of free amino acids [128]. Furthermore, many lactic acid cultures are able to convert amino acids into biogenic amines, such as *Lactococcus* [129, 130, 131] and *Lactobacillus* [126, 131] through amino acid decarboxylation, or transamination of aldehydes or ketones [132].

Also, is important the determination of hemolytic activity if the evaluated strain belongs to a species with known hemolytic potential [111, 124]. Hemolytic activity is an important factor in the selection of bioprotective and probiotic cultures, as it is associated with the ability of the strains to use the iron ions of red blood cells; which can trigger anemia and edema in the host [133]. The most common test for the determination of hemolytic activity in bacteria is based on the inoculation of these strains on to blood agar. The formation of halos around the colonies indicates a positive reaction for hemolytic activity; with clear halos indicating β-hemolysis, green halos to α-hemolysis and the absence of halos determines γ-hemolysis [134, 135]. Finally, the production of gelatinase which is considered a metalloendopeptidase – a proteolytic enzyme capable of hydrolyzing collagen, gelatin, insulin, casein and other peptides [136, 137]. The gelatinase substrates are identified in order to understand the function of these enzymes in the execution of their regulation, in which the main objective is to supply nutrients for the bacteria to cause different physiological and pathological responses in the host such as vascular diseases, tumors, inflammation, infectious and degenerative diseases [138].

3.4. *In vitro* and *in situ* tests

To validate if LAB strains are able to produce antagonistic substances, the adoption of *in vitro* tests is the simplest way to evaluate typical pathogenic and spoilage microorganisms

[19, 39]. The *in vitro* tests are relatively easy, fast, and cheap to perform; however, no information is generated regarding the interaction between the antimicrobial substances and the food matrix [101].

After *in vitro* evaluation, the selected LAB strains should be analyzed by means of *in situ* inhibition bioassays, assessing if they have the capacity for biocontrol [35, 101, 139]. Regarding *in situ* inhibition, the assay can be experimentally designed to apply the potential bioprotective culture directly to the food or to incorporate during its processing.

Aljasir *et al.* [140] evaluated the efficiency of individual and combined bioprotective cultures of *P. acidilactici*, *L. curvatus*, *L .plantarum*, *Carnobacterium* spp. using a direct test on raw milk; they concluded that both the individual and combined culture tests had an antimicrobial effect on *L. monocytogenes*. Macieira *et al.* [14] applied *L. plantarum* onto Traditional Portuguese Sausage and verified that the strain had an antagonistic effect on *L. monocytogenes*. Siroli *et al.* [13] demonstrated that *L. plantarum* was able to increase the shelf-life of minimally processed sliced apples and lamb's lettuce for up to 9 days when used alone, and up to 16 days when combined with other natural antimicrobials.

Besides incorporating the LAB strains directly into food, other *in situ* testing strategies involve the reproduction of the food matrix in a model system. This model system is normally created to optimize assays to reduce the time and price of analyses. Garnier *et al.* [141] tested the antifungal capacity of LAB cultures in a model system that mimics cheese (mini cheeses) in 24-well plates. The authors verified that there was antifungal activity in the tested LAB. However, as there were many tests performed as a way of optimizing analyses, it was noticed that this activity can vary according to the batches, manufacturing method, and care, among others. In the same contexts, Salas *et al.* [4] created models that imitated dairy cheese and yogurt, to evaluate the antifungal activity of LAB combinations on some types of fungi. They

found that two types of combinations were effective on fungi such as *Penicillium commune*, *Mucor racemosus* and *Rhodotorula mucilaginosa*, both in *in vitro* and *in situ* tests [4].

Despite the substantial number of studies, few bioprotective cultures are available in the market due to restrictions such as the differences in effectiveness between *in vitro* and *in situ* assays, sensorial impacts on the food, safety and maintenance of cell viability during commercialization [4, 14]. Therefore, tests at a pilot scale are essential to formulate an idea about a real industrial biocontrol scenario exercised by the culture and, thus, scale up its application for an industrial production.

As an example of the discrepancies between *in vitro* and *in situ* tests, Delavenne *et al.* [142] detected 11 LAB strains with good antagonistic activity against fungi in *in vitro* tests. However, among the evaluated strains, only one showed high antagonistic activity, when the *in situ* test was performed directly on the yogurt. Although, some parameters can influence the behavior of a bioprotective culture in the *in vitro* and *in situ* tests. In the case of the *in vitro* test the conditions, parameters such as time and incubation temperature, as well as the composition and/or modifications of the culture medium can be preponderant factors for the strain that has good antagonistic effects [4]. Therefore, adjusting the time/temperature binomial and providing diverse cultivation conditions may or may not favor the bioprotective effect of the selected strain. Le Lay *et al.* [35] tested different compositions of culture media to verify the bioprotective activity of LAB. They identified great differences in antifungal activity between the evaluated culture media and also in the different sugar concentrations tested, they reported that the media with a greater addition of concentrated sugars also increased the production of organic acids that have a high antifungal effect.

Regarding *in vivo* tests, if a culture with bioprotective potential does not behave well as a bioprotective, one of the parameters that must be evaluated is whether this culture is in fact interacting with the food matrix; either for reasons of polarity or if something has been

intentionally added, as some additives inactivate the bioprotective culture. In addition, another extremely important factor to consider is the concentration of the added bioprotective culture in the test, which can also strongly influence the bioprotective effect [35]. In these *in vitro* and *in situ* tests, any adverse factor must be eliminated for the culture to perform its bioprotection role well. And if in fact the culture presents a good bioprotective effect in both tests, the directive is that the next step is carried out, which is the application of tests in a pilot plant. In this way, the conditions for formulating the food product and application of the culture with bioprotective potential are closer to real conditions [4].

3.5. Pilot tests

Once the bioprotective potential of a strain has been verified and proven in both *in vitro* and *in situ* tests, the last step is to carry out the tests on a pilot scale. This stage is carried out as one of the last phases of the strategy to identify a bioprotective culture. This step is performed last because it involves a greater amount of resources; where it aims evaluate the potential that the culture will in fact meet the needs of the market. The pilot test tries to mimic the conditions of a large-scale industrial production, but is carried out on smaller-scale equipment (size and energy demand), also with the aim of reducing analysis costs and preventing the industrial processing plant from being paralyzed, even temporarily, to carry out the test. In these cases, the bioprotective culture can be added during product processing or inoculated during the final stages of processing, depending on the food product.

The main LAB cultures currently available on the market are for the production of cheese (Table 1B), because these bacteria can produce acids that, when combined with the acids already naturally present in the product, will not significantly interfere with its sensorial characteristics [12]. Using cheese production as an example, in a pilot scale, following each step of the process is essential for maintaining the characteristics of the final product, as well as for the effectiveness of the protective culture. Some care is required when adding the

protective culture, whether lyophilized or not; this includes considerations such as the temperature of the milk being close to the ideal action temperature of the culture (between 30 and 36°C), pH should be suitable, and the absence of antibiotics and contaminants that can influence the development of the culture. In general, the time for the adaptive response of the culture must also be considered. Therefore, before the addition of ingredients and the coagulation process, a minimum period of time (20 to 30 minutes) must be respected, to adapt the culture to the environment. During production, some process parameters can also be evaluated (yield, fermentation time, curd and pH) to assess the effect of the bioprotective culture on the process [9]. During ripening and storage time, some parameters can also be evaluated (degree of proteolysis, texture, ripening time if it is a ripening cheese, enumeration of fungi and LAB).

The determination of shelf life is extremely important in this process and the comparison between the product with the added and non-added culture must be carefully carried out to prove the effectiveness at a larger scale. Also, as proof of effectiveness, the sensory aspects need to be evaluated, as the bioprotective culture should not, or as little as possible, alter these attributes [9]. In this way, texture, color, aroma, flavor and taste are prioritized in sensorial tests. Li *et al.* [12] evaluated the potential use of a strain of *L. casei* as a bioprotective culture in yogurt and, in addition to the bioprotective effect, the authors also evaluated whether the culture influenced the attributes of color, texture, flavor and taste. According to the sensorial test performed, no alteration in these attributes was significantly perceived. Cosentino *et al.* [143] evaluated attributes such as taste and aroma and found no significant differences between Caciotta cheese samples with or without bioprotective strains of lactobacilli. In another work, the authors verified that combinations of LAB cultures promoted significant differences in the perception of the acidity attribute of cheese and sour cream. However, the samples were accepted by consumers, indicating that although some

differences were noticed, the final product was not completely modified and accepted [4]. Therefore, when adding a bioprotective culture, or combinations of them, checking the recommended dosage is essential so that an excess of metabolites are not produced to the point of modifying the sensorial characteristics of the product.

After these evaluations, if the protective characteristics of the culture are confirmed by the pilot tests, and it is also verified that it does not influence the sensorial aspects of the food, the next step is to carry out the certification of the strains for commercialization; whose proceedings can vary from one country to another [144]. In the United States, the agency responsible for certifying microbial cultures for food application is the FDA, granting the well-known expression, GRAS Status [11]. The countries of America, as a whole, for culture certification follow the recommendations governed by the FDA. In Europe, certification is made by the EFSA (European Food Safety Authority), which grants QPS (Qualified Presumption of Safety) Status [144].

A review carried out by Laulund *et al.* [145] presented some countries that have their own regulations for marketing within their own country, such as Japan, Thailand, China and Malaysia. The first nation with national legislation that required safety approval for cultures applied to food was Denmark and, in 2010, it no longer required approval for the marketing of culture, but notification of a new strain (taxonomy) is required [144]. It is still a global challenge to certify ideal bioprotective cultures in the world market, considering that LAB cultures provide benefits to the food product, whether in fermentation or for some probiotic potential and improvement of sensorial properties; and if safe, these can already then be certified and marketed. However, current cultures with the bioprotective effect designation can be certified for food application not have the necessary efficacy. This certification step is the last of the development of bioprotective cultures in food preservation process and, therefore, after certification, the process of sales and marketing begins.

4. Conclusion

Independent of the studies and complete characterization on the compound profiles, the protective effect of biopreserving cultures can suffer variations in different and complex food matrices, and with the different species and their combinations. It is still a challenge to develop bioprotective cultures with significant effects, due to the complexity of interactions and food matrices. In addition, granting GRAS or QPS status requires complete studies that are often not in accordance with what the law requires. Thus, more research is needed to understand the performance of metabolites in bioprotection in the complex environment of food matrices. In addition, cultures can be combined with other methods of preservation and synergy as a way to ensure the effectiveness.

The current challenge in the development of effective protective cultures lies in identifying the compounds produced in the different food matrices, and the action/efficacy on different types of target microorganisms. Many of the compounds were not detected by the techniques employed; however, advancement in the sensibility and precision of the analytical tools can be the key factor in isolating and identifying potential compounds with antimicrobial activity.

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. References

[1] Mordor Intelligence, “Clean Label Ingredient Market – Growth, Trends, Covid-19 impact, and forecast 2021-2026”, Accessed November 22, 2021. https://www.mordorintelligence.com/industry-reports/clean-label-ingredients-market#; https://www.globenewswire.com/news-release/2021/07/21/2266211/0/en/The-clean-label-ingredient-market-is-expected-to-reach-an-estimated-75-2-billion-by-2026-with-a-CAGR-of-6-7-from-2020-to-2026.html /

[2] Y. Miyah, M. Benjelloun, S. Lairini and A. Lahrichi, “COVID-19 Impact on Public Health, Environment, Human Psychology, Global Socioeconomy, and Education”, *The Scientific World Journal*, *2022*. https://doi.org/10.1155/2022/5578284

[3] B. Katz and L. A. Williams, “Cleaning up processed foods”. Food Technology, vol.65, no. 12, 2011.

[4] M. L. Salas, A. Thierry, M. Lemaître et al., “Antifungal Activity of Lactic Acid Bacteria Combinations in Dairy Mimicking Models and Their Potential as Bioprotective Cultures in Pilot Scale Applications”, *Frontiers in microbiology*, vol. 9, pp. 1–18, 2018. https://doi.org/10.3389/fmicb.2018.01787

[5] M. Ebrahimi, A. Sadeghi and S.A. Mortazavi, “The use of cyclic dipeptide producing LAB with potent anti-aflatoxigenic capability to improve techno-functional properties of clean-label bread”, *Annals of microbiology*, vol. 70, no. 1, pp. 1-12, 2020. https://doi.org/10.1186/s13213-020-01571-y

[6] F.K. Lücke. Fermented meat products. *Food research international*, vol. 27, no. 3, pp. 299-30, 1994. https://doi.org/10.1016/0963-9969(94)90098-1

[7] W.H. Holzapfel, R. Geisen and U. Schillinger, “Biological preservation of foods with reference to protective cultures, bacteriocins and food-grade enzymes”, *International*

journal of food microbiology, vol. 24, pp. 343-362, 1995. https://doi.org/10.1016/0168-1605(94)00036-6

[8] M.E. Stiles, "Biopreservation by lactic acid bacteria", *Antonie van Leeuwenhoek*, vol. 70, pag. 331-345, 1996. https://doi.org/10.1007/BF00395940

[9] G. Vignolo and S. Fadda, "Starter cultures: Bioprotective cultures", *Handbook of fermented meat and poultry*, pp.147-157, 2015. https://doi.org/10.1002/9780470376430.ch14

[10] European Food Safety Authority (EFSA), "Opinion of the Scientific Committee on a request from EFSA related to a generic approach to the safety assessment by EFSA of microorganisms used in food/feed and the production of food/feed additives", *EFSA Journal*, vol. 3, pp. 226, 2005. https://doi.org/10.2903/j.efsa.2005.226

[11] Food & Drug Administration (FDA), *CRF – Code of Federal Regulations. Title 21. Food and Drugs. Chapter I – Departament of health and human services. Subchapter F – Biologics. Part 610 – General Biological Products Standars – Subpart B – General provisions, Sec. 610.18 Cultures*, 2019. https://www.accessdata.fda.gov/scripts/cdrh/cfdocs/cfcfr/CFRSearch.cfm?fr=610.18/

[12] H. Li, L. Liu, S. Zhang et al., "Potential use of *Lactobacillus casei* AST18 as a bioprotective culture in yogurt", *Food Control*, vol. 34, no.2, pp. 675-680, 2013. https://doi.org/10.1016/j.foodcont.2013.06.023

[13] L. Siroli, F. Patrignani, D. I. Serrazanetti et al., "Lactic acid bacteria and natural antimicrobials to improve the safety and shelf-life of minimally processed sliced apples and lamb's lettuce", *Food Microbiology*, vol. 47, pp. 74–84, 2015. https://doi.org/10.1016/j.fm.2014.11.008

[14] A. Macieira, D. Barros, M. Vaz-Velho et al, "Effects of *Lactobacillus plantarum* Bacteriocinogenic Culture on Physicochemical , Microbiological , and Sensorial Characteristics of " Chouriço Vinha d ´ Alhos ", a Traditional Portuguese Sausage", *Journal*

of food quality and hazards control, vol. 5, pp. 118–127, 2018. https://doi.org/10.29252/jfqhc.5.4.2

[15] H. Mathur, T. P. Beresford and P. D. Cotter, "Health benefits of lactic acid bacteria (LAB) fermentates", *Nutrients*, vol. 12, no. 6, 2020. https://doi.org/10.3390/nu12061679

[16] J. Zheng, S. Wittouck, E. Salvetti et al, "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*, vol. 70, no. 4, pp. 2782-2858, 2020. https://doi.org/10.7939/r3-egnz-m294

[17] L. Schaefer, T. A. Auchtung, K. E. Hermans, K. E. et al., "The antimicrobial compound reuterin (3-hydroxypropionaldehyde) induces oxidative stress via interaction with thiol groups", *Microbiology*, vol. 156, pp. 1589-1599, 2010. https://doi.org/10.1099/mic.0.035642-0

[18] A. Guimarães, A. Santiago, J. A.,Teixeira, A. Venâncio and L. Abrunhosa, "Anti-aflatoxigenic effect of organic acids produced by *Lactobacillus plantarum", International Journal of Food Microbiology*, vol. 264, pp. 31–38, 2018a. https://doi.org/10.1016/j.ijfoodmicro.2017.10.025

[19] R. Trias,E. Badosa, E. Montesinos and L. Bañeras, "Bioprotective *Leuconostoc* strains against *Listeria monocytogenes* in fresh fruits and vegetables", *International Journal of Food Microbiology*, vol.127, pp. 91-98, 2008. https://doi.org/10.1016/j.ijfoodmicro.2008.06.011

[20] N. Mirkovic, J. Kulas, Z. Miloradovic et al., "Lactolisterin BU-producer *Lactococcus lactis* subsp. *lactis* BGBU1-4: Bio-control of *Listeria monocytogenes* and *Staphylococococcus aureus* in fresh soft cheese and effect on immunological response of

rats", *Food Control*, vol. 111, no. 107076, 2020. https://doi.org/10.1016/j.foodcont.2019.107076

[21] T. Aymerich, M. Rodríguez, M. Garriga, M. and S. Bover-cid, "Assessment of the bioprotective potential of lactic acid bacteria against *Listeria monocytogenes* on vacuum-packed cold-smoked salmon stored at 8 ° C", *Food Microbiology*, vol. 83, pp. 64–70, 2019. https://doi.org/10.1016/j.fm.2019.04.011

[22] P.M. Kaktcham, L.T. Piame, G. Sileu et al., "Bacteriocinogenic *Lactococcus lactis* subsp. *lactis* 3MT isolated from freshwater Nile Tilapia: isolation, safety traits, bacteriocin characterisation, and application for biopreservation in fish pâté", *Archives of Microbiology*, vol. 201, pp. 1249–1258, 2019. https://doi.org/10.1007/s00203-019-01690-4

[23] J. C. C. P. Costa, S. Bover-Cid, A. Bolívar, G. Zurera and F. Pérez-Rodríguez, "Modelling the interaction of the sakacin-producing *Lactobacillus sakei* CTC494 and *Listeria monocytogenes* in filleted gilthead sea bream (*Sparus aurata*) under modified atmosphere packaging at isothermal and non-isothermal conditions. International", *Journal of Food Microbiology*, vol. 297, pp. 72–84, 2019. https://doi.org/10.1016/j.ijfoodmicro.2019.03.002

[24] A. Danza, A. Lucera, P. Lavermicocca et al., "Tuna Burgers Preserved by the Selected *Lactobacillus paracasei* IMPC 4.1 Strain", *Food and Bioprocess Technology*, vol. 11, pp. 1651–1661, 2019. https://doi.org/10.1007/s11947-018-2129-y

[25] M. Ramaroson, S. Guillou, A. Rossero et al., "Selection procedure of bioprotective cultures for their combined use with High Pressure Processing to control spore-forming bacteria in cooked ham", *International Journal of Food Microbiology*, vol. 276, pp. 28–38, 2018. https://doi.org/10.1016/j.ijfoodmicro.2018.04.010

[26] N. P. A. de Castilho, S.D. Todorov, L.L. Oliveira, L. S. Bersot and L. A. Nero, "Inhibition of *Listeria monocytogenes* in fresh sausage by bacteriocinogenic *Lactobacillus*

curvatus UFV-NPAC1 and its semi-purified bacteriocin", *LWT - Food Science and Technology*, vol. 118, no. 108757, 2020. https://doi.org/10.1016/j.lwt.2019.108757

[27] O. B. Braïek, S. Smaoui, K. Ennouri et al., "RAPD-PCR characterisation of two *Enterococcus lactis* strains and their potential on *Listeria monocytogenes* growth behaviour in stored chicken breast meats: Generalised linear mixed-effects approaches", *LWT - Food Science and Technology*, vol. 99, pp. 244–253, 2019. https://doi.org/10.1016/j.lwt.2018.09.053

[28] G. K. İncili, P. Karatepe and O. İ. İlhak, "Effect of chitosan and *Pediococcus acidilactici* on *E. coli* O157:H7, *Salmonella Typhimurium* and *Listeria monocytogenes* in meatballs", LWT - *Food Science and Technology*, vol. 117, no. 108706, 2020. https://doi.org/10.1016/j.lwt.2019.108706

[29] M. Giello, A. La Storia, F. De Filippis, D. Ercolini, and F. Villani, "Impact of *Lactobacillus curvatus* 54M16 on microbiota composition and growth of *Listeria monocytogenes* in fermented sausages", *Food Microbiology*, vol. 72, pp. 1–15, 2018. https://doi.org/10.1016/j.fm.2017.11.003

[30] L. Iacumin, M. Manzano, D. Andyanto and G. Comi, G, "Biocontrol of ochratoxigenic moulds (*Aspergillus ochraceus* and *Penicillium nordicum*) by *Debaryomyces hansenii* and *Saccharomycopsis fibuligera* during speck production", *Food Microbiology*, vol. 62, pp. 188–195, 2017. https://doi.org/10.1016/j.fm.2016.10.017

[31] S. M. Osés, A. M. Diez, E. M. Gómez et al., "Control of *Escherichia coli* and *Listeria monocytogenes* in suckling-lamb meat evaluated using microbial challenge tests". *Meat Science*, vol. 110, pp. 262–269, 2015. https://doi.org/10.1016/j.meatsci.2015.08.004

[32] A. Sadeghi, M. Ebrahimi, S. A. Mortazavi and A. Abedfar, "Application of the selected antifungal LAB isolate as a protective starter culture in pan whole-wheat sourdough

bread", *Food Control*, vol. 95, pp. 298–307, 2019. https://doi.org/10.1016/j.foodcont.2018.08.013

[33] R. F. Stefanello, E. H. Nabeshima, A. O. Garcia et al., "Stability, sensory attributes and acceptance of panettones elaborated with *Lactobacillus fermentum* IAL 4541 and *Wickerhamomyces anomallus* IAL 4533", *Food Research International*, vol. 116, pp. 973–984, 2019. https://doi.org/10.1016/j.foodres.2018.09.035

[34] I. Mantzourani, S. Plessas, M. Odatzidou et al., "Effect of a novel *Lactobacillus paracasei* starter on sourdough bread quality", *Food Chemistry*, vol. 271, pp. 259–265, 2019. https://doi.org/10.1016/j.foodchem.2018.07.183

[35] C. Le Lay, J. Mounier, V. Vasseur et al., "*In vitro* and *in situ* screening of lactic acid bacteria and propionibacteria antifungal activities against bakery product spoilage molds", *Food Control*, vol. 60, pp. 247–255, 2016. https://doi.org/10.1016/j.foodcont.2015.07.034

[36] P. Lavermicocca, L. Angiolillo, S. L. Lonigro et al., "*Lactobacillus plantarum* 5BG survives during refrigerated storage bio-preserving packaged Spanish-style table olives (cv. Bella di Cerignola)", *Frontiers in Microbiology*, vol. 9, pp. 1–10, 2018. https://doi.org/10.3389/fmicb.2018.00889

[37] Q. Dong, W. Zhang, L. Guo et al., "Influence of *Lactobacillus plantarum* individually and in combination with low O2-MAP on the pathogenic potential of *Listeria monocytogenes* in cabbage", *Food Control*, vol. 107, no. 106765, 2020. https://doi.org/10.1016/j.foodcont.2019.106765

[38] Y. Rao, Y. Tao, Y. Li et al., "Characterization of a probiotic starter culture with anti-: *Candida* activity for Chinese pickle fermentation", *Food and Function*, vol. 10, pp. 6936–6944, 2019. https://doi.org/10.1039/c9fo01191a

[39] J. Ma, Y. Hong, L. Deng, L. Yi and K. Zeng, “Screening and characterization of lactic acid bacteria with antifungal activity against *Penicillium digitatum* on citrus”, *Biological Control*, vol. 138, no.104044, 2019. https://doi.org/10.1016/j.biocontrol.2019.104044

[40] B. Taroub, L. Salma, Z. Manel et al.,“Isolation of lactic acid bacteria from grape fruit: antifungal activities, probiotic properties, and in vitro detoxification of ochratoxin A”, *Annals of Microbiology*, vol. 69, pp.17–27, 2019. https://doi.org/10.1007/s13213-018-1359-6

[41] Z.M. Moghanjougi, M.R. Bari, M. A. Khaledabad, H. Almasi, and S. Amiri, S, “Bio-preservation of white brined cheese (Feta) by using probiotic bacteria immobilized in bacterial cellulose : Optimization by response surface method and characterization”, *LWT - Food Science and Technology*, vol. 117, no. 108603, 2020. https://doi.org/10.1016/j.lwt.2019.108603

[42] M. Ouiddir, G. Bettache, M. L. Salas et al., “Selection of Algerian lactic acid bacteria for use as antifungal bioprotective cultures and application in dairy and bakery products”, *Food Microbiology*, vol. 82, pp. 160–170, 2019. https://doi.org/10.1016/j.fm.2019.01.020

[43] S. Mieszkin, N. Hymery, S. Debaets et al., “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*, vol. 248, pp. 47–55, 2017 https://doi.org/10.1016/j.ijfoodmicro.2017.02.013

[44] S. Morandi, T. Silvetti, G. Battelli and M. Brasca, M. “Can lactic acid bacteria be an efficient tool for controlling *Listeria monocytogenes* contamination on cheese surface? The case of Gorgonzola cheese”, *Food Control*, vol. 96, pp. 499–507, 2019 https://doi.org/10.1016/j.foodcont.2018.10.012

[45] E. Tirloni, C. Bernardi, E. Ghelardi et al., “Biopreservation as a potential hurdle for *Bacillus cereus* growth in fresh cheese”, *Journal of Dairy Science*, vol. 103, pp. 150–160, 2020. https://doi.org/10.3168/jds.2019-16739

[46] R. Anastasiou, A. Aktypis, M. Georgalaki et al., “Inhibition of *Clostridium tyrobutyricum* by *Streptococcus macedonicus* ACA-DC 198 under conditions mimicking Kasseri cheese production and ripening”, *International Dairy Journal*, vol. 19, pp. 330–335, 2019. https://doi.org/10.1016/j.idairyj.2008.12.001

[47] E. R. Kashket, “Bioenergetics of lactic acid bacteria: cytoplasmic pH and osmotolerance”, *FEMS Microbiology Reviews*, vol. 3, pp. 233-244, 1987. https://doi.org/10.1111/j.1574-6968.1987.tb02463.x

[48] S. E. Lindgren and W. J. Dobrogosz, “Antagonistic activities of lactic acid bacteria in food and feed fermentations”, *FEMS microbiology reviews*, vol. 7, no.1-2, pp. 149-163, 1990. https://doi.org/10.1111/j.1574-6968.1990.tb04885.x

[49] P. A. Vandenbergh, “Lactic acid bacteria, their metabolic products and interference with microbial growth”, *FEMS Microbiology Reviews*, vol. 12, no.1-3, pp. 221-237, 1993. https://doi.org/10.1111/j.1574-6976.1993.tb00020.x

[50] V. K. Batish, U. Roy, R. Lal and S. Grower, “Antifungal attributes of lactic acid bacteria—a review”, *Critical reviews in biotechnology*, vol. 17, no. 3, pp. 209-225, 1997. https://doi.org/10.3109/07388559709146614

[51] U. Schillinger and J. V. Villarreal, “Inhibition of *Penicillium nordicum* in MRS medium by lactic acid bacteria isolated from foods”, *Food control*, vol. 21, no.2, pp. 107-111, 2010. https://doi.org/10.1016/j.foodcont.2008.11.010

[52] N. V. Narendranath, K. C. Thomas and W. M. Ingledew, “Effects of acetic acid and lactic acid on the growth of *Saccharomyces cerevisiae* in a minimal medium”, *Journal of*

Industrial Microbiology and Biotechnology, vol. 26, pp. 171–177, 2001. https://doi.org/10.1038/sj.jim.7000090

[53] S. Condon, "Responses of lactic acid bacteria to oxygen", *FEMS Microbiology Reviews*, vol. 3, no. 3, pp. 269-280, 1987. https://doi.org/10.1016/0378-1097(87)90112-1

[54] J. A. Imlay, S. M. Chin and S. Linn, "Toxic DNA damage by hydrogen peroxide through the Fenton reaction in vivo and in vitro", *Science*, vol. 240, no. 4852, pp. 640-642, 1988. https://doi.org/10.1126/science.2834821

[55] B. Cesselin, C. Henry, A. Gruss, K. Gloux and P. Gaudu, "Mechanisms of Acetoin Toxicity and Adaptive Responses in an Acetoin-Producing Species, *Lactococcus lactis*", *Applied and Environmental Microbiology*, vol. 87, no. 24, 2021. https://doi.org/10.1128/AEM.01079-21

[56] M. G. Gänzle and R. F. Vogel, "Studies on the Mode of Action of Reutericyclin", *Applied and Environmental Microbiology*, vol. 69, pp. 1305–1307, 2003. https://doi.org/10.1128/AEM.69.2.1305-1307.2003

[57] T. R. Klaenhammer, "Genetics of bacteriocins produced by lactic acid bacteria", *FEMS microbiology reviews*, vol. no. 12, pp. 39-85, 1993. https://doi.org/10.1111/j.1574-6976.1993.tb00012.x

[58] P. D. Cotter, C. Hill and R. P. Ross, "Bacteriocins: developing innate immunity for food", *Nature Reviews Microbiology*, vol. 3, no. 10, pp. 777-788, 2005. https://doi.org/10.1038/nrmicro1273

[59] P.G. Arnison, M.J. Bibb, G. Bierbaum et al., "Ribosomally synthesized and post-translationally modified peptide natural products: Overview and recommendations for a universal nomenclature", *Natural Product Reports*, vol. 30, pp. 108–160, 2013. https://doi.org/10.1039/c2np20085f

[60] P. Alvarez-Sieiro, M. Montalbán-López, D. Mu and O. P. Kuipers, "Bacteriocins of lactic acid bacteria: extending the family", *Applied microbiology and biotechnology*, vol. 100, pp. 2939-2951, 2016. https://doi.org/10.1007/s00253-016-7343-9

[61] S. Soltani, E. Biron, L. Ben Said, M. Subirade and I. Fliss, "Bacteriocin-Based Synergetic Consortia: a Promising Strategy to Enhance Antimicrobial Activity and Broaden the Spectrum of Inhibition", *Microbiology spectrum*, vol. 10, no. 1, e00406-21, 2022. https://doi.org/10.1128/spectrum.00406-21

[62] J. Lozo, L. Topisirovic and M. Kojic, "Natural bacterial isolates as an inexhaustible source of new bacteriocins", *Applied Microbiology and Biotechnology*, vol. 105, no. 2, pp. 477-492, 2021. https://doi.org/10.1007/s00253-020-11063-3

[63] Food & Drug Administration (FDA). *Nisin preparation: affirmation of GRAS status as a direct human food ingredient*. Fed. Reg. 53, pp. 11247–11251, 1988.

[64] European Commission (EC). *Commission Directive 83/463/EEC 22 of July introducing temporary measures for the designation of certain ingredients in the labelling of foodstuffs for sale to the ultimate consumer*. Official J. Eur. Commun, vol. 255, pp. 1–6, 1983.

[65] E. Chollet, I. Sebti, A. Martial-Gros and P. Degraeve, "Nisin preliminary study as a potential preservative for sliced ripened cheese: NaCl, fat and enzymes influence on nisin concentration and its antimicrobial activity", *Food Control*, vol. 19, pp. 982-989, 2008. https://doi.org/10.1016/j.foodcont.2007.10.005

[66] P. D. Cotter, R. P. Ross and C. Hill, "Bacteriocins—a viable alternative to antibiotics?", *Nature Reviews Microbiology*, vol. 11, no. 2, pp. 95-105, 2013. https://doi.org/10.1038/nrmicro2937

[67] C. V. Prudêncio, M. T. Dos Santos and M. C. D. Vanetti, M. C. D, "Strategies for the use of bacteriocins in Gram-negative bacteria: relevance in food microbiology", *Journal*

of food science and technology, vol. 52, no. 9, pp. 5408-5417, 2015. https://doi.org/10.1007/s13197-014-1666-2

[68] Y. Wang, J. Wang, D. Bai et al., "Synergistic inhibition mechanism of pediocin PA-1 and L-lactic acid against *Aeromonas hydrophila*", *Biochimica et Biophysica Acta (BBA)-Biomembranes*, vol. 1862, no. 10, pp. 183346, 2020. https://doi.org/10.1016/j.bbamem.2020.183346

[69] S. Zhao, X. Hao, F. Yang et al., "Antifungal Activity of *Lactobacillus plantarum* ZZUA493 and Its Application to Extend the Shelf Life of Chinese Steamed Buns", *Foods*, vol. 11, no. 2, p. 195, 2022. https://doi.org/10.3390/foods11020195

[70] A. Guimarães, A. Venancio and L. Abrunhosa, L, "Antifungal effect of organic acids from lactic acid bacteria on *Penicillium nordicum", Food Additives & Contaminants: Part A*, vol. 35, pp. 1803-1818, 2018b. https://doi.org/10.1080/19440049.2018.1500718

[71] J. Schnürer and J. Magnusson, J, "Antifungal lactic acid bacteria as biopreservatives", *Trends in Food Science & Technology*, vol.16, pp. 70-78, 2005. https://doi.org/10.1016/j.tifs.2004.02.014

[72] P. Russo, C. Fares, A. Longo, G. Spano and V. Capozzi, "*Lactobacillus plantarum* with Broad Antifungal Activity as a Protective Starter Culture for Bread Production", *Foods*, vol. 6, no. 110, 2017. https://doi.org/10.3390/foods6120110

[73] L. Topisirovic, M. Kojic, D. Fira, "Potential of lactic acid bacteria isolated from specific natural niches in food production and preservation", *International journal of food microbiology*, vol. 112, no. 3, pp. 230-235, 2006. https://doi.org/10.1016/j.ijfoodmicro.2006.04.009

[74] M.P. Fenton, "An investigation into the sources of lactic acid bacteria in grass silage", *Journal of applied bacteriology*, vol. 62, no. 3, pp. 181-188, 1987. https://doi.org/10.1111/j.1365-2672.1987.tb02397.x

[75] S. H. Duncan, P. Louis and H. J. Flint, “Lactate-utilizing bacteria, isolated from human feces, that produce butyrate as a major fermentation product”, *Applied and environmental microbiology*, vol. 70, no. 10, pp. 5810-5817, 2004. https://doi.org/10.1128/AEM.70.10.5810-5817.2004

[76] J. L. Balcázar, D. Vendrell, I. de Blas et al., “Characterization of probiotic properties of lactic acid bacteria isolated from intestinal microbiota of fish”, *Aquaculture*, vol. 278, pp. 188-19, 2008. https://doi.org/10.1016/j.aquaculture.2008.03.014

[77] M. Colombo, N. P. Castilho, S. D. Todorov and L. A. Nero, “Beneficial properties of lactic acid bacteria naturally present in dairy production”, *BMC microbiology*, vol. 18, no. 1, pp. 1-12, 2018. https://doi.org/10.1186/s12866-018-1356-8

[78] A. Terzić-Vidojević, K. Veljović, M. Tolinački et al., “Diversity of non-starter lactic acid bacteria in autochthonous dairy products from Western Balkan Countries-technological and probiotic properties”, *Food Research International*, vol. 136, no. 109494, 2020. https://doi.org/10.1016/j.foodres.2020.109494

[79] I. Lačanin, J. Mounier, A. Pawtowski et al., “Assessment of the antifungal activity of *Lactobacillus* and *Pediococcus* spp. for use as bioprotective cultures in dairy products”, *World Journal of Microbiology and Biotechnology*, vol. 33, pp. 1-8, 2017. https://doi.org/10.1007/s11274-017-2354-y

[80] N. Da Silva, M. H. Taniwaki, V. C. A. Junqueira et al., “*Microbiological examination methods of food and water: a laboratory manual,*CRC Press, 2018. https://doi.org/10.1201/9781315165011

[81] E. E. Snell, “The nutritional requirements of the lactic acid bacteria and their application to biochemical research”, *Journal of bacteriology*, vol. 50, no. 4, pp. 373-382, 1945. https://doi.org/10.1128/jb.50.4.373-382.1945

[82] E.M. Hébert, R. R. Raya and G. S. Giori, " Evaluation of minimal nutritional requirements of lactic acid bacteria used in functional foods", In *Environmental Microbiology*, pp. 139-148, 2004a. https://doi.org/10.1385/1-59259-765-3:139

[83] E.M. Hébert, R. R. Raya and G. S. Giori, "Nutritional requirements of *Lactobacillus delbrueckii* subsp. *lactis* in a chemically defined medium", *Current microbiology*, vol. 49, pp. 341-345, 2004b. https://doi.org/10.1007/s00284-004-4357-9

[84] E. Vera Pingitore, E. M. Hebert, F. Sesma and M. E. Nader-Macías, "Influence of vitamins and osmolites on growth and bacteriocin production by *Lactobacillus salivarius* CRL 1328 in a chemically defined medium", *Canadian Journal of Microbiology*, vol. 55, pp. 304-310, 2009. https://doi.org/10.1139/W08-092

[85] M. M. O'Donnell, B. M. Forde, B. Neville, P. R. Ross and P. W. O'Toole, "Carbohydrate catabolic flexibility in the mammalian intestinal commensal *Lactobacillus ruminis* revealed by fermentation studies aligned to genome annotations", In *Microbial cell factories*, vol. 10, pp. 1-11, 2011. https://doi.org/10.1186/1475-2859-10-S1-S12

[86] J. C. De Man, D. Rogosa and M. E. Sharpe, "A medium for the cultivation of lactobacilli", *Journal of applied Bacteriology*, vol. 23, no. 1, pp. 130-135, 1960. https://doi.org/10.1111/j.1365-2672.1960.tb00188.x

[87] B. E. Terzaghi and W. Sandine, "Improved medium for lactic streptococci and their bacteriophages", *Applied microbiology*, vol. 29, no. 6, pp. 807-813, 1975 https://doi.org/10.1128/am.29.6.807-813.1975

[88] J. V. Mayeux, J.V., W. E. Sandine and P. R. Elliker, "A selective medium for detecting *Leuconostoc* in mixed-strain starter cultures", *J. Dairy Science*, vol. 45, pp. 655-656, 1962.

[89] C. G. Vinderola and J. A. Reinheimer, "Culture media for the enumeration of *Bifidobacterium bifidum* and *Lactobacillus acidophilus* in the presence of yoghurt bacteria",

International dairy journal, vol. 9, pp. 497-505, 1999. https://doi.org/10.1016/S0958-6946(99)00120-X

[90] D. M. Saccaro, C. Y. Hirota, A. Y. Tamime and N. de Oliveira, "Evaluation of different selective media for enumeration of probiotic micro-organisms in combination with yogurt starter cultures in fermented milk", *African Journal of Microbiology Research*, vol. 5, pp. 3901-3906, 2011. https://doi.org/10.5897/AJMR11.598

[91] P. M. Moraes, L. M. Perin, M. B. T. Ortolani et al., "Protocols for the isolation and detection of lactic acid bacteria with bacteriocinogenic potential", *LWT-Food Science and Technology*, vol. 43, no. 9, pp. 1320-1324, 2010. https://doi.org/0.1016/j.lwt.2010.05.005

[92] N. Noordiana, A. B. Fatimah and A. S. Mun, A. S, "Antibacterial agents produced by lactic acid bacteria isolated from Threadfin Salmon and Grass Shrimp", *International Food Research Journal*, vol. 20, no. 1, pp. 127 – 124, 2013.

[93] I. Sanchez, L. Palop and C. Ballesteros, "Biochemical characterization of lactic acid bacteria isolated from spontaneous fermentation of 'Almagro'eggplants", *International Journal of Food Microbiology*, vol. 59, pp. 9-17, 2000. https://doi.org/10.1016/S0168-1605(00)00256-7

[94] K. S. Makarova and E. V. Koonin, "Evolutionary genomics of lactic acid bacteria", *Journal of bacteriology*, vol. 189, no. 4, pp. 1199-1208, 2007. https://doi.org/10.1128/JB.01351-06

[95] M. Malesevic, N. Stanisavljevic, M. Miljkovic et al., "The large plasmidome of *Lactococcus lactis* subsp. *lactis* bv. *diacetylactis* S50 confers its biotechnological properties", *International Journal of Food Microbiology*, vol. 337, no. 108935, 2021. https://doi.org/10.1016/j.ijfoodmicro.2020.108935

[96] L. Marché, T. Saraoui, B. Remenant et al., “Complete genome sequence of *Lactococcus piscium* CNCM I-4031, a bioprotective strain for seafood products”, *Genome announcements*, vol. 4, pp. 1-5, 2017. https://doi.org/10.1128/genomeA.01510-16

[97] National Center for Biotechnology Information (NCBI) information Genome Brower: https://www.ncbi.nlm.nih.gov/genome/browse/#!/overview/. Accessed 2022.

[98] A. Fusieger, L. M. Perin, C. G. Teixeira, A. F. Carvalho and L. A. Nero, “The ability of *Lactococcus lactis* subsp. *lactis* bv. *diacetylactis* strains in producing nisin”, Antonie van Leeuwenhoek, *International Journal of General and Molecular Microbiology*, vol. 6, pp. 1-12, 2019. https://doi.org/10.1007/s10482-019-01373-6

[99] R. Urso, K. Rantsiou, C. Cantoni, G.Comi, L. Cocolin, “Sequencing and expression analysis of the sakacin P bacteriocin produced by a *Lactobacillus sakei* strain isolated from naturally fermented sausages”, *Applied microbiology and biotechnology*, vol. 71, pp. 480-485, 2006. https://doi.org/10.1007/s00253-005-0172-x

[100] T. Qi, S. Wang, L. Deng, L. Yi and K. Zeng, “Controlling pepper soft rot by *Lactobacillus paracasei* WX322 and identification of multiple bacteriocins by complete genome sequencing”, *Food Control*, vol. 121, no. 107629, 2021. https://doi.org/10.1016/j.foodcont.2020.107629

[101] M. L. Salas, J. Mounier, M. B. Maillard et al., “Identification and quantification of natural compounds produced by antifungal bioprotective cultures in dairy products”, *Food Chemistry*, vol. 301, no. 125260, 2019. https://doi.org/10.1016/j.foodchem.2019.125260

[102] A. Mosbah, E. Delavenne, Y. Souissi et al., “Novel antifungal compounds, spermine-like and short cyclic polylactates, produced by *Lactobacillus harbinensis* K V9.3.1Np in Yogurt”, *Frontiers in Microbiology*, vol. 9, pp. 1–10, 2018. https://doi.org/10.3389/fmicb.2018.02252

[103] J. Pezzatti, J. Boccard, S. Codesido et al., “Implementation of liquid chromatography–high resolution mass spectrometry methods for untargeted metabolomic analyses of biological samples: a tutorial”, *Analytica Chimica Acta*, vol. 1105, pp. 28 – 44, 2020. https://doi.org/10.1016/j.aca.2019.12.062

[104] H. Tian, Y. Shi, Y. Zhang et al., “Screening of aroma-producing lactic acid bacteria and their application in improving the aromatic profile of yogurt”, J*ournal of food biochemistry*, vol. 43, no. 12837, 2019. https://doi.org/10.1111/jfbc.12837

[105] S. Y. Mun, S. K. Kim, E. R. Woo and H. C. Chang, “Purification and characterization of an antimicrobial compound produced by *Lactobacillus plantarum* EM showing both antifungal and antibacterial activities”, *LWT- Food Science and Technology*, vol. 114, no. 108403, 2019. https://doi.org/10.1016/j.lwt.2019.108403

[106] B. Fernandez, A. Vimont, É. Desfossés-Foucault et al., “Antifungal activity of lactic and propionic acid bacteria and their potential as protective culture in cottage cheese”, *Food Control*, vol. 78, pp. 350–356, 2017. https://doi.org/10.1016/j.foodcont.2017.03.007

[107] J. Pei, W. Jin, A. M. Abd El-Aty et al., “Isolation, purification, and structural identification of a new bacteriocin made by *Lactobacillus plantarum* found in conventional kombucha”, *Food Control*, vol. 110, no. 106923, 2020. https://doi.org/10.1016/j.foodcont.2019.106923

[108] M. G. Shehata, A. N. Badr, S. A. El Sohaimy, D. Asker and T. S. Awad, “Characterization of antifungal metabolites produced by novel lactic acid bacterium and their potential application as food biopreservatives”, *Annals of Agricultural Sciences*, vol. 64, pp. 71–78, 2019. https://doi.org/10.1016/j.aoas.2019.05.002

[109] O. M. Sharaf, M. S. Al-Gamal, G. A. Ibrahim et al., “Evaluation and characterization of some protective culture metabolites in free and nano-chitosan-loaded

forms against common contaminants of Egyptian cheese", *Carbohydrate Polymers*, vol. 223, no. 115094, 2019. https://doi.org/10.1016/j.carbpol.2019.115094

[110] M. Saarela, G. Mogensen, R. Fondén, J. Mättö and T. Mattila-Sandholm, "Probiotic bacteria: safety, functional and technological properties", *Journal of biotechnology*, vol. 84, pp. 197-215, 2000. https://doi.org/10.1016/S0168-1656(00)00375-8

[111] Food and Agriculture Organization/World Health Organization - FAO/WHO, *Guidelines for the Evaluation on Probiotics in Food*. London, Ontario, Canada. 11p, 2002.

[112] M. P. Vélez, K. Hermans, T. L. A. Verhoeven, "Identification and characterization of starter lactic acid bacteria and probiotics from Columbian dairy products", *Journal of Applied Microbiology*, vol. 103, pp. 666-674, 2007. https://doi.org/10.1111/j.1365-2672.2007.03294.x

[113] A.A. Argyri, G. Zoumpopoulou, K. A. G. Karatzas et al., "Selection of potential probiotic lactic acid bacteria from fermented olives by *in vitro* tests", *Food microbiology*, vol. 33, pp. 282-291, 2013. https://doi.org/10.1016/j.fm.2012.10.005

[114] M. V. Arasu, N. A. Al-Dhabi, T. S. Rejiniemon et al., "Identification and characterization of *Lactobacillus brevis* P68 with antifungal, antioxidant and probiotic functional properties", *Indian journal of microbiology*, vol. 55, no. 1, pp. 19-28, 2015. https://doi.org/10.1007/s12088-014-0495-3

[115] M. G. Besselink, H. C. Van Santvoort, E. Buskens et al., "Probiotic prophylaxis in predicted severe acute pancreatitis: a randomised, double-blind, placebo-controlled trial", *The Lancet,* v. 371, p. 651 – 659, 2008. https://doi.org/10.1016/S0140-6736(08)60207-X

[116] T. Didari, S. Solki, S. Mozaffari, S. Nikfar, M. Abdollahi, "A systematic review of the safety of probiotics", *Expert opinion on drug safety*, vol. 13, no. 2, pp. 227-239, 2014. https://doi.org/10.1517/14740338.2014.872627

[117] S. Topcuoglu, T. Gursoy, F. Ovalı, O. Serce, G. Karatekin, “A new risk factor for neonatal vancomycin-resistant *Enterococcus* colonisation: bacterial probiotics”, *The Journal of Maternal-Fetal & Neonatal Medicine*, vol. 28, no. 12, pp. 1491-1494, 2015. ttps://doi.org/10.3109/14767058.2014.958462

[118] S. R. Hayes and A. J. Vargas, A. J, “ Probiotics for the prevention of pediatric antibiotic-associated diarrhea”, *Explore*, vol. 12, no. 6, pp. 463-466, 2016. https://doi.org/10.1016/j.explore.2016.08.015

[119] M. L. Carvour, S. L. Wilder, K. L. Ryan et al., “Predictors of *Clostridium difficile* infection and predictive impact of probiotic use in a diverse hospital-wide cohort”, *American journal of infection control*, vol. 47, no. 1, pp. 2-8, 2019. https://doi.org/10.1016/j.ajic.2018.07.014

[120] J. Suez, N. Zmora and E. Elinav, “Probiotics in the next-generation sequencing era”, *Gut Microbes*, vol. 11, no. 1, pp. 77-93, 2020. https://doi.org/10.1080/19490976.2019.1586039

[121] J. Barbosa, P. A. Gibbs and P.Teixeira, P. “Virulence factors among enterococci isolated from traditional fermented meat products produced in the North of Portugal”, *Food Control*, vol. 21, no. 5, pp. 651-656, 2010. https://doi.org/10.1016/j.foodcont.2009.10.002

[122] P.M. Moraes, L. M. Perin, S. D. Todorov et al., “Bacteriocinogenic and virulence potential of *Enterococcus* isolates obtained from raw milk and cheese”, *Journal of Applied Microbiology*, vol. 113, no. 2, pp. 318-328, 2012 https://doi.org/10.1111/j.1365-2672.2012.05341.x

[123] A. Terzić-Vidojević, K. Veljović, J. Begović et al., “Diversity and antibiotic susceptibility of autochthonous dairy enterococci isolates: are they safe candidates for autochthonous starter cultures?”, *Frontiers in Microbiology*, vol. *6*, no. 954, 2015. https://doi.org/10.3389/fmicb.2015.0095

[124] V. Sagheddu, E. Guidesi, S. Galletti and M. Elli, “Selection and characterization criteria of probiotics intended for human use from the past to the future”, *Food Science and Nutrition Studies*, vol. 3, no.10.22158, 2019. http://dx.doi.org/10.22158/fsns.v3n2p73

[125] S. Bardócz, T. J. Duguid, D.S. Brown et al., “The importance of dietary polyamines in cell regeneration and growth”, *British Journal of Nutrition*, vol. 73, no. 6, pp. 819-828, 1995. https://doi.org/10.1079/BJN19950087

[126] D. M. Linares, M. Martín, V. Ladero, M. A. Alvarez and M. Fernández, “Biogenic amines in dairy products”, *Critical reviews in food science and nutrition*, vol. 51, no. 7, pp. 691-703, 2011. https://doi.org/10.1080/10408398.2011.582813

[127] L.M. Perin, R. O. Miranda, S. D. Todorov, B. D. G. de Melo Franco, L. A. Nero, “Virulence, antibiotic resistance and biogenic amines of bacteriocinogenic lactococci and enterococci isolated from goat milk”, *International journal of food microbiology*, vol. 185, pp. 121-126, 2014. https://doi.org/10.1016/j.ijfoodmicro.2014.06.001

[128] J. Karovičová and Z. Kohajdova, “Biogenic amines in food”, *Chemical Papers,* vol. 59, pp. 70-79, 2005.

[129] V. Ladero, F. P. Rattray, B. Mayo et al., “Sequencing and transcriptional analysis of the biosynthesis gene cluster of putrescine-producing *Lactococcus lactis”, Applied and environmental microbiology*, vol. 77, no. 18, pp. 6409-6418, 2011. https://doi.org/10.1128/AEM.05507-11

[130] R. Flasarová, V. Pachlová, L. Buňková et al., “Biogenic amine production by *Lactococcus lactis* subsp. *cremoris* strains in the model system of Dutch-type cheese”, *Food Chemistry,* vol. 194, pp. 68–75, 2016. https://doi.org/10.1016/j.foodchem.2015.07.069

[131] J. F. Martín and M. Coton, “Blue cheese: microbiota and fungal metabolites”, In *Fermented foods in health and disease prevention*, pp. 275-303, 2017. https://doi.org/10.1016/B978-0-12-802309-9.00012-1

[132] M. Papageorgiou, D. Lambropoulou, C. Morrison et al., “Literature update of analytical methods for biogenic amines determination in food and beverages”, *TrAC Trends in Analytical Chemistry*, vol. 98, pp. 128-142, 2018. https://doi.org/10.1016/j.trac.2017.11.001

[133] S. Vesterlund, V. Vankerckhoven, M. Saxelin et al., “Safety assessment of *Lactobacillus* strains: presence of putative risk factors in faecal, blood and probiotic isolates”, *International journal of food microbiology*, vol. 116, no. 3, pp. 325-331, 2007. https://doi.org/10.1016/j.ijfoodmicro.2007.02.002

[134] N. P. Mangia, L. Saliba and P. Deiana, “Functional and safety characterization of autochthonous *Lactobacillus paracasei* FS103 isolated from sheep cheese and its survival in sheep and cow fermented milks during cold storage”, *Annals of Microbiology*, vol. 69, no. 2, pp. 161-170, 2019. https://doi.org/10.1007/s13213-018-1416-1

[135] S. Y. Lim, K. W. Loo and W. L. Wong, “Synergistic antimicrobial effect of a seaweed-probiotic blend against acute hepatopancreatic necrosis disease (AHPND)-causing *Vibrio parahaemolyticus”, Probiotics and antimicrobial proteins*, vol. 12, no. 3, pp. 906-917, 2020. https://doi.org/10.1007/s12602-019-09616-8

[136] P.L. Mäkinen, D. B. Clewell, F. An and K. K. Mäkinen, “Purification and substrate specificity of a strongly hydrophobic extracellular metalloendopeptidase (“gelatinase”) from *Streptococcus faecalis* (strain 0G1-10)”, *Journal of Biological Chemistry*, vol. 264, no. 6, pp. 3325-3334, 1989. https://doi.org/10.1016/S0021-9258(18)94069-X

[137] K. Kanemitsu, T. Nishino, H. Kunishima et al., “Quantitative determination of gelatinase activity among enterococci”, *Journal of microbiological methods*, vol. 47, no. 1, pp. 11-16, 2001. https://doi.org/10.1016/S0167-7012(01)00283-4

[138] N. Cui, M. Hu and R. A. Khalil, “Biochemical and biological attributes of matrix metalloproteinases”, *Progress in molecular biology and translational science*, vol. 147, pp. 1-73, 2017. https://doi.org/10.1016/bs.pmbts.2017.02.005

[139] R. Xu, R. Sa, J. Jia et al., “Screening of Antifungal Lactic Acid Bacteria as Bioprotective Cultures in Yogurt and a Whey Beverage”, *Journal of Food Protection*, vol. 84, pp. 953-961, 2021. https://doi.org/10.4315/JFP-20-441

[140] S. F. Aljasir, C. Gensler, L. Sun and D. J. D´Amico, “The efficacy of individual and combined commercial protective cultures against *Listeria monocytogenes* , *Salmonella* , O157 and non-O157 shiga toxin- producing *Escherichia coli* in growth medium and raw milk”, *Food Control*, vol. 109, no. 106924, 2020. https://doi.org/10.1016/j.foodcont.2019.106924

[141] L. Garnier, M. L. Salas, N. Pinon et al., “High-throughput method for antifungal activity screening in a cheese-mimicking model”, *Journal of dairy science*, vol. 101, no. 6, pp. 4971-4976, 2018. https://doi.org/10.3168/jds.2017-13518

[142] E. Delavenne, R. Ismail, A. Pawtowski et al., “Assessment of lactobacilli strains as yogurt bioprotective cultures”, *Food Control*, vol. 30, no. 1, pp. 206-213, 2013. https://doi.org/10.1016/j.foodcont.2012.06.043

[143] S. Cosentino, S. Viale, M. Deplano, M. E. Fadda and M. B. Pisano, “Application of autochthonous *Lactobacillus* strains as biopreservatives to control fungal spoilage in Caciotta cheese”, *BioMed Research International*, 2018. https://doi.org/10.1155/2018/3915615

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

[145] S. Laulund, A. Wind, P. M. Derkx and V. Zuliani, “Regulatory and safety requirements for food cultures”, *Microorganisms*, vol. 5, no. 2, pp. 28, 2017. https://doi.org/10.3390/microorganisms5020028

[146] *Commission Regulation (EU) No 1129/2011 of 11 November 2011 amending Annex II to Regulation (EC) No 1333/2008 of the European Parliament and of the Council by establishing a Union list of food additives*. Avaiable from https://eur-lex.europa.eu/legal-content/EN/TXT/PDF/?uri=CELEX:32011R1129&from=EN

CHAPTER III.

RESEARCH ARTICLE TWO

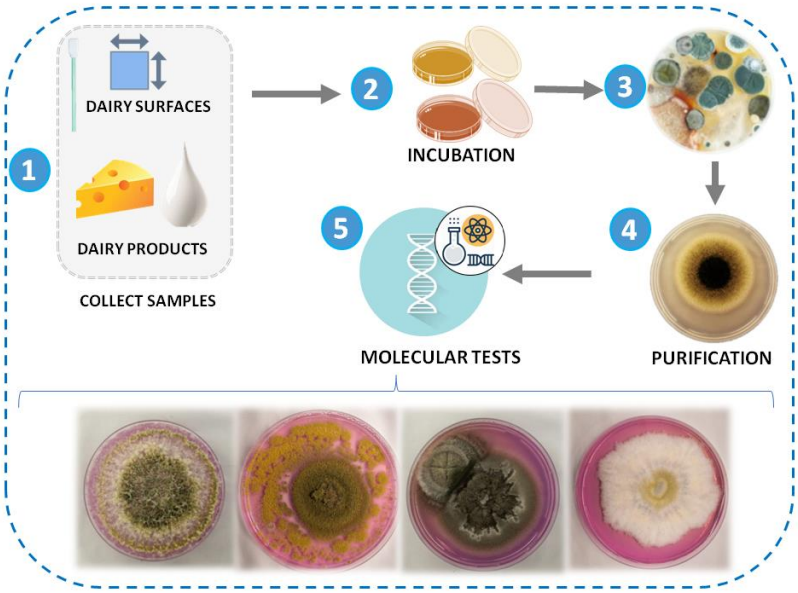
Diversity of Filamentous Fungi Associated with Dairy Processing Environments and Spoiled Products in Brazil

SOUZA et al.

Manuscript published in *Foods* (IF 5.561: 2021), 2022.
DOI: https://doi.org/10.3390/foods12010153

Chapter presented according to the final format of the journal.

All analyzes described in this chapter were conducted at InovaLeite, Department of Food Technology, Federal University of Viçosa, Viçosa, MG, Brazil



Title page

Diversity of Filamentous Fungi Associated with Dairy Processing Environments and Spoiled Products in Brazil

Luana Virgínia Souza [1], Rafaela da Silva Rodrigues [1,2], Andressa Fusieger [1,2,3] , Raiane Rodrigues da Silva [1], Sidney Rodrigues de Jesus Silva [1], Evandro Martins [1], Solimar Gonçalves Machado [1], Cinzia Caggia [3], Cinzia Lucia Randazzo [3] and Antonio Fernandes de Carvalho [1,*]

[1] InovaLeite – Laboratório de Pesquisa em Leites e Derivados, Departamento de Tecnologia de Alimentos, Universidade Federal de Viçosa, Viçosa 36570 900, MG, Brazil; andressa.fusieger@ufv.br (A.F.); raiane.r.silva@ufv.br (R.R.d.S.); rafaela.s.rodrigues@ufv.br (R.d.S.R.); jaqueline.honorato@ufv.br (J.A.H.); sid-ney.silva@ufv.br (S.R.d.J.S.); mariana.pena@ufv.br (M.L.P.)

[2] InsPOA – Laboratório de Inspeção de Produtos de Origem Animal, Departamento de Veterinária, Universidade Federal de Viçosa, Viçosa 36570 900, MG, Brazil; nero@ufv.br

[3] Di3A – Dipartimento di Agricoltura, Alimentazione e Ambiente, Università degli Studi di Catania, 95123 Catania, CT, Italy; ccaggia@unict.it

* Correspondence: antoniofernandes@ufv.br

Abstract

Few studies have investigated the diversity of spoilage fungi from the dairy production chain in Brazil, despite their importance as spoilage microorganisms. In the present study, 109 filamentous fungi were isolated from various spoiled dairy products and dairy production environments. The isolates were identified through sequencing of the internal transcribed spacer (ITS) region. In spoiled products, *Penicillium* and *Cladosporium* were the most frequent genera of filamentous fungi and were also present in the dairy environment, indicating that they may represent a primary source of contamination. For dairy production environments, the most frequent genera were *Cladosporium*, *Penicillium*, *Aspergillus,* and *Nigrospora*. Four species (*Hypoxylon griseobrunneum*, *Rhinocladiella similis*, *Coniochaeta rosae,* and *Paecilomyces maximus*) were identified for the first time in dairy products or in dairy production environment. Phytopathogenic genera were also detected, such as *Montagnula*, *Clonostachys*, and *Riopa*. One species isolated from the dairy production environment is classified as the pathogenic fungi, *R. similis*. Regarding the phylogeny, 14 different families were observed and most of the fungi belong to the Ascomycota phylum. The understanding of fungal biodiversity in dairy products and environment can support the development of conservation strategies to control food spoilage. This includes the suitable use of preservatives in dairy products, as well as the application of specific cleaning and sanitizing protocols designed for a specific group of target microorganisms.

Keywords: air factory contamination; *Cladosporium*; dairy chain; food safety; spoilage fungi.

1. Introduction

One of the main global concerns for humans, in regard to food safety and security, is the loss of food through waste and spoilage during production [1]. Food Waste Index report prepared by the United Nations (UNEP) shows that 931 million tons of edible food were wasted globally in 2019, which represents 17% of all food produced worldwide [2]. Currently, one of the main goals of the United Stated Department of Agriculture (USDA) and Environmental Protection Agency (EPA) is to halve global retail and consumer food waste by 2030, as well as reduce food losses in production and supply chains [3].

Concerning food spoilage, food products can be chemically, biochemically, physically, or microbiologically spoiled. Bacteria and/or fungi are the main agents that cause microbial spoilage [4]. Filamentous fungi are estimated to account for 5–10% of all food waste and loss in developing countries [5]. Furthermore, fungal contamination represents a major problem for industry and consumers. Fungi may modify the desired characteristics of products and/or have the ability to produce mycotoxins that are a concern for public health [5–7].

Filamentous fungi are ubiquitously found in nature and are also common contaminants of raw foods, such as fruits and vegetables and food products of an animal origin, as well as processed foods, mainly dairy products [7,8]. The food production environment is one of the main sources of contamination, with airborne fungal spores being disseminated by aerosolization throughout the processing plant [8].

Contamination by fungi can lead to the appearance of mycelia, which eventually leads to the deterioration of the product [5]. The presence of black, white, green, pink, or yellow spots can also be associated with the development of fungal colonies and, generally, results in the elimination of the entire product at the industrial or consumer level. Other defects

associated with fungal development in food matrices include the production of gas, off-flavors and/or off-odors and changes in texture [9,10].

Regarding the ability of some species of fungi to produce mycotoxins, many species of the *Aspergillus* and *Penicillium* genera are capable of producing aflatoxins, ochratoxins, gliotoxin, fumonisins, sterigmatocystin, patulin, fumigaclavine, roquefortine C, mycophenolic acid, and zearalenone [7,11–13]. Although mycotoxins in food are well documented, the effect of mycotoxins on the human body are not fully understood. However, some consequences of ingesting fungal toxins have been reported and include liver carcinomas and renal dysfunction [7]. None of these toxins have been linked to outbreaks of dairy products, but their presence in these products cannot be ruled out.

The Brazilian dairy industry represents a large part of the national economy, with milk production estimated at approximately 25 billion liters in 2021 [14]. The fungi associated with dairy product spoilage can cause significant losses for the dairy industry, and several studies have already addressed contamination in different types of products, such as cheese, yogurt, butter, cream, and milk [6,13,15–18]. Due to the capacity of certain spoilage fungi to growth in low pH and cold temperature (below 10 °C) environments, fermented dairy products can easily be contaminated. Certain fungi can even tolerate low oxygen concentrations; hence, vacuum-packed cheeses are at risk of contamination [5,17].

Considering the importance of the dairy sector to the Brazilian economy and the negative impact that can be caused by fungal microorganisms, understanding the fungal biodiversity in dairy products and in the production environment is the first step to develop strategies to reduce food waste. Although numerous studies have reported the isolation of fungi from foods [16,17,19,20], there is a lack of information on the characterization of fungi isolated from dairy products in Brazil. Therefore, in this study we aimed to isolate and

identify filamentous fungi from dairy processing environments and spoiled dairy products from the Zona da Mata region of Minas Gerais, Brazil.

2. Materials and Methods

2.1. Isolation of Filamentous Fungi

The diversity of spoilage fungi was evaluated using different "preservative-free" dairy products that were spoiled and from dairy processing environments. The cheese evaluated were Mozzarella (3 samples), Reino (2 samples), Montanhês (3 samples), Parmesan (2 samples), Gorgonzola (1 sample), Provolone (2 samples), Coalho (1 sample), Processed cheese (2 samples), and butter Ghee (2 samples). The samples were obtained from 2 different dairy manufacturers from the state of Minas Gerais, Brazil, and diversity was evaluated over a period of six months.

Isolation of spoilage fungi from dairy products was carried out according to Le Lay et al. [21], with modifications. A contaminated portion of each dairy product was removed with sterile forceps and deposited onto the surface of Dichloran Rose Bengal Chlortetracycline Agar (DRBC; Merck, Darmstadt, Germany), Potato Dextrose Agar (PDA; Merck), and Malt Extract Agar (MEA; Merck), and incubated for 5–7 days at 25 °C.

Airborne fungal spore collections were taken from the warehouse, cold chamber, ghee storage room, product refrigerators, cheese ripening room, gorgonzola ripening room, processing room, cheese packing room, and brine room, using a passive sampling technique. Petri dishes containing DRBC agar, PDA, and MEA were opened for 30 min in each of the previously mentioned environments of the dairy plant. Fungal collection from equipment and tools including the production tables, cheese containers, cheese packaging, and cheese tank was performed using the swab method [22]. A cotton swab was scrubbed across the surfaces using horizontal, unidirectional, and parallel strokes and then streaked onto Petri dishes

containing DRBC, PDA, and MEA media. After this procedure, all the plates were incubated for 5 to 7 days at 25 °C.

After incubation, fungal colonies with distinct morphological characteristics were selected. The selected fungi were purified twice using the same agar medium on which they were isolated and confirmed according to colony uniformity after incubation. For maintenance and subsequent use, a sterile inoculation loop was used to remove a few spores, or a tuft of mycelium from the pure cultures, and inoculated onto Petri dishes and inclined tubes with the defined medium and then incubated for 7 days at 25 °C.

2.2. Phenotypic Characterization

The isolates were phenotypically characterized using macro-observations. For macroscopic characterization, fungi were subjected to the inoculum point technique, where a fungal fragment was inoculated onto MEA media at a central point on a Petri dish, sealed and incubated inverted for 7 days at 25 °C. The colony characteristics were observed, including coloring (on both sides), growth rate via colony diameter measurements in millimeters from the reverse side, and observation of presence of pigments and exudates [23].

2.3. Molecular Identification of Fungal Isolates

2.3.1. Extraction of Fungal DNA

After phenotypic characterization, molecular identification of the isolated fungal strains was performed. The DNA was extracted from mycelial plugs using the Wizard® Genomic DNA Purification kit, according to the manufacturer's instructions (Promega Madison, WI, USA). The quality and concentration of the extracted DNA was measured using a NanoDrop™ Lite Spectrophotometer (Thermo Scientific, Waltham, MA, USA).

2.3.2. PCR Amplification, DNA Sequencing, and BLAST Identification

For PCR amplification and DNA sequencing, the rDNA internal transcribed spacer (ITS) region (for all isolates) was PCR-amplified using primers ITS4 (5′-

TCCTCCGCTTATTGATATGC-3′) and ITS5 (5′- GGAAGTAAAAGTCGTAACAAGG-3′) [24]. PCR amplification and DNA sequencingwere performed in a Thermocycler (MaxyGene® Gradiente Thermal Cycler, Axygen Scientific, Union City, CA, USA).

PCR reactions were performed using a final volume of 25 μL, containing 1X Go Taq® Flexi Buffer [5X] (Promega); 1.5 mM $MgCl_2$ [25 mM]; 0.2 mM of each dNTP [10 mM dNTP] (Promega); 0.2 μM of the ITS4 oligonucleotide; 0.2 μM of the ITS5 oligonucleotide; 1.25 U of Go Taq® DNA Polymerase (Promega) and 20 ng/μL of genomic DNA; and made to volume with autoclaved ultrapure water. A negative control (without the DNA) was included. The PCR protocol utilized had an initial denaturation step at 95 °C for 2 min, followed by 39 cycles at 95 °C for 1 min (denaturation), 50 °C for 1 min (annealing) and 72 °C for 1 min (extension), and a final extension step at 72 °C for 7 min. After amplification, corresponding amplicons were analyzed by electrophoresis on 1% agarose gel (*w*/*v*) for 2 h at a constant voltage of 60 mV in 0.5 X TBE buffer. The gels were stained using GelRed (Biotium Inc., Hayward, CA, USA) and developed using an LPIX transilluminator (Loccus Biotecnologia, São Paulo, Brazil).

PCR products were sequenced by a commercial sequencing service (ACTGene, Porto Alegre, Brazil) and the obtained sequences were edited using MEGA (Molecular Evolutionary Genetics Analysis) software version 11.0.11 (USA) [25]. Isolate identification was performed using the BLAST (Basic Local Alignment Search Tool) (http://www.ncbi.nlm.nih.gov/blast/) (accessed on 10 July 2022) against the internal transcribed spacer (ITS) region from fungi type and reference material database of NCBI (National Center for Biotechnology Information). Fungal strains were deposited in the culture collection of InovaLeite (Laboratory of Milk and Dairy Products, Federal University of Viçosa, Viçosa, Minas Gerais, Brazil). Sequences were deposited in GenBank under accession numbers OP584542 to OP584650.

2.4. Phylogenetic Analysis

To demonstrate evolutionary relationships between fungal isolates, the ITS sequences from this study, along with other ITS sequences from the National Center for Biotechnology Information (NCBI) Reference Sequence (RefSeq) database, were used. Phylogenetic analysis was performed using MEGA (Molecular Evolutionary Genetics Analysis) software version 11.0.11 (USA) [25]. Sequences were aligned using the MUSCLE algorithm [26]; the phylogeny was inferred using Maximum Likelihood (ML) [27]. The phylogenetic trees were constructed using the Kimura 2-parameter [28] and Tamura 3-parameter [29] substitution models, with a discrete Gamma distribution (+*G*) with 5 rate categories. Gaps or missing data were treated as partial deletion with a site coverage cut-off value of 95%. Branch support was determined through bootstrapping using 1000 replicates [30]. Trees were viewed and edited using the Interactive Tree Of Life (iTOL v.6.5.4, (Germany) web-based tool [31].

3. Results

3.1. Isolation of Filamentous Fungi

A total of 109 filamentous fungi were isolated, with 37.6% being isolated from spoiled dairy products and 62.4% from the dairy plant environment (Figure 1). Fungi isolated from dairy products ranged from 1.8% to 6.4% of the total number of isolated fungi, with Montanhês and Reino cheese having the highest occurrence (6.4%) and Ghee butter with the lowest percentage (1.8%).

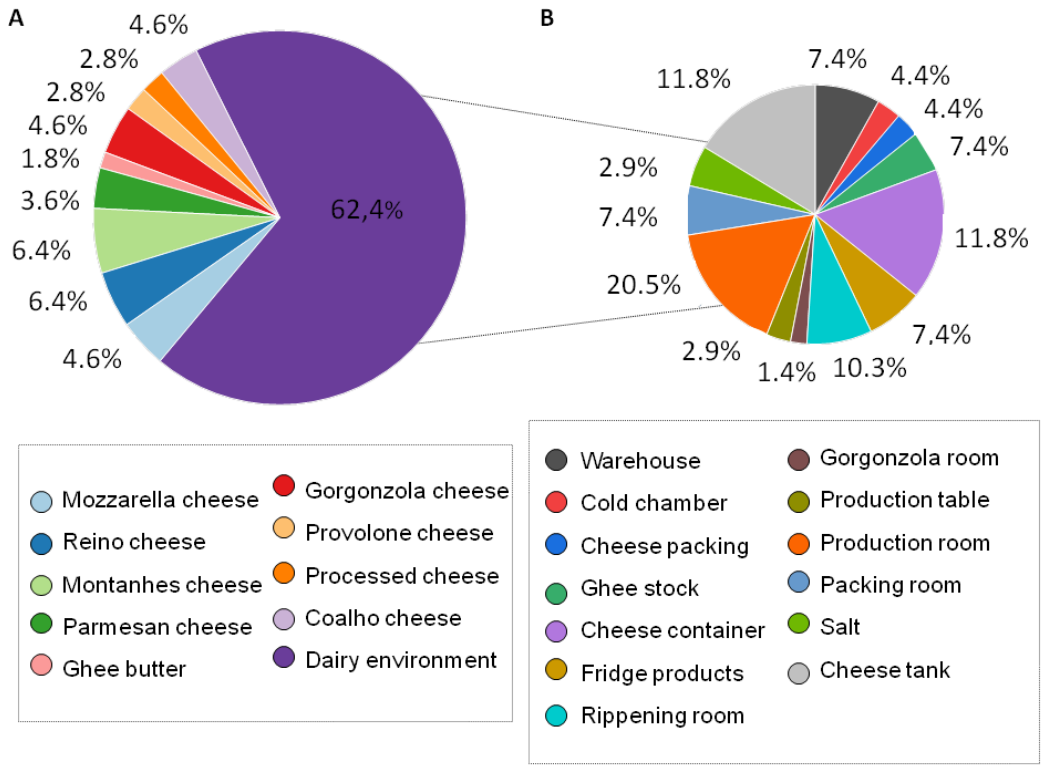


Figure 1. Percentage of filamentous fungi isolated from spoiled dairy products (**A**) and dairy processing environment, equipment, and tools (**B**).

The percentage of the biodiversity fungi isolated from the environment had a varied distribution, with the production room (20.5%), cheese containers, and cheese tank surfaces having the highest proportion (11.8%) of filamentous fungi and the gorgonzola ripening room having the lowest proportion (1.4%).

3.2. Identification of Fungal Isolates

A total of 85 isolates (59.44%) were identified to the genus level through ITS amplification and sequencing. From these, 16 different genera were identified as: *Penicillium* (36 isolates), *Cladosporium* (23 isolates), *Nigrospora* (5 isolates), *Riopa* (5 isolates), *Aspergillus* (4 isolates), *Hipoxylon* (2 isolates), *Fusarium* (1 isolate), *Montagnula* (1 isolate), *Clonostachys* (1 isolate), *Phaeosphaeria* (1 isolate), *Rhinocladiella* (1 isolate), *Coniochaeta* (1 isolate), *Trichoderma* (1 isolate), *Paecilomyces* (1 isolate), *Didymella* (1 isolate), and *Bipolaris* (1 isolate). A total of 24 isolates (16.78%) could not be identified at genus level

(Supplementary Table S1). A further 34 fungal isolates (23.78%) could not be amplified using ITS primers. Despite the variety of fungi identified, it is very difficult to distinguish between them, due to the high similarity in morphological and biological characteristics between the different groups. The ITS region that was used in this study is the most used marker for filamentous fungi identification, considering that most fungi have this specific region [18].

Regarding the identified genera, at least one fungus was found in each of the 8 evaluated products (Table 1). The *Penicillium* genus was the most prevalent (22.94%) and was found in all spoiled dairy products, except processed cheese. The *Phaeosphaeria* and *Fusarium* genera were found (0.92%) in only one type of product, coalho and montanhês cheese, respectively. The *Cladosporium* genus was found (5.5%) in three types of cheese: montanhês, coalho, and processed cheese. Only these 4 genera (*Penicillium*, *Cladosporium*, *Phaeosphaeria,* and *Fusarium*) appeared in dairy products. The other genera found in this study were isolated from the environment, equipment, and tools (Table 2). At least one fungal genus was isolated (Table 2) from each dairy environment (air, equipment, and tools). The *Cladosporium* genus was the most frequent (15.60%), being present in most of the sampled environments, followed by *Penicillium* (10.09%), *Nigrospora* (4.58%) and *Riopa* (4.58%). Unidentified genera were found in 14.68% of the sampled environments. A greater diversity of genera was found in the production room (8 genera), followed by the cheese tanks (6 genera). Only one fungal genus was found in the ripening gorgonzola room and production tables, *Trichoderma* and *Cladosporium*, respectively.

Identification and number of isolates	Mozzarela Cheese (n=3)	Reino Cheese (n=2)	Montanhês Cheese (n=3)	Parmesan Cheese (n=2)	Ghee Butter (n=2)	Gorgonzola Cheese (n=1)	Provolone Cheese (n=2)	Processed Cheese (n=2)	Coalho Cheese (n=1)	Total % in spoiled dairy products	Total % in air, equipment and tools
Penicillium (36)	2	5	2	4	2	5	3	-	2	25 (22.94%)	11 (10.09%)
Cladosporium (23)	-	-	2	-	-	-	-	2	2	6 (5.50%)	17 (15.60%)
Nigrospora (5)	-	-	-	-	-	-	-	-	-	-	5 (4.58%)
Riopa (5)	-	-	-	-	-	-	-	-	-	-	5 (4.58%)
Aspergillus (4)	-	-	-	-	-	-	-	-	-	-	4 (3.67%)
Hipoxylon (2)	-	-	-	-	-	-	-	-	-	-	2 (1.83%)
Fusarium (1)	-	-	1	-	-	-	-	-	-	1 (0.92%)	-
Montagnula (1)	-	-	-	-	-	-	-	-	-	-	1 (0.92%)
Clonostachys (1)	-	-	-	-	-	-	-	-	-	-	1(0.92%)
Phaeosphaeria (1)	-	-	-	-	-	-	-	-	1	1(0.92%)	-
Rhinocladiella (1)	-	-	-	-	-	-	-	-	-	-	1(0.92%)
Coniochaeta (1)	-	-	-	-	-	-	-	-	-	-	1(0.92%)
Trichoderma (1)	-	-	-	-	-	-	-	-	-	-	1(0.92%)
Paecilomyces (1)	-	-	-	-	-	-	-	-	-	-	1(0.92%)
Didymella (1)	-	-	-	-	-	-	-	-	-	-	1(0.92%)
Bipolaris (1)	-	-	-	-	-	-	-	-	-	-	1(0.92%)
Not identified (24)	3	2	2	-	-	-	-	1	-	8 (7.33%)	16(14.68%)
Total	5	7	7	4	5	3	3	3	5	41	68
Total (%)	12.19	17.07	17.07	9.76	12.19	7.33	7.33	7.33	12.19	37.61	62.39

Table 1. Diversity and number of fungi isolated from spoiled dairy products (n = number of samples).

Table 2. Diversity of spoilage fungi isolated from dairy environments (factory air, equipment and tools).

Identification	Ware house	Cold chamber	Ghee stock room	Product refrigerators	Cheese ripening room	Gorgonzola Ripening room	Production room	Cheese packing room	Brine room	Production tables	Cheese containers	Cheese packaging	Cheese tank
Penicillium sp.	-	1	-	-	1	-	-	1	-	-	6	-	2
Cladosporium sp.	1	1	2	2	4	-	2	1	1	1	-	1	1
Nigrospora sp.	-	-	1	-	-	-	1	2	-	-	-	-	1
Riopa sp.	-	1	-	1	1	-	1	-	-	-	-	-	1
Aspergillus sp.	1	-	-	1	-	-	1	-	-	-	-	-	1
Hipoxylon sp.	-	-	-	-	-	-	-	-	-	-	-	-	2
Fusarium sp.	-	-	-	-	-	-	-	-	-	-	-	-	-
Montagnula sp.	-	-	-	-	-	-	1	-	-	-	-	-	-
Clonostachys sp.	-	-	1	-	-	-	-	-	-	-	-	-	-
Phaeosphaeria sp.	-	-	-	-	-	-	-	-	-	-	-	-	-
Rhinocladiella sp.	-	-	-	-	-	-	1	-	-	-	-	-	-
Coniochaeta sp.	-	-	-	-	-	-	1	-	-	-	-	-	-
Trichoderma sp.	-	-	-	-	-	1	-	-	-	-	-	-	-
Paecilomyces sp.	-	-	-	-	-	-	-	-	1	-	-	-	-
Didymella sp.	-	-	-	-	-	-	-	-	-	-	-	1	-
Bipolaris sp.	-	-	-	-	-	-	-	-	-	-	1	-	-
Not identified	3	-	2	-	1	-	6	1	-	1	1	1	-
Total	5	3	6	4	7	1	14	5	2	2	8	3	8
Total (%)	7.35	4.42	8.82	5.89	10.29	1.47	20.59	7.35	2.94	2.94	11.76	4.42	11.76

Cladosporium and *Penicillium*, which were the most frequent in the environment, were also detected in dairy products (Table 1 and 2). *Fusarium* and *Phaeosphaeria*, found in the dairy products, were not isolated from the dairy environments.

At the species level, 4 isolates were identified (Table 3). The species identified through BLAST of the NCBI database were filtered according to the material type selection criteria.

Table 3. Species identified according to BLAST of the NCBI database.

			BLASTN		
Sample	**Length (nt)**	**Identification**	**Query Coverage**	**Percent Identify**	**Accession Number**
1.21	570	*H. griseobrunneum*	97%	98.92%	NR_155184.1
4.24	640	*R. similis*	96%	100.00%	NR_166008.1
4.27	600	*C. rosae*	94%	95.13%	NR_157509.1
4.38	630	*P. maximus*	90%	96.84%	NR_149329.1

In addition to molecular identification, the phenotypic characteristics of the isolates were also considered very similar to the accessioned species previously deposited in NCBI, and the morphological characteristics (Figure 2) were similar to images available in the literature that described the species of *H. griseobrunneum* [32], *R. similis* [33], *C. rosae* [34], and *P. maximus* [35].

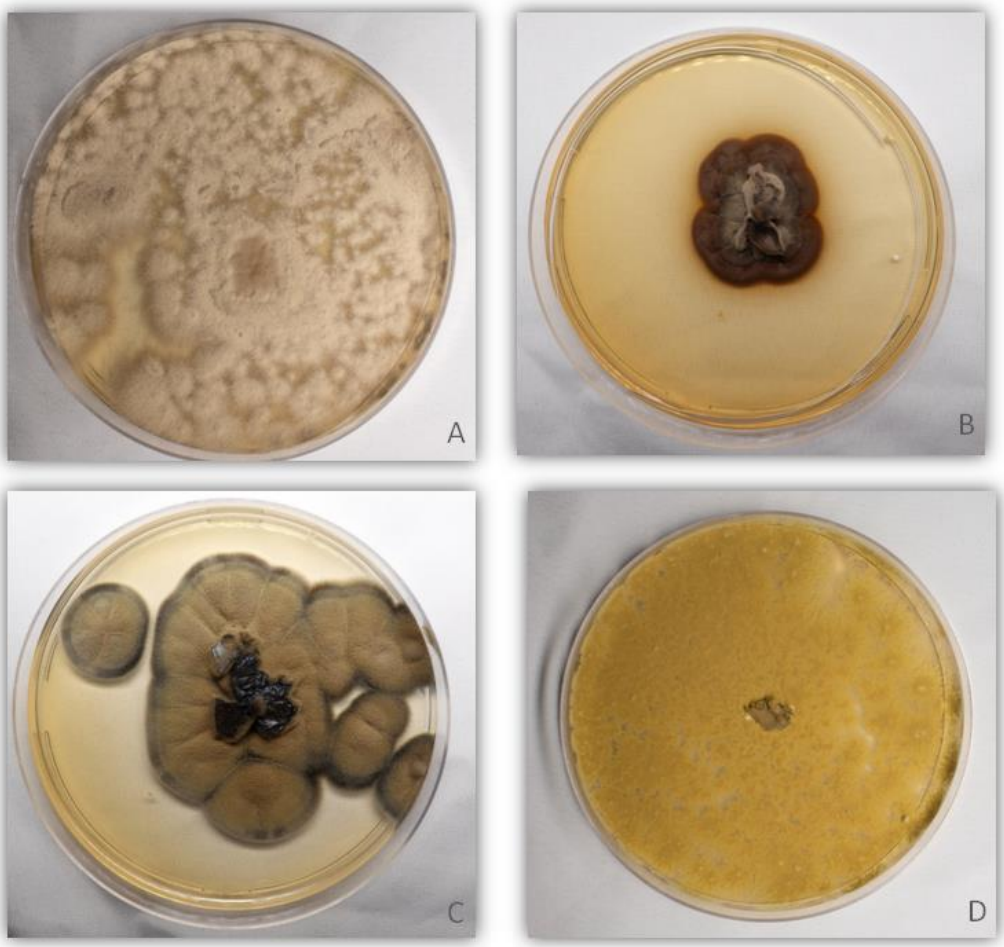


Figure 2. Morphological characteristics of the isolates identified at the species level: (**A**) *H. griseobrunneum,* (**B**) *R. similis,* (**C**) *C.rosae,* and (**D**) *P. maximus*.

3.3. Phylogenetic Analysis

Among the 109 isolates, 85 isolates were identified to at least the genus level. Phylogenetic analysis was conducted using the isolated samples' ITS sequences and with ITS reference sequences from the NCBI database (Supplementary Figure S1). The 85 isolates belonged to six orders (Eurotiales, Xylariales, Coniochaetales, Hypocreales, Chaetothyriales, Cladosporiales, Pleosporales, and Polyporales) and 14 fungal families (Aspergillaceae, Thermoascaceae, Apiosporaceae, Hypoxylaceae, Coniochaetaceae, Nectriaceae, Bionectriaceae, Hypocreaceae, Herpotrichiellaceae, Cladosporiaceae, Didymosphaeriaceae,

Didymellaceae, Phaeosphaeriaceae, Pleosporaceae, and Phanerochaetaceae). In addition, the isolates belonging to the genus *Riopa* were the only isolates found for the phylum Basidiomycota, while the other isolates belonged to the phylum Ascomycota.

It was not possible to identify 24 sequenced isolates, and eight of them had no similarity with any of the ITS reference sequences. However, 11 had query coverage and percent identity above 95% with three species of different genera: *Epicoccum phragmospora*, *Didymella keratinophila*, and *Ascochyta phacae*, all belonging to the Pleosporales order, and Didymellaceae family. An inferred phylogeny, using the unidentified isolates and reference sequences with greater similarity from the NCBI database, showed that these 11 isolates; the fungi type; two other isolates with similarity to other species, but of the same order and family (4.22 and 3.13); and one with no similarity to any reference sequence (4.7) clustered together. However, no pattern in the source of isolation and fungal color was observed (Figure 3). More information about these isolates is described in the Supplementary Table S1.

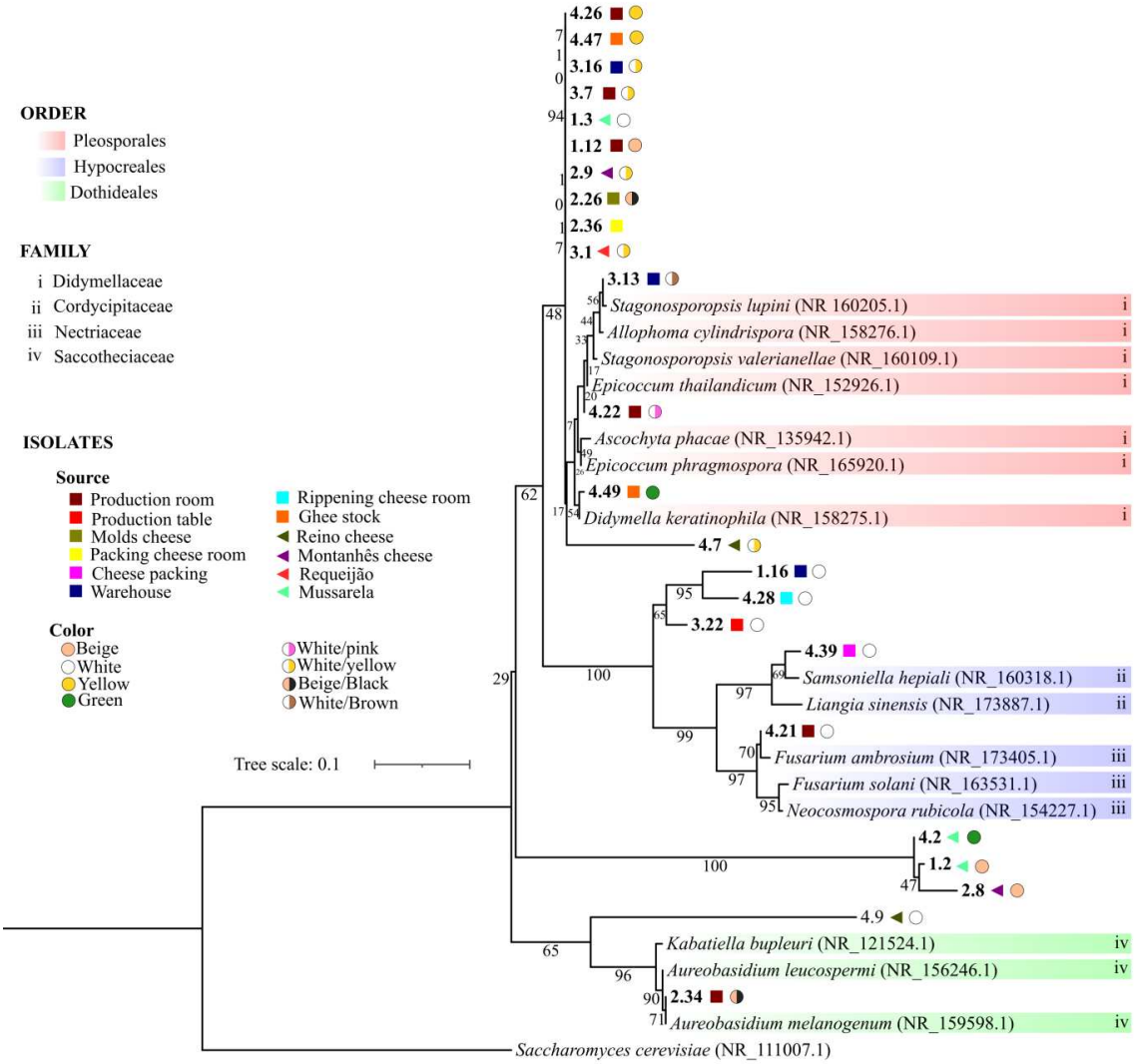


Figure 3. Phylogenetic analysis of not identified isolates.

Phylogeny based on ITS region nucleotide sequences, inferred using Maximum Likelihood and Tamura 3-parameter substitution model [29]. The tree with the highest log likelihood is shown. A discrete Gamma distribution was used to model evolutionary rate differences among sites. All positions with less than 95% site coverage were eliminated (partial deletion option). There was a total of 352 positions in the final dataset. Numbers at branches indicate percentages of bootstrap values obtained from 1000 replicates. *Saccharomyces cerevisiae* was used as an out group. The codes 4.26, 4.47, 3.16, 3.7, 1.3, 1.12, 2.9, 2.26, 2.36, 3.1, 3.13, 4.22, 4.49, 4.7, 1.16, 4.28, 3.22, 3.22, 4.39 4.21, 4.2, 1.2, 2.8, and 2.34 are the isolates that were not identified.

4. Discussion

The current results highlight the great diversity of fungi that are associated with dairy products and dairy processing environments in the Zona da Mata region of Minas Gerais (Brazil), identifying a total of 16 genera. It is worth mentioning that the majority of fungi was isolated from the environment; however, a considerable amount of fungi was still isolated from spoiled dairy products, such as semi-hard (Coalho, Montanhês, Reino, and Provolone) and hard cheese (parmesan cheese). The designation of cheese as semi-hard or hard is related to the moisture content, where cheeses with moisture content between 49% to 56% and 54% to 69% are classified as hard and semi-hard, respectively [36]. These cheeses can be ripened for a specific period until reaching the desired and particular characteristics of each type of cheese. Throughout storage, several factors can influence cheese spoilage, if conservation techniques are not applied effectively [37]. Even though these types of cheeses are considered as having a relatively long shelf life, they can still be spoiled by filamentous fungi in storage, as highlighted in this study.

Garnier et al. [17] also identified filamentous fungi from spoiled dairy products, and the greatest diversity was found in hard and semi-hard cheeses. Furthermore, Decontardi et al. [38] reported the presence of fungi in hard cheese and detected the presence of the mycotoxin ochratoxin A, indicating that the identified species was a mycotoxin producer. Considering that it is common to isolate fungi from the production environment, management strategies that prevent environmental contamination in dairy industries are essential for maintaining product quality and food safety, since many of these fungi can represent a source of contamination and can generate economic losses for the industry [10].

Regarding production environments, locations where a greater variety of fungal genera were detected included the production room and the cheese manufacturing tank, which reinforces the importance of applying quality management systems, such as GMP (Good

Manufacturing Practice) and HACCP (Hazard Analysis Critical Control Point), to minimize problems associated with microbiological contamination.

In the present study, we identified 85 isolates and distributed in 16 genera belonging to the Ascomycota phylum, which are the most frequently described in dairy products [5,39]. Among all the genera identified, only one does not belong to the Ascomycota phylum (*Riopa* = Basidiomycota). The genera most commonly and frequently found in dairy products are *Penicillium* and *Cladosporium*, of the Eurotiomycetes class [13,16,17,40], which were also the main genera identified in this study. One of the biggest problems of these genera in cheeses is that they can degrade compounds such as sorbic acid, potassium sorbate, and 1,3 pentadiene, which causes a strong off-flavor and unpleasant odor, called "kerosene odor" [10]. In addition, the visible growth of an undesirable fungus on any type of cheese results in immediate consumer dissatisfaction.

The predominant presence of *Penicillium* in several types of semi-hard and hard cheeses can be attributed to an adaptive response to water stress, mainly because these cheeses generally have water activity below the optimal level required for fungal growth [41]. On the other hand, the prevalence of *Cladosporium* in the dairy production environment, rather than in dairy products, is largely due to the species of this genus generally being slow growing and commonly disseminated through air. They can also be psychrotolerant and xerotolerant [5].

Another genus largely reported and associated with dairy product contamination is *Aspergillus* [40,42,43]. However, despite being widely reported in the literature, this genus was not identified in the spoiled products, only from the production environment. On the other hand, the genera *Phaeosphaeria* and *Fusarium* were identified as contaminants in the dairy products, but they were not identified in the dairy processing environment, most likely due to the great diversity of genera found. One of the major problems associated with

the occurrence of filamentous fungi in food products is mycotoxin contamination. Mycotoxins are toxic secondary metabolites produced mainly by the species in the genera of *Aspergillus*, *Penicillium,* and *Fusarium*, among others [13], all of which were identified in the current study.

As these genera were identified at great frequency in this study, the possible problems associated with mycotoxin contamination must be considered. The most dangerous mycotoxins reported thus far in cheese are Ochratoxin A and Aflatoxin M1 (AFM1), and are produced by fungi through direct or indirect contamination of milk (such as contamination of animal feed), respectively. This has been well reviewed by Hymery et al. [44]. Akinyemi et al. [45] reported the presence of several mycotoxins in three types of milk (camel, cow, and goat milk) and detected aflatoxins, alternariol, monomethyl ether, citrinin, dihydrocitrinone, enniatins, ochratoxin, and sterigmatocystin. Fontaine et al. [11] detected the presence of mycotoxins in cheeses, such as roquefortine C and mycophenolic acid; however, they did not detect the presence of AFM1. Although mycotoxin management in cheese is important, identifying and detecting mycotoxigenic fungal strains is fundamental; in order to track the possible origins of metabolite production. A study carried out by Anelli et al. [13] detected mycotoxigenic species of *Aspergillus* and *Penicillium* isolated from cheese and was able to correlate these species with the production of mycotoxins in cheese rinds during ripening.

The main effects of mycotoxins in the human body are liver and kidney toxicity, immune system destabilization, fetal toxicity, and carcinogenicity; however, it is not easy to prove that fungal species can produce mycotoxin or whether the mycotoxin will cause any damage if detected in a product. Nevertheless, recent models and some epidemiological data are enough to conclude that mycotoxins pose a danger, and that more studies on the risk of exposure to toxins should be further studied and validated [46,47].

Other genera that were identified in this study have also been reported by other authors as contaminants of dairy products and production environments, such as *Didymella* and *Fusarium*, which were identified by Cenci-Goga et al. [48] at low prevalence (1.5% each one). Garnier et al. [17] also identified the genera mentioned above and found *Phaeosphaeria* at a lower frequency (1 isolated), as in the present study. In addition, *Trichoderma* has been described in dairy production environments and products [15] and was isolated from the gorgonzola ripening room in the current work. The genus *Paecilomyces* was also described [16]. In fact, the diversity of genera and some species of filamentous fungi in dairy products and plants is well recognized. However, it is known that the biodiversity of a given region can favor the prevalence of one type of fungi over others. As an example, some studies report the prevalence of *Penicillium* in certain regions, such as in Spanish, French, and Italian dairy products [17,49,50]. Marín et al. [41] reported that *Geotrichum* and *Fusarium* were the genera most frequently isolated from Spanish milk samples. Other studies consider *Aspergillus*, *Cladosporium, Mucor,* and *Peniciliium* as the major contaminants in Egytian dairy products [42,51]. Nevertheless, the genera identified in this work, such as *Riopa, Hypoxylon*, *Montagnula*, *Clonostachys*, *Rhinocladiella*, *Coniochaeta,* and *Bipolaris*, are not commonly found in dairy production environments. In this present work, *Riopa, Hypoxylon*, *Montagnula*, *Clonostachys,* and *Coniochaeta* have been identified for the first time from a dairy production environment. *Bipolaris* and *Rhinocladiella* genera were also identified in a study conducted by Mbareche et al. [52] and by Moubasher et al. [53] from a dairy farm and Roquefort cheese, respectively. The latter was found over four decades ago and has not been reported further.

Bipolaris is a genus of dark conidia fungi in the phylum Ascomycota and is generally reported as a plant and animal pathogen [54]. The genus *Riopa*, belonging to the Basidiomycota phylum, contains two species, *Riopa metamorphosa* and *Riopa pudens*. This genus has already been classified as a synonym of another genus *Ceriporia* because they are

extremely similar phenotypically and genotypically [55]. They are a common soil borne fungi and are decomposers of tree wood and can cause white rot [56]. This may be the first report of this genus being associated with dairy environments, and despite not being reported in food, the current study frequently isolated this genus from various types of dairy production environments.

H. griseobrunneum was reported in our results, and it is an atypical fungus isolated from dairy products and the dairy environment, probably due to it being an endophytic filamentous fungus. The morphological characteristics of this species are well known, and the brown color on the surface of the colony facilitates its identification at the species level [32]. In addition, *Montagnula* was also described in our results. *Montagnula* is a genus that in the last 10 years has undergone several changes in its taxonomy and with many new species inclusions [57]. Perhaps this is the reason for not having a greater knowledge of the genus being associated with dairy products and environments, considering that the inclusion of the new species into molecular datasets is recent. This genus is associated with growth on dead wood, branches, stems, bark, and leaves [57] and is therefore a contaminant present in the dairy industries.

Clonostachys spp. are commonly found in temperate climates, typically on decomposing plant material; however, it is a non-pathogenic genus that may even have biocontrol characteristics for phytopathogens [58,59]. *C. rosae* was recently described by Wanasinghe et al. [34], but it was not associated with dairy and food environments. *R. similis*, another species identified in this study, was described by De Hoog et al. [60] and originally isolated from a chronic cutaneous ulcer of a patient in the state of Minas Gerais, Brazil. It is considered a pathogenic fungus, and its colonies present characteristics of a medium with a black, dry, and velvety center; the budding cells are abundant, with the development of germ cells and brown hyphae. The morphological characteristics described coincide with our study

(Figure 3) and can be compared with other studies [33,61]. Although *R. similis* is a recently described species, several studies are being carried out in America and Europe to better understand this filamentous fungus and its pathogenicity, mainly in the medical field [33,61,62]. However, this is the first report of *R. similis* associated with food or the food environment. We consider this fact important to carry out further studies in this area, as the discovery of new species and genera with no previous association with the food industry may have negative consequences.

In recent years, the taxonomy of fungi has increasingly changed, and many species are being reclassified [17]. New phylogenetic species are recognized as "species complexes" because they have few morphological differences, often making identification to the species level or even to genera difficult. In this way, our phylogeny shows that the isolates that were not identified display great similarity between them, such as, the representatives from Didymellaceae, which include *Epicoccum* and *Didymella*. These genera are extremely similar even in their morphological characteristics [63]. The inclusion of several species into these families and a new DNA marker (*rpb2*) for better identification at the species level were suggested [63]. It is important to use genetic markers for several species-level identifications. However, for an initial and general tracking of the presence of fungi in certain environments, it is important to begin from a starting point, generally the ITS region. Now, for specific identification to the species level and/or specific genera, the use of multiple or different DNA markers is essential.

Finally, considering the importance of filamentous fungi diversity present in dairy processing plants, it is important to emphasize that, clean/sterile areas must be designated and maintained, to avoid possible fungal contamination of the entire production environment and dairy products. With the present study, it was possible to find a biodiversity of filamentous

fungi in dairy products and environments, and some strains were found for the first time in the dairy environment.

In this way, understanding the specific biodiversity of fungi from each region and stage of the production system is very important, in order to develop new strategies to control spoilage. Furthermore, more studies are required to evaluate new fungi species identified within the production environment, to better understand their role.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/2304-8158/12/1/153. Table S1: Identified fungal isolates based on BLASTN search on the RefSeq curated dataset for Internal transcribed spacer region (ITS) from Fungi and reference material, on NCBI; Figure S1: The evolutionary history, using ITS region nucleotide sequences, was inferred using the Maximum Likelihood method and Kimura 2-parameter model [28]. The tree with the highest log likelihood is shown. A discrete Gamma distribution was used to model evolutionary rate differences among sites. All positions with less than 95% site coverage were eliminated (partial deletion option). There was a total of 438 positions in the final dataset. Numbers at branches indicate percentages of bootstrap values (>20%) obtained from 1000 replicates. Entorrhiza citriformis and E. parvula were used as outgroup.

Author Contributions: Conceptualization, L.V.S. and A.F.d.C.; methodology, L.V.S.; validation, L.V.S., R.d.S.R., A.F., R.R.d.S., and S.R.d.J.S.; formal analysis, L.V.S. and R.d.S.R.; investigation, L.V.S., R.d.S.R., A.F., R.R.d.S., and S.R.d.J.S.; resources, A.F.d.C.; writing—original draft preparation, L.V.S..; writing—review and editing, R.d.S.R., A.F., E.M., S.G.M., C.C., C.LR., and A.F.d.C.; visualization, L.V.S. and A.F.d.C.; supervision, E.M., S.G.M., C.C., C.L.R., and A.F.d.C.; project administration, L.V.S. All authors have read and agreed to the published version of the manuscript.

Funding: This research received no external funding.

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Data Availability Statement: Data is contained within the article or supplementary material.

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

Conflicts of Interest: The authors declare no conflicts of interest.

References

1. Food and Agriculture Organization of the United Nations *The State of Food and Agriculture. Moving Forward on Food Loss and Waste Reduction*; FAO: Rome, Italy, 2019; pp. 2–13.
2. United Nations Environment Programme UNEP Food Waste Index—Report 2021. Available online: https://www.unep.org/resources/report/unep-food-waste-index-report-2021 (accessed on 13 November 2022).
3. United States Department of Agriculture (USDA); Environmental Protection Agency (EPA) U.S. Food Loss & Waste 2030 Champions. Milestones Report—May 2021. Available online: https://www.usda.gov/sites/default/files/documents/food-loss-waste-champions-report.pdf (accessed on 12 November 2022).
4. Brooks, J.C.; Martinez, B.; Stratton, J.; Bianchini, A.; Krokstrom, R.; Hutkins, R. Survey of Raw Milk Cheeses for Microbiological Quality and Prevalence of Foodborne Pathogens. *Food Microbiol.* 2012, *31*, 154–158. https://doi.org/10.1016/j.fm.2012.03.013.
5. Pitt John, I.; Hocking, A.D. The Ecology of Fungal Food Spoilage. In *Fungi and Food Spoilage*; Springer: Boston, MA, USA, 2009; pp. 3–9, ISBN 978-0-387-92207-2.
6. Pattono, D.; Grosso, A.; Stocco, P.P.; Pazzi, M.; Zeppa, G. Survey of the Presence of Patulin and Ochratoxin A in Traditional Semi-Hard Cheeses. *Food Control* 2013, *33*, 54–57. https://doi.org/10.1016/j.foodcont.2013.02.019.
7. Ráduly, Z.; Szabó, L.; Madar, A.; Pócsi, I.; Csernoch, L. Toxicological and Medical Aspects of *Aspergillus*-Derived Mycotoxins Entering the Feed and Food Chain. *Front. Microbiol.* 2020, *10*, 2908. https://doi.org/10.3389/fmicb.2019.02908.

8. Parussolo, G.; Bernardi, A.O.; Garcia, M.V.; Stefanello, A.; dos Santos Silva, T.; Copetti, M.V. Fungi in Air, Raw Materials and Surface of Dry Fermented Sausage Produced in Brazil. *LWT* 2019, *108*, 190–198. https://doi.org/10.1016/j.lwt.2019.03.073.
9. Pinches, S.E.; Apps, P. Production in Food of 1,3-Pentadiene and Styrene by *Trichoderma* Species. *Int. J. Food Microbiol.* 2007, *116*, 182–185. https://doi.org/10.1016/j.ijfoodmicro.2006.12.001.
10. Leyva Salas, M.; Mounier, J.; Valence, F.; Coton, M.; Thierry, A.; Coton, E. Antifungal Microbial Agents for Food Biopreservation—A Review. *Microorganisms* 2017, *5*, 37. https://doi.org/10.3390/microorganisms5030037.
11. Fontaine, K.; Passeró, E.; Vallone, L.; Hymery, N.; Coton, M.; Jany, J.-L.; Mounier, J.; Coton, E. Occurrence of Roquefortine C, Mycophenolic Acid and Aflatoxin M1 Mycotoxins in Blue-Veined Cheeses. *Food Control* 2015, *47*, 634–640. https://doi.org/10.1016/j.foodcont.2014.07.046.
12. Dobson, A.D.W. Chapter 23—Mycotoxins in Cheese. In *Cheese*, 4th edition; McSweeney, P.L.H., Fox, P.F., Cotter, P.D., Everett, D.W., Eds.; Academic Press: San Diego, CA, USA, 2017; pp. 595–601, ISBN 978-0-12-417012-4.
13. Anelli, P.; Haidukowski, M.; Epifani, F.; Cimmarusti, M.T.; Moretti, A.; Logrieco, A.; Susca, A. Fungal Mycobiota and Mycotoxin Risk for Traditional Artisan Italian Cave Cheese. *Food Microbiol* 2019, *78*, 62–72. https://doi.org/10.1016/j.fm.2018.09.014.
14. Instituto Brasileiro de Geografia e Estatística (IBGE) Pesquisa Trimestral Do Leite. Available online: https://www.ibge.gov.br/estatisticas/economicas/agricultura-e-pecuaria/9209-pesquisa-trimestral-do-leite.html?=&t=destaques (accessed on 12 November 2022).

15. Kure, C.F.; Wasteson, Y.; Brendehaug, J.; Skaar, I. Mould Contaminants on Jarlsberg and Norvegia Cheese Blocks from Four Factories. *Int. J. Food Microbiol.* 2001, *70*, 21–27. https://doi.org/10.1016/S0168-1605(01)00520-7.

16. Kure, C.F.; Skaar, I.; Brendehaug, J. Mould Contamination in Production of Semi-Hard Cheese. *Int. J. Food Microbiol.* 2004, *93*, 41–49. https://doi.org/10.1016/j.ijfoodmicro.2003.10.005.

17. Garnier, L.; Valence, F.; Pawtowski, A.; Auhustsinava-Galerne, L.; Frotté, N.; Baroncelli, R.; Deniel, F.; Coton, E.; Mounier, J. Diversity of Spoilage Fungi Associated with Various French Dairy Products. *Int. J. Food Microbiol.* 2017, *241*, 191–197. https://doi.org/10.1016/j.ijfoodmicro.2016.10.026.

18. Buehler, A.J.; Evanowski, R.L.; Wiedmann, M.; Martin, N.H. Internal Transcribed Spacer (ITS) Sequence-Based Characterization of Fungal Isolates from Multiple Yogurt Facilities—A Case Study. *J. Dairy Sci.* 2019, *102*, 3646–3653. https://doi.org/10.3168/jds.2018-15636.

19. Snyder, A.B.; Churey, J.J.; Worobo, R.W. Association of Fungal Genera from Spoiled Processed Foods with Physicochemical Food Properties and Processing Conditions. *Food Microbiol.* 2019, *83*, 211–218. https://doi.org/10.1016/j.fm.2019.05.012.

20. de Souza, T.P.; Evangelista, S.R.; Passamani, F.R.F.; Bertechini, R.; de Abreu, L.R.; Batista, L.R. Mycobiota of Minas Artisanal Cheese: Safety and Quality. *Int. Dairy J.* 2021, *120*, 105085. https://doi.org/10.1016/j.idairyj.2021.105085.

21. le Lay, C.; Mounier, J.; Vasseur, V.; Weill, A.; le Blay, G.; Barbier, G.; Coton, E. In Vitro and in Situ Screening of Lactic Acid Bacteria and Propionibacteria Antifungal Activities against Bakery Product Spoilage Molds. *Food Control* 2016, *60*, 247–255. https://doi.org/10.1016/j.foodcont.2015.07.034.

22. Legnani, P.; Leoni, E.; Berveglieri, M.; Mirolo, G.; Alvaro, N. Hygienic Control of Mass Catering Establishments, Microbiological Monitoring of Food and Equipment. *Food Control* 2004, *15*, 205–211. https://doi.org/10.1016/S0956-7135(03)00048-3.

23. Visagie, C.M.; Hirooka, Y.; Tanney, J.B.; Whitfield, E.; Mwange, K.; Meijer, M.; Amend, A.S.; Seifert, K.A.; Samson, R.A. *Aspergillus, Penicillium* and *Talaromyces* Isolated from House Dust Samples Collected around the World. *Stud. Mycol.* 2014, *78*, 63–139. https://doi.org/10.1016/j.simyco.2014.07.002.

24. White, T.J.; Bruns, T.D.; Lee, S.; Taylor, J.W. Amplification and Direct Sequencing of Fungal Ribosomal RNA Genes for Phylogenetics. In *PCR Protocols: A Guide to Methods and Applications*; Innis, M.A., Gelfand, D.H., Sninsky, J., White, T.J., Eds.; Academic Press: San Diego, CA, USA, 1990; pp. 315–322.

25. Tamura, K.; Stecher, G.; Kumar, S. MEGA11: Molecular Evolutionary Genetics Analysis Version 11. *Mol. Biol. Evol.* 2021, *38*, 3022–3027. https://doi.org/10.1093/molbev/msab120.

26. Edgar, R.C. MUSCLE: Multiple Sequence Alignment with High Accuracy and High Throughput. *Nucleic Acids Res.* 2004, *32*, 1792–1797. https://doi.org/10.1093/nar/gkh340.

27. Felsenstein, J. Journal of Molecular Evolution Evolutionary Trees from DNA Sequences: A Maximum Likelihood Approach. *J Mol. Evol.* 1981, *17*, 368–376. https://doi.org/10.1007/BF01734359.

28. Kimura, M. A Simple Method for Estimating Evolutionary Rates of Base Substitutions Through Comparative Studies of Nucleotide Sequences. *J. Mol. Evol.* 1980, *16*, 111–120. https://doi.org/10.1007/BF01731581.

29. Tamura, K. Estimation of the Number of Nucleotide Substitutions When There Are Strong Transition-Transversion and G+C-Content Biases. *Mol. Biol. Evol.* 1992, *9*, 678–687. https://doi.org/10.1093/oxfordjournals.molbev.a040752.

30. Felsenstein, J. Confidence Limits on Phylogenies: An Approach Using the Bootstrap. *Evolution* 1985, *39*, 783–791. https://doi.org/10.1111/j.1558-5646.1985.tb00420.x.

31. Letunic, I.; Bork, P. Interactive Tree Of Life (ITOL) v5: An Online Tool for Phylogenetic Tree Display and Annotation. *Nucleic Acids Res.* 2021, *49*, 293–296. https://doi.org/10.1093/nar/gkab301.

32. Kuhnert, E.; Fournier, J.; Peršoh, D.; Luangsa-ard, J.J.D.; Stadler, M. New *Hypoxylon* Species from Martinique and New Evidence on the Molecular Phylogeny of *Hypoxylon* Based on ITS RDNA and β-Tubulin Data. *Fungal Divers.* 2014, *64*, 181–203. https://doi.org/10.1007/s13225-013-0264-3.

33. Abdolrasouli, A.; Gibani, M.M.; de Groot, T.; Borman, A.M.; Hoffman, P.; Azadian, B.S.; Mughal, N.; Moore, L.S.P.; Johnson, E.M.; Meis, J.F. A Pseudo-Outbreak of *Rhinocladiella Similis* in a Bronchoscopy Unit of a Tertiary Care Teaching Hospital in London, United Kingdom. *Mycoses* 2021, *64*, 394–404. https://doi.org/10.1111/myc.13227.

34. Wanasinghe, D.N.; Phukhamsakda, C.; Hyde, K.D.; Jeewon, R.; Lee, H.B.; Gareth Jones, E.B.; Tibpromma, S.; Tennakoon, D.S.; Dissanayake, A.J.; Jayasiri, S.C.; et al. Fungal Diversity Notes 709–839: Taxonomic and Phylogenetic Contributions to Fungal Taxa with an Emphasis on Fungi on *Rosaceae*. *Fungal Divers.* 2018, *89*, 1–236. https://doi.org/10.1007/s13225-018-0395-7.

35. Zhang, Y.-J.; Herrera-Balandrano, D.D.; Shi, X.-C.; Wang, S.-Y.; Laborda, P. Biocontrol of *Colletotrichum Brevisporum* in Soybean Using a New Genistein-Producing

Paecilomyces Strain. *Biol. Control* 2022, *169*, 104877. https://doi.org/10.1016/j.biocontrol.2022.104877.

36. Codex Alimentarius Commission General Standar for Cheese (CXS 283-1978). Available online: https://www.fao.org/fao-who-codexalimentarius/sh-proxy/en/?lnk=1&url=https%253A%252F%252Fworkspace.fao.org%252Fsites%252Fcodex%252FStandards%252FCXS%2B283-1978%252FCXS_283e.pdf (accessed on 13 November 2022).
37. Nájera, A.I.; Nieto, S.; Barron, L.J.R.; Albisu, M. A Review of the Preservation of Hard and Semi-Hard Cheeses: Quality and Safety. *Int. J. Environ. Res. Public Health* 2021, *18*, 9789. https://doi.org/10.3390/ijerph18189789.
38. Decontardi, S.; Mauro, A.; Lima, N.; Battilani, P. Survey of Penicillia Associated with Italian Grana Cheese. *Int. J. Food Microbiol.* 2017, *246*, 25–31. https://doi.org/10.1016/j.ijfoodmicro.2017.01.019.
39. Lund, F.; Filtenborg, O.; Frisvad, J.C. Associated Mycoflora of Cheese. *Food Microbiol.* 1995, *12*, 173–180. https://doi.org/10.1016/S0740-0020(95)80094-8.
40. Kandasamy, S.; Park, W.S.; Yoo, J.; Yun, J.; Kang, H.B.; Seol, K.-H.; Oh, M.-H.; Ham, J.S. Characterisation of Fungal Contamination Sources for Use in Quality Management of Cheese Production Farms in Korea. *Asian-Australas. J. Anim. Sci.* 2020, *33*, 1002–1011. https://doi.org/10.5713/ajas.19.0553.
41. Marín, P.; Palmero, D.; Jurado, M. Occurrence of Moulds Associated with Ovine Raw Milk and Cheeses of the Spanish Region of Castilla La Mancha. *Int. J. Dairy Technol.* 2015, *68*, 565–572. https://doi.org/10.1111/1471-0307.12208.
42. Moubasher, A.-A.H.; Abdel-Sater, M.A.; Soliman, Z.S.M. Yeasts and Filamentous Fungi Associated with Some Dairy Products in Egypt. *J. Mycol. Med.* 2018, *28*, 76–86. https://doi.org/10.1016/j.mycmed.2017.12.003.

43. Torkar, K.G.; Vengušt, A. The Presence of Yeasts, Moulds and Aflatoxin M1 in Raw Milk and Cheese in Slovenia. *Food Control* 2008, *19*, 570–577. https://doi.org/10.1016/j.foodcont.2007.06.008.

44. Hymery, N.; Vasseur, V.; Coton, M.; Mounier, J.; Jany, J.L.; Barbier, G.; Coton, E. Filamentous Fungi and Mycotoxins in Cheese: A Review. *Compr. Rev. Food Sci. Food Saf.* 2014, *13*, 437–456. https://doi.org/10.1111/1541-4337.12069.

45. Akinyemi, M.O.; Braun, D.; Windisch, P.; Warth, B.; Ezekiel, C.N. Assessment of Multiple Mycotoxins in Raw Milk of Three Different Animal Species in Nigeria. *Food Control* 2022, *131*, 108258. https://doi.org/10.1016/j.foodcont.2021.108258.

46. Pinhão, M.; Tavares, A.M.; Loureiro, S.; Louro, H.; Alvito, P.; Silva, M.J. Combined Cytotoxic and Genotoxic Effects of Ochratoxin A and Fumonisin B1 in Human Kidney and Liver Cell Models. *Toxicol Vitr.* 2020, *68*, 104949. https://doi.org/10.1016/j.tiv.2020.104949.

47. Claeys, L.; de Saeger, S.; Scelo, G.; Biessy, C.; Casagrande, C.; Nicolas, G.; Korenjak, M.; Fervers, B.; Heath, A.K.; Krogh, V.; et al. Mycotoxin Exposure and Renal Cell Carcinoma Risk: An Association Study in the EPIC European Cohort. *Nutrients* 2022, *14*, 3581. https://doi.org/10.3390/nu14173581.

48. Cenci-Goga, B.; Cruciani, D.; Crotti, S.; Karama, M.; Ylldlrlm, G.; Bulut, M.; Marino, C.; Grispoldi, L. Diversity of Yeasts and Moulds in Dairy Products from Umbria, Central Italy. *J. Dairy Res.* 2021, *88*, 217–220. https://doi.org/10.1017/S002202992100042X.

49. Barrios, M.J.; Medina, L.M.; Lopez, M.C.; Jordano, R. Fungal Biota Isolated from Spanish Cheeses. *J. Food Saf.* 1998, *18*, 151–157. https://doi.org/10.1111/j.1745-4565.1998.tb00210.x.

50. Montagna, M.T.; Santacroce, M.P.; Spilotros, G.; Napoli, C.; Minervini, F.; Papa, A.; Dragoni, I. Investigation of Fungal Contamination in Sheep and Goat Cheeses in

Southern Italy. *Mycopathologia* 2004, *158*, 245–249. https://doi.org/10.1023/b:myco.0000041897.17673.2c.

51. Seddek, N.H.; H.Gomah, N.; Osman, Dina.M. Fungal Flora Contaminating Egyptian Ras Cheese with Reference to Their Toxins and Enzymes. *Food Sci. Technol.* 2016, *4*, 64–68. https://doi.org/10.13189/fst.2016.040403.
52. Mbareche, H.; Veillette, M.; Bilodeau, G.J.; Duchaine, C. Fungal Aerosols at Dairy Farms Using Molecular and Culture Techniques. *Sci. Total Environ.* 2019, *653*, 253–263. https://doi.org/10.1016/j.scitotenv.2018.10.345.
53. Moubasher, A.H.; Abdel-Kader, M.I.A.; El-Kady, I.A. Toxigenic Fungi Isolated from Roquefort Cheese. *Mycopathologia* 1979, *66*, 187–190. https://doi.org/10.1007/BF00683970.
54. Giri, D.K.; Sims, W.P.; Sura, R.; Cooper, J.J.; Gavrilov, B.K.; Mansell, J. Cerebral and Renal Phaeohyphomycosis in a Dog Infected With *Bipolaris* Species. *Vet. Pathol* .2011, *48*, 754–757. https://doi.org/10.1177/0300985810375239.
55. Miettinen, O.; Spirin, V.; Vlasák, J.; Rivoire, B.; Stenroos, S.; Hibbett, D.S. Polypores and Genus Concepts in Phanerochaetaceae (*Polyporales*, Basidiomycota). *MycoKeys* 2016, *17*, 1–46. https://doi.org/10.3897/mycokeys.17.10153.
56. Justo, A.; Miettinen, O.; Floudas, D.; Ortiz-Santana, B.; Sjökvist, E.; Lindner, D.; Nakasone, K.; Niemelä, T.; Larsson, K.-H.; Ryvarden, L.; et al. A Revised Family-Level Classification of the *Polyporales* (*Basidiomycota*). *Fungal Biol.* 2017, *121*, 798–824. https://doi.org/10.1016/j.funbio.2017.05.010.
57. Mapook, A.; Hyde, K.D.; McKenzie, E.H.C.; Jones, E.B.G.; Bhat, D.J.; Jeewon, R.; Stadler, M.; Samarakoon, M.C.; Malaithong, M.; Tanunchai, B.; et al. Taxonomic and Phylogenetic Contributions to Fungi Associated with the Invasive Weed *Chromolaena*

Odorata (Siam Weed). *Fungal Divers.* 2020, *101*, 1–175. https://doi.org/10.1007/s13225-020-00444-8.

58. Schroers, H.-J. A Monograph of Bionectria (Ascomycota, Hypocreales, Bionectriaceae) and Its Clonostachys Anamorphs. *Stud. Mycol.* 2001, *46*, 1–214.

59. Moraga-Suazo, P.; Opazo, A.; Zaldúa, S.; González, G.; Sanfuentes, E. Evaluation of *Trichoderma* Spp. and *Clonostachys* Spp. Strains to Control *Fusarium Circinatum* in *Pinus Radiata* Seedlings. *Chil. J. Agric. Res.* 2011, *71*, 412–417. https://doi.org/10.4067/S0718-58392011000300011.

60. De Hoog, G.S.; Vicente, V.; Caligiorne, R.B.; Kantarcioglu, S.; Tintelnot, K.; van den Ende, A.H.G.G.; Haase, G. Species Diversity and Polymorphism in the *Exophiala Spinifera* Clade Containing Opportunistic Black Yeast-Like Fungi. *J. Clin. Microbiol.* 2003, *41*, 4767–4778. https://doi.org/10.1128/JCM.41.10.4767-4778.2003.

61. Heidrich, D.; González, G.M.; Pagani, D.M.; Ramírez-Castrillón, M.; Scroferneker, M.L. Chromoblastomycosis Caused by *Rhinocladiella Similis*: Case Report. *Med. Mycol. Case Rep.* 2017, *16*, 25–27. https://doi.org/10.1016/j.mmcr.2017.04.002.

62. De Andrade, T.S.; de Almeida, A.M.Z.; Basano, S. de A.; Takagi, E.H.; Szeszs, M.W.; Melhem, M.S.C.; Albuquerque, M.; Camargo, J. de S.A.A.; Gambale, W.; Camargo, L.M.A. Chromoblastomycosis in the Amazon Region, Brazil, Caused by *Fonsecaea Pedrosoi, Fonsecaea Nubica*, and *Rhinocladiella Similis*: Clinicopathology, Susceptibility, and Molecular Identification. *Med. Mycol.* 2020, *58*, 172–180. https://doi.org/10.1093/mmy/myz034.

63. Hou, L.W.; Groenewald, J.Z.; Pfenning, L.H.; Yarden, O.; Crous, P.W.; Cai, L. The Phoma-like Dilemma. *Stud. Mycol.* 2020, *96*, 309–396. https://doi.org/10.1016/j.simyco.2020.05.001.

CHAPTER IV.

RESEARCH ARTICLE THREE

Evaluation of antifungal activity of lactic acid bacteria against fungi in simulated cheese matrix

SOUZA et al.

Manuscript to be submitted *LWT* (IF 6.056: 2021), 2022.

Chapter presented according to the final format of the journal.

All analyzes described in this chapter were conducted at InovaLeite, Department of Food Technology, Federal University of Viçosa, Viçosa, MG, Brazil

Title page

Evaluation of antifungal activity of lactic acid bacteria against fungi in simulated cheese matrix

Luana Virgínia Souza[a, b], Raiane Rodrigues da Silva[a], Andressa Falqueto[c], Andressa Fusieger[a], Evandro Martins[a], Cinzia Caggia[b], Cinzia Lucia Randazzo[b], Antonio Fernandes de Carvalho[a*]

[a] InovaLeite - Laboratório de Pesquisa em Leites e Derivados, Departamento de Tecnologia de Alimentos, 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] Lampoah - Laboratório de Microbiologia de Patógenos de Origem Alimentar e Hídrica, Departamento de Microbiologia Agrícola, Universidade Federal de Viçosa, 36570 900, Viçosa, MG, Brazil

*Corresponding author: Antonio Fernandes de Carvalho. InovaLeite - Laboratório de Pesquisa em Leites e Derivados, Departamento de Tecnologia de Alimentos, Universidade Federal de Viçosa, 36570 900, Viçosa, MG, Brazil.

* Correspondence: antoniofernandes@ufv.br

Abstract

This short communication reports the antifungal activity of lactic acid bacteria (LAB) in media culture and in simulated cheese matrix. In the *in vitro* test, 52 isolates of LAB were tested against *Aspergillus niger* and *Penicillium chrysogenum*. Based on the antifungal performance, 5 LAB strains were selected for *in situ* test in a simulated cheese matrix. The selected strains exhibited antifungal effect in simulated cheese matrix; however, the antifungal effect was lost when the LABs were combined with starter culture. The commercial culture tested showed antifungal effect when combined and not combined with the starter culture.

Despite this latter data, results of the present report suggest that these LAB strains may have great potential in foods obtained without starter culture. In addition, they can produce antifungal compounds that can be characterized and potentially be used as new adjunct cultures in the future.

Keywords: *Aspergillus niger*, biocontrol, dairy products, *Penicillium chrysogenum*.

1. Introduction

In the dairy industry, filamentous fungi can cause problems that range from visible mycelium growth on product surface to production of bitter flavors or gas. In addition, some fungi are capable to produce mycotoxins (e.g. aflatoxins, ochratoxins, trichothecenes, zearalenone, and fumonisins) considered a public health concern (Pitt John I. and Hocking, 2009; Ráduly et al., 2020). In 2019, it was estimated that around 931 tons/year of food were wasted globally, with fungi spoilage being the main reason (United Nations Environment Programme, 2021). As a control tool for fungal growth, food industries react adopting the good manufacturing practices and generally using chemical preservatives such as natamycin, potassium sorbate or sodium benzoate (Leyva Salas et al., 2017).

However, in recent years, following the consumer trend for "clean label" foods, there has been a growing demand for more natural and healthy foods obtained through fewer ingredients and through the replacement of chemical preservatives by natural compounds (Maruyama et al., 2021). In this scenario, scientific interest for microbial cultures with bioprotective functionality has been intensified and the market for this type of cultures is continuously rising (Souza et al., 2023).

Among microorganisms most commonly used for bioprotection purposes, lactic acid bacteria (LAB) stand out based on their capacity to produce several antimicrobial compounds, such as organic acids, fatty and volatile acids, reuterin, diacetyl, bacteriocins, peptides and/or low molecular weight proteinaceous compounds (Ibrahim et al., 2021). For antifungal effects, many molecules are likely to act synergistically as lactic and acetic acids, and in cheese and yogurt, LAB produce a very large array of antifungal compounds (Leyva Salas et al., 2019).

For antifungal LAB, many molecules are likely to act synergistically with lactic and acetic acids and, and when these LAB are added to cheese and yogurt, they are capable to

produce a very large array of antifungal compounds, mainly organic acids and fatty acids (Leyva Salas et al., 2019).

Considering the importance of reducing the use of chemical preservatives and the rinsing demand for new microbial cultures with bioprotective effects, the aim of this study was to compare the antifungal effect of LAB strains with those of and compare their antifungal activity with commercial bioprotective cultures.

2. Materials and methods

2.1. Strains and growth conditions

Fifty two (n=52) LAB strains from the Laboratório de Pesquisa em Leites e Derivados (InovaLeite, Universidade Federal de Viçosa) were included in this study. The strains were isolated from dairy environment samples from four different Brazilian regions producing artisanal cheeses (Figure 1) and were previous identified at the species level by 16S rDNA sequencing (Martins et al., 2020; Teixeira et al., 2021). The strains were stored at -80 ºC in de Man Rogosa and Sharpe (MRS) broth (Kasvi, São José dos Pinhais, PR, Brazil), supplemented with glycerol at 20% (v/v; Exôdo Cientifica, Sumaré, SP, Brazil). To carry out the study, stock strains were transferred into 10 mL of MRS broth and strains of *Lactiplantibacillus* spp., *Weissella* spp., *Lactococcus* spp., *Streptococcus* spp. and *Pediococcus* spp. (1% v/v) were incubated at 37 ºC for 24 h.

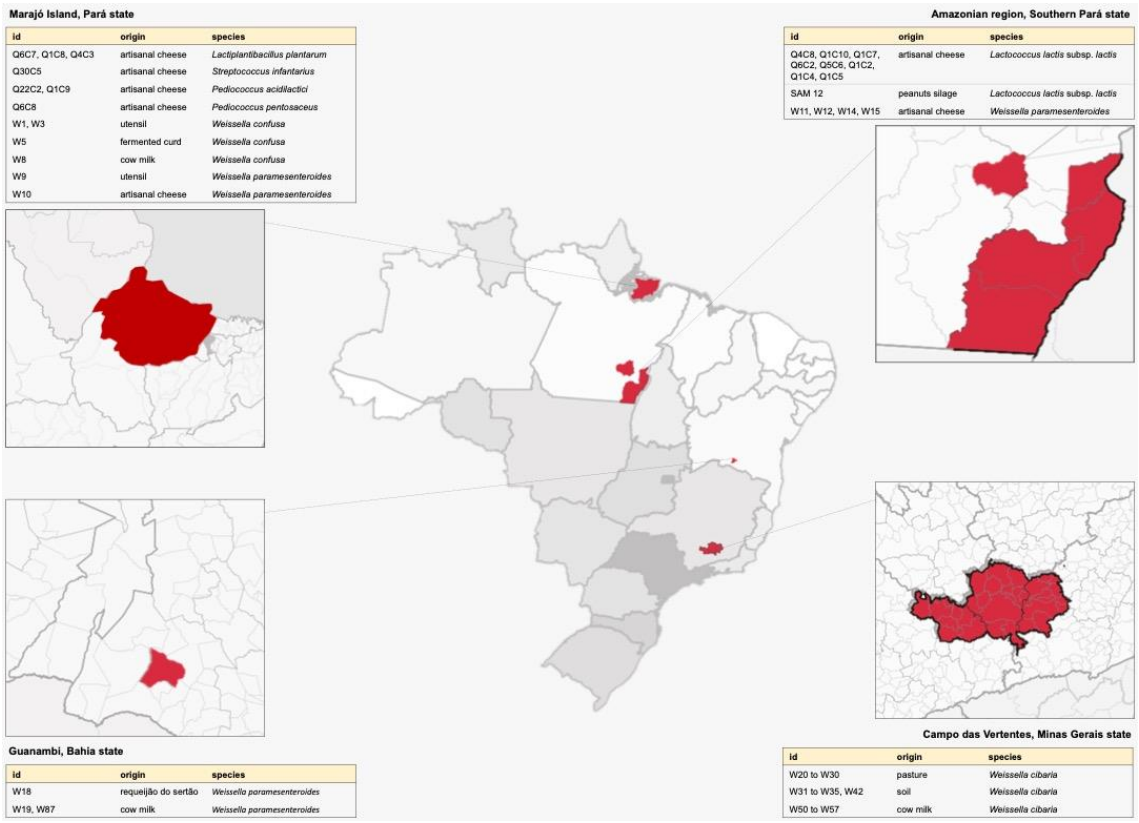


Figure 1. Strains, geographical origins and identification of LAB isolates obtained from Brazilian artisanal cheese-producing regions.

To test the antifungal activity of LAB strains, *Aspergillus niger* IOC 207 and *Penicillium chrysogenum* IOC 132, obtained from Fundação Oswaldo Cruz (Fiocruz, Rio de Janeiro, RJ, Brazil), were selected as targets, based on their high incidence as food spoiler in Brazil (Parussolo et al., 2019; Souza et al., 2023). The preparation of fungal spore suspension was carried out according to the procedure described by (Roy et al., 1996). The fungi were grown on Potato Dextrose Agar (PDA; Merck, Darmstadt, HE, Germany) tilted at 25 °C until they formed spores. Subsequently, spores were harvested with sterile distilled water supplemented with 0.1% of Tween-80 (Exôdo Cientifica), and the suspension agitated using a vortex mixer to break up the spore clusters. The spore count was performed using an optical microscope (Olympus, Melville, NY, USA) listed by a Neubauer cell and the stock suspensions were standardized to a final concentration of 1.5×10^7 spores/mL, supplemented with glycerol at 10% (v/v) and stored at -80 °C until the use.

2.2. In vitro *screening of LAB strains for antifungal activity*

LAB cells cultures were centrifuged (Heraeus Megafuge 8R, Thermo Fisher Scientific, Waltham, MA, USA) at 3260 × g for 15 min at 7 ºC, and filtrated (Ø = 0.22 µm pore size, Kasvi) to obtain a cell-free supernatant (CFS). The inhibitory activity was determined by microtiter plate well assay according to (Zhao et al., 2022), with modifications. Spores suspension (100 µL) of *A. niger* or *P. chrysogenum* at 1.5×10^7 spores/mL were used undiluted and diluted (4- and 14-fold in sterile water) and added to 96-well microtiter plate. Then, 100 µL of LAB-CFS were added to each well and the microtiter plate incubated at 30 °C for 48 h. The fungal growth was determined by optical density (OD) measured every 1 h (λ = 600 nm) in a microplate spectrophotometer (Multiskan™ GO Microplate Spectrophotometer, Thermo Fisher Scientific, Madison, WI, USA). Growth controls were prepared in wells added with the target cultures (100 µL) and MRS broth (100 µL). Negative controls were prepared in wells added with MRS (200 µL) alone. Inhibition assays were conducted in triplicate and over two independent repetitions. The antifungal activity of the CFS was expressed as the fungal growth inhibitions in percentage (%), based on the following equation:

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

Where OD_{LAB} is the OD of suspension of fungal spores in presence of CFS, and OD_{CONTROL} is the OD of suspension of fungal spores in MRS broth. Recorded ODs were corrected based on negative controls, and OD mean values calculated over the reading time. CFS with antifungal activity higher than 70% against the undiluted target fungal spore suspension were selected for the *in situ* analysis (le Lay et al., 2016).

2.3. In situ *screening of LAB strains for antifungal activity*

The high-performance method for antifungal screening was applied according to the model proposed by (Garnier et al., 2018), with adaptations. For the preparation of the cheese-mimicking matrix, standardized ultrafiltered milk retentate was prepared according to (Hannon et al., 2006) with adaptations. Raw milk was heated up to 50 °C, skimmed using a centrifugal separator (Westfalia, Cuijk, The Netherlands) and microfiltered (Membralox® tubular membrane pore size 1.4 μm; Pall Corp., Washington, NY, USA) at 50 °C (WGM, Brazil). The retentate was ultrafiltered at 0.02 μm at 50 °C, added with sterile sodium chloride (0.7%), and heated at 95 °C for 2 min. Starter culture at 10^5 CFU/mL (Delvo® cheese CW-100; DSM Food Specialties, Delft, The Netherlands) and 1.5 mL/L of rennet were 5 × diluted and filtered (0.22 μm, Kasvi) (Maxiren 180; DSM Food Specialties) and added to the retentate. After, homogenization for 1 min, the retentate was distributed in 24-well plates (2 mL/well) and LAB suspensions in milk were added (final concentration of 1.5×10^5 CFU/mL in each well). As controls, wells without starter culture or LAB cultures were performed.

In order to compare the antifungal performance of LAB cultures to commercial bioprotective culture (Delvo® Guard 301; DSM Food Specialties), wells with the commercial bioprotective culture (10^5 CFU/mL in each well) combined or not with the starter culture were performed. The plates were incubated at 30 °C for 1 h to perform a fermented "mini-cheese" (pH 5.0) in each well. After 24 hours, the exudate on the surface of the mini-cheeses was removed with a sterile dropper and 5×10^2 spores/mL of the fungal targets were deposited, separately, at the center of each well of mini-cheese (1 fungi tested/plate). Antifungal activity was visually assessed by assessment of fungal growth compared to the negative control (without fungal spores inoculation) after 5 days at 25 °C. Scoring of antifungal activity was

performed using a qualitative scheme with a daily record of total inhibition until visible fungal growth.

3. Results and Discussion

3.1. In vitro *screening of LAB strains for antifungal activity*

Overall results showed that when spore suspension was diluted, the antifungal effect of bacterial strains was greater (Table 1). Strains as *L. lactis* subsp. *lactis* (Q1C4, Q1C2, and SAM12), *L. plantarum* (Q6C7), *S. infantarius* (Q22C2), *W. paramesenteroides* (W14, W15, W18, W19) and *W. cibaria* (W20, W31, W32, W34 e W35) exhibited a high antifungal activity (more than 85% in undiluted fungal suspension) against *P. chrysogenum*; while a lower activity was observed against *A. niger.* This result could be related to the spore structure of *A. niger,* composed of polysaccharides, such as chitin and glucans, covered by an outer layer of hydrophobins and pigments of melanin. In detail, the presence of these outer layers make spores highly hydrophobic, and the melanin seems involved in different mechanisms, like protection of spores or virulence, being implicated in adhesion mechanisms and in generation of reactive oxygen species (ROS) (Beauvais et al., 2014; Cortesão et al., 2020). These morphological specificities explain the higher resistance of *A. niger* to LAB metabolites, compared to *P. chrysogenum.* Furthermore, *A. niger* has been already characterized by resistance to weak acids produced by some bacteria such as sorbic acid and other types of organic acids (Geoghegan et al., 2020).

Fernandez et al. (2017) found *Lactiplantibacillus* spp. strains with strong *in vitro* antifungal activity against *P. chrysogenum*, while Cosentino et al. (2018) and Zhao et al. (2022) described the antifungal activity of autochthonous *Lactiplantibacillus* spp. strains against *A. niger*. Moreover, strains of *Lactococcus* spp. and *Leuconostoc* spp. were also reported as able to *in vitro* inhibit *A. niger* and *P. chrysogenum* (Varsha et al., 2014, 2015). According to Cosentino et al. (2018), although several studies on the *in vitro* antifungal activity of LAB

strains are available, the comparison of results is often difficult due to different strains, conditions assays, and methods used. However, in the present study, the highest inhibition percentages were detected for *Weissella* spp. (W5, W8, W9, and W25). These findings are in agreement with results previously reported about the *in vitro* antifungal activity of *W. cibaria*, *W. confusa* and *W. paramesenteroides* (Ndagano et al., 2011; Ogunremi et al., 2022).

In the present study these strains showed high inhibition rates when fungal spore suspension was closer to the real reality, since high fungal concentrations (approximately 10^7 spores/g) in dairy products are not expected (Penland et al., 2021; Rychlik et al., 2017). To successfully reduce fungal growth in dairy products, the most effective strategy remains to associate preventive measures to reduce the contamination risk with measures that inhibit or reduce fungal growth. In this way, although some LAB strains were not selected for the *in situ* study, those that showed high antifungal rates may be proposed for application as bioprotective cultures, mainly if lowest spore contaminations (as 10^4 or 10^3 spores/g, as represented by 4 and 14 times spore suspensions dilutions) are considered.

Table 1. Inhibitory potential (%) of the CFS of LAB strains against undiluted and diluted spore suspension of *A.niger* IOC 207 and *P. chrysogenum* IOC 132.

Code	Species identification	Undiluted		Diluted 4X		Diluted 16X	
		A.niger IOC 207	*P. chrysogenum* IOC 132	*A.niger* IOC 207	*P. chrysogenum* IOC 132	*A.niger* IOC 207	*P. chrysogenum* IOC 132
Q4C8	*L. lactis* subsp. *lactis*	44.4 ± 0.08	74.1 ± 0.03	64.6 ± 0.17	88.7± 0.01	70.7± 0.19	92.5± 0.01
Q1C10	*L. lactis* subsp. *lactis*	1.4 ± 0.15	69.8 ± 0.12	7.1 ± 0.10	71.4 ± 0.03	45.0 ± 0.51	88.9 ± 0.05
Q1C7	*L. lactis* subsp. *lactis*	18.6 ± 0.10	51.1 ± 0.51	28.5 ± 0.09	78.5 ± 0.09	36.9 ± 0.41	91.4 ± 0.01
Q6C2	*L. lactis* subsp. *lactis*	11.1 ± 0.08	83.8 ± 0.06	15.9 ± 0.10	90.2 ± 0.01	18.8 ± 0.03	92.4 ± 0.01
Q5C6	*L. lactis* subsp. *lactis*	2.4 ± 0.13	83.7 ± 0.03	20.7 ± 0.07	84.5 ± 0.05	43.7 ± 0.31	90.4 ± 0.01
Q1C4	*L. lactis* subsp. *lactis*	44.8 ± 0.11	85.5 ± 0.01	59.7 ± 0.26	90.8 ± 0.01	61.3 ± 0.20	94.6 ± 0.01
Q1C2	*L. lactis* subsp. *lactis*	8.3 ± 0.13	85.7 ± 0.02	26.9 ± 0.05	86.6 ± 0.02	9.4 ± 0.10	92.1 ± 0.01
Q1C5	*L. lactis* subsp. *lactis*	7.4 ± 0.12	82.6 ± 0.01	20.4 ± 0.07	81.3 ± 0.01	6.1 ± 0.01	89.3 ± 0.01
SAM12	*L. lactis* subsp. *lactis*	38.3 ± 0.22	87.4 ± 0.02	79.3 ± 0.11	87.9 ± 0.03	81.1 ± 0.20	92.2 ± 0.01
Q1C8	*L. plantarum*	16.8 ± 0.04	29.3 ± 0.19	33.8 ± 0.05	57.7 ± 0.35	37.7 ± 0.07	86.8 ± 0.16
Q4C3	*L. plantarum*	75.0 ± 0.19	80.6 ± 0.02	89.5 ± 0.01	90.9 ± 0.01	92.0 ± 0.03	92.9 ± 0.01
Q6C7	*L. plantarum*	28.9 ± 0.16	88.3 ± 0.09	28.8 ± 0.08	81.8 ± 0.02	64.1 ± 0.47	89.3 ± 0.04
Q6C8	*P. pentasaceus*	46.1 ± 0.09	73.1 ± 0.01	57.2 ± 0.15	88.9 ± 0.01	64.8 ± 0.23	92.7 ± 0.01
Q1C9	*P.acidilactici*	27.3 ± 0.09	81.0 ± 0.02	16.6 ± 0.15	84.2 ± 0.05	29.8 ± 0.05	88.5 ± 0.03
Q22C2	*P. acidilactici*	39.2 ± 0.13	79.2 ± 0.03	81.3 ± 0.12	86.8 ± 0.01	90.0 ± 0.03	94.7 ± 0.01
Q30C5	*S. infantarius*	27.5 ± 0.24	70.7 ± 0.12	23.6 ± 0.23	86.6 ± 0.02	48.4 ± 0.17	86.2 ± 0.07
W1	*W. confusa*	12.2 ± 0.06	77.1 ± 0.03	39.6 ± 0.07	89.0 ± 0.02	48.5 ± 0.20	93.7 ± 0.01
W3	*W. confusa*	59.5 ± 0.32	84.8 ± 0.02	84.7 ± 0.14	91.0 ± 0.05	88.9 ± 0.08	95.2 ± 0.01
W5	*W. confusa*	89.5 ± 0.04	85.5 ± 0.01	94.1 ± 0.01	93.3 ± 0.01	97.4 ± 0.01	95.2 ± 0.01
W8	*W. confusa*	90.2 ± 0.07	82.9 ± 0.01	94.5 ± 0.01	88.4 ± 0.04	97.1 ± 0.01	95.3 ± 0.01
W9	*W. paramesenteroides*	87.6 ± 0.08	85.1 ± 0.04	94.0 ± 0.01	93.0 ± 0.01	97.1 ± 0.01	95.6 ± 0.01
W10	*W. paramesenteroides*	38.5 ± 0.04	79.7 ± 0.05	42.4 ± 0.34	86.1 ± 0.11	81.4 ± 0.12	93.0 ± 0.01
W11	*W. paramesenteroides*	16.7 ± 0.22	64.2 ± 0.45	28.7 ± 0.13	88.1 ± 0.06	32.3 ± 0.41	89.1 ± 0.04
W12	*W. paramesenteroides*	42.6 ± 0.40	80.6 ± 0.13	43.7 ± 0.17	88.3 ± 0.06	54.6 ± 0.19	90.0 ± 0.03
W14	*W. paramesenteroides*	41.4 ± 0.18	87.6 ± 0.01	42.2 ± 0.03	82.7 ± 0.01	48.4 ± 0.17	88.2 ± 0.04
W15	*W. paramesenteroides*	32.0 ± 0.60	87.3 ± 0.25	47.6 ± 0.10	73.1 ± 0.09	30.2 ± 0.24	89.9 ± 0.01
W18	*W. paramesenteroides*	31.1 ± 0.09	88.8 ± 0.42	20.9 ± 0.29	74.9 ± 0.03	80.2 ± 0.02	83.4 ± 0.05
W19	*W. paramesenteroides*	24.0 ± 0.11	87.6 ± 0.03	57.8 ± 0.07	87.8 ± 0.03	75.1 ± 0.01	90.2 ± 0.04
W20	*W. cibaria*	42.3 ± 0.13	87.4 ± 0.06	79.4 ± 0.01	88.7 ± 0.02	74.6 ± 0.02	88.8 ± 0.01

W21	*W. cibaria*	30.6 ± 0.14	84.9 ± 0.08	74.9 ± 0.02	89.6 ± 0.03	73.9 ± 0.03	91.0 ± 0.03
W22	*W. cibaria*	0.02 ± 0.17	11.5 ± 0.19	9.2 ± 0.12	33.1 ± 0.51	8.4 ± 0.08	71.0 ± 0.02
W23	*W. cibaria*	45.9 ± 0.27	81.4 ± 0.05	58.4 ± 0.26	84.1 ± 0.01	69.4 ± 0.13	86.5 ± 0.06
W25	*W. cibaria*	89.5 ± 0.01	84.2 ± 0.01	95.9 ± 0.01	91.8 ± 0.03	97.3 ± 0.01	96.8 ± 0.01
W26	*W. cibaria*	4.77 ± 0.15	75.1 ± 0.19	4.73 ± 0.12	78.7 ± 0.09	82.5 ± 0.01	83.5 ± 0.02
W27	*W. cibaria*	Full	78.2 ± 0.09	36.7 ± 0.26	79.3 ± 0.05	58.2 ± 0.43	85.1 ± 0.03
W28	*W. cibaria*	Full	65.6 ± 0.08	82.2 ± 0.01	79.5 ± 0.05	83.5 ± 0.04	83.6 ± 0.01
W29	*W. cibaria*	59.6 ± 0.31	83.4 ± 0.05	63.9 ± 0.20	86.1 ± 0.01	67.7 ± 0.08	88.4 ± 0.05
W30	*W. cibaria*	17.3 ± 0.22	72.8 ± 0.05	40.7 ± 0.08	83.8 ± 0.14	32.5 ± 0.53	88.8 ± 0.02
W31	*W. cibaria*	28.8 ± 0.15	87.6 ± 0.06	25.5 ± 0.05	89.9 ± 0.01	42.5 ± 0.11	91.9 ± 0.01
W32	*W. cibaria*	42.2 ± 0.13	87.3 ± 0.01	42.8 ± 0.15	91.3 ± 0.01	48.9 ± 0.18	93.1 ± 0.01
W33	*W. cibaria*	56.6 ± 0.20	84.9 ± 0.02	48.1 ± 0.24	87.0 ± 0.14	39.1 ± 0.23	93.2 ± 0.01
W34	*W. cibaria*	34.6 ± 0.06	87.3 ± 0.02	41.1 ± 0.06	91.7 ± 0.01	35.9 ± 0.23	92.7 ± 0.01
W35	*W. cibaria*	39.2 ± 0.16	86.2 ± 0.09	39.3 ± 0.15	91.6 ± 0.01	62.7 ± 0.24	93.7 ± 0.01
W42	*W. cibaria*	0.1 ± 0.01	0.3 ± 0.03	43.9 ± 1.09	25.7 ± 1.01	92.9 ± 0.14	50.1 ± 0.74
W50	*W. cibaria*	45.0 ± 0.07	88.0 ± 0.02	32.3 ± 0.05	89.0 ± 0.08	31.8 ± 0.25	91.6 ± 0.02
W51	*W. cibaria*	14.5 ± 0.15	84.4 ± 0.07	20.3 ± 0.08	88.0 ± 0.02	31.7 ± 0.30	92.2 ± 0.01
W53	*W. cibaria*	52.0 ± 0.13	86.6 ± 0.01	43.1 ± 0.17	89.1 ± 0.02	37.1 ± 0.12	92.3 ± 0.04
W54	*W. cibaria*	32.3 ± 0.29	87.7 ± 0.02	33.6 ± 0.04	88.8 ± 0.02	59.5 ± 0.19	92.8 ± 0.01
W55	*W. cibaria*	33.5 ± 0.33	87.6 ± 0.01	33.6 ± 0.01	90.8 ± 0.01	42.8 ± 0.10	90.5 ± 0.01
W56	*W. cibaria*	49.5± 0.11	87.3 ± 0.01	33.7 ± 0.18	89.1 ± 0.03	17.8 ± 0.20	90.6 ± 0.01
W57	*W. cibaria*	26.6 ± 0.23	91.5 ± 0.13	38.3 ± 0.17	88.1 ± 0.13	32.1 ± 0.26	90.5 ± 0.01
W87	*W. paramesenteroides*	48.5 ± 0.43	85.3 ± 0.02	71.6 ± 0.04	89.6 ± 0.01	55.0 ± 0.18	93.1 ± 0.01

Values were obtained as detailed in section 2.2.

In addition, the *in vitro* results can be useful to explain how artisanal products can be preserved for a longer time, considering that they are produced without addition of preservatives against fungi. The compounds produced by these LAB can be identified bringing possibilities for the discovery of new antifungal compounds.

Thus, autochthonous LAB strains can be selected for applications that have greater potential for the production of antifungal compounds. Among the 52 tested LAB strains, 5 strains showed antifungal activity higher than 70% against both fungal species at all tested spore concentrations. It is interesting to highlighted that the 6 strains belonged to different species, as: *W. confusa* (W5, and W8), *W. paramesenteroides* (W9), *W. cibaria* (W25), *L. plantarum* (Q4 C3) (Table 1). Thus, these six strains were selected for the *in situ* test. Furthermore, different strains within the same species showed different antifungal activity, confirming that the antifungal activity is a strain-related trait, probably linked to the expression of genes encoding for specific metabolites that make each strain a unique strain (Venegas-Ortega et al., 2019).

3.2. In situ screening of LAB strains for antifungal activity

Results related to the antifungal effect of the five selected strains against *A. niger* and *P. chrysogenum* inoculated in the cheese matrix are shown in Figure 2. Data confirmed that the commercial bioprotective culture showed antifungal action also when combined with the starter culture, whereas *W. confusa* (W5, W8), *L. plantarum* (Q4C3) and *W. paramesenteroides* (W9) lost their antifungal activity when combined with the starter culture. Some hypotheses may infer that the antifungal effect was not effective when the strains were combined with the starter culture: (i) the starter culture may inhibit the production of acids or other antifungal compounds (Blaya et al., 2018) and; (ii) both microbial cultures may compete

for nutrients, for which starter cultures may have an advantage, considering that the tested cultures showed antifungal activity when applied without starter culture (Irlinger et al., 2017).

According to work carried out by Teixeira et al. (2021) the same strains of *W. confusa* (W5, W8) that were used in our study already showed considerable antimicrobial effects against pathogens such as *L. monocytogenes, S. aureus, S. Typhimurium* e *E. coli* and *W. paramesenteroides* (W9) against *L. monocytogenes* on *in vitro* tests. This confirms that these strains have also an antimicrobial potential and that further tests on food matrices can be performed to prove their potential.

Among tested strains, *W. cibaria* (W25) showed some antifungal activity against *A. niger*, even when combined with the starter culture, reinforcing the importance of applying tests simulating different strains and combinations. The strain (W25) was previously characterized also on study carried out by Teixeira et al. (2021) as for its technological property, in addition to producing compounds such as diacetyl; this strain was capable of performing homogeneous milk coagulation, potentially being a strain that may in the future be an adjunct and/or starter culture. Also, this strain is reported as safe (Teixeira et al., 2023). With our results, we can suggest that this strain has great antifungal and technological potential and can be selected for further tests related to the identification of its antifungal compounds, a pilot scale test on different dairy matrices and a complete characterization of its genome.

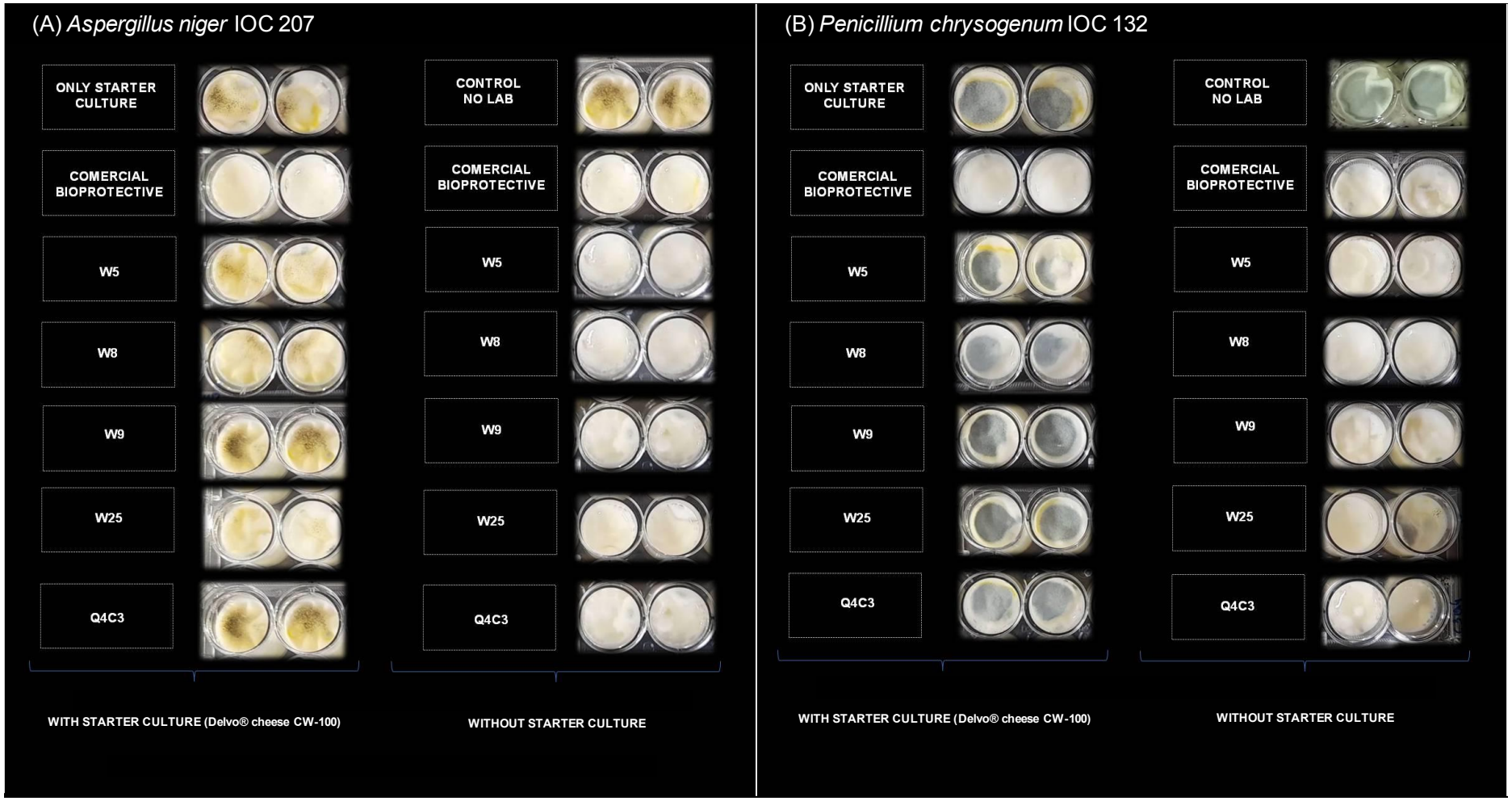


Figure 2. Antifungal activity of LAB on simulated cheese matrix against *A. niger* (A) and *P. chrysogenum* (B).

The relationship and the interaction between strains and starter cultures need to be characterized for commercial applications. Studies that focus into the metabolome of microbial interactions are being explored to elucidate the ability of bacterial strains to produce compounds, whether under adverse conditions related to intrinsic or extrinsic factors of the food or in the presence of other types of bacteria, whether within the same group, or against pathogens, spoilage and filamentous fungi (Honoré et al., 2016). Honoré et al (2016) detected types of active antifungal compounds produced by LAB strains and although were found at very low concentrations to explain the observed antifungal effect, the authors hypothesized that the production of metabolites associated to the consumption of nutrients during the bacterial growth can supply a basis for the antifungal effect, i.e. the composition of its exometabolome.

4. Conclusions

As demonstrated by our *in vitro* results, LAB isolated from artisanal cheeses or dairy environment showed inhibitory activity against *A. niger* and *P. chrysogenum*. The combination of different LAB in the same cheese helps to explain how cheese is preserved even without the addition of chemical preservatives or the application of thermal treatments. In *in situ* tests, the antifungal activity of the LAB strains was strongly affected by the commercial starter culture, confirming that both nutrient assets and culture conditions may negatively interfere in antifungal compounds production. More in-depth research on the best parameters combination to produce antifungal compounds in considerable quantity can be useful in food technology, whether in modifying food composition or process parameters. This knowledge can also direct the LAB to specific foods whose composition and processing favor the production of the antifungal compound. Considering that one strain showed highest rate of fungal inhibition when combined with the starter culture, it is relevant to further

explore their technological potential to propose them simultaneously as starter and bioprotective cultures in cheese processing. Thus, complementary studies regarding the genome and metabolome are recommended.

References

Beauvais, A., Fontaine, T., Aimanianda, V., & Latgé, J.-P. (2014). *Aspergillus* cell wall and biofilm. *Mycopathologia*, *178*(5), 371–377. https://doi.org/10.1007/s11046-014-9766-0

Blaya, J., Barzideh, Z., & LaPointe, G. (2018). Symposium review: Interaction of starter cultures and nonstarter lactic acid bacteria in the cheese environment1. *Journal of Dairy Science*, *101*(4), 3611–3629. https://doi.org/https://doi.org/10.3168/jds.2017-13345

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*. https://doi.org/10.3389/fmicb.2020.00560

Cosentino, S., Viale, S., Deplano, M., Fadda, M. E., & Pisano, M. B. (2018). Application of autochthonous lactobacillus strains as biopreservatives to control fungal spoilage in caciotta cheese. *BioMed Research International*, *2018*. https://doi.org/10.1155/2018/3915615

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/https://doi.org/10.1016/j.foodcont.2017.03.007

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

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

Hannon, J. A., Deutsch, S.-M., Madec, M.-N., Gassi, J.-Y., Chapot-Chartier, M.-P., & Lortal, S. (2006). Lysis of starters in UF cheeses: Behaviour of mesophilic lactococci and thermophilic lactobacilli. *International Dairy Journal*, *16*(4), 324–334. https://doi.org/https://doi.org/10.1016/j.idairyj.2005.04.003

Honoré, A. H., Aunsbjerg, S. D., Ebrahimi, P., Thorsen, M., Benfeldt, C., Knøchel, S., & Skov, T. (2016). Metabolic footprinting for investigation of antifungal properties of Lactobacillus paracasei. *Analytical and Bioanalytical Chemistry*, *408*(1), 83–96. https://doi.org/10.1007/s00216-015-9103-6

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. In *Foods* (Vol. 10, Issue 12). MDPI. https://doi.org/10.3390/foods10123131

Irlinger, F., Helinck, S., & Jany, J. L. (2017). Chapter 11 - Secondary and Adjunct Cultures. In P. L. H. McSweeney, P. F. Fox, P. D. Cotter, & D. W. Everett (Eds.), *Cheese (Fourth Edition)* (Fourth Edition, pp. 273–300). Academic Press. https://doi.org/https://doi.org/10.1016/B978-0-12-417012-4.00011-9

le Lay, C., Mounier, J., Vasseur, V., Weill, A., le Blay, G., Barbier, G., & Coton, E. (2016). *In vitro* and *in situ* screening of lactic acid bacteria and propionibacteria antifungal activities against bakery product spoilage molds. *Food Control*, *60*, 247–255. https://doi.org/10.1016/j.foodcont.2015.07.034

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*. https://doi.org/10.1016/j.foodchem.2019.125260

Leyva Salas, M., Mounier, J., Valence, F., Coton, M., Thierry, A., & Coton, E. (2017). Antifungal Microbial Agents for Food Biopreservation—A Review. *Microorganisms*, *5*(3), 37. https://doi.org/10.3390/microorganisms5030037

Martins, M. C. D. F., Fusieger, A., Freitas, R. D., Valence, F., Nero, L. A., & Carvalho, A. F. (2020). Novel sequence types of Lactococcus lactis subsp. lactis obtained from Brazilian dairy production environments. *LWT*, *124*. https://doi.org/10.1016/j.lwt.2020.109146

Maruyama, S., Streletskaya, N. A., & Lim, J. (2021). Clean label: Why this ingredient but not that one? *Food Quality and Preference*, *87*. https://doi.org/10.1016/j.foodqual.2020.104062

Ndagano, D., Lamoureux, T., Dortu, C., Vandermoten, S., & Thonart, P. (2011). Antifungal Activity of 2 Lactic Acid Bacteria of the Weissella Genus Isolated from Food. *Journal of Food Science*, *76*(6). https://doi.org/10.1111/j.1750-3841.2011.02257.x

Ogunremi, O. R., Freimüller Leischtfeld, S., Mischler, S., & Miescher Schwenninger, S. (2022). Antifungal activity of lactic acid bacteria isolated from kunu-zaki, a cereal-based Nigerian fermented beverage. *Food Bioscience*, *49*. https://doi.org/10.1016/j.fbio.2022.101648

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

Penland, M., Falentin, H., Parayre, S., Pawtowski, A., Maillard, M. B., Thierry, A., Mounier, J., Coton, M., & Deutsch, S. M. (2021). Linking Pélardon artisanal goat cheese microbial communities to aroma compounds during cheese-making and ripening. *International Journal of Food Microbiology*, *345*. https://doi.org/10.1016/j.ijfoodmicro.2021.109130

Pitt John I. and Hocking, A. D. (2009). The Ecology of Fungal Food Spoilage. In *Fungi and Food Spoilage* (pp. 3–9). Springer US. https://doi.org/10.1007/978-0-387-92207-2_2

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

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), 27–34. https://doi.org/https://doi.org/10.1016/0168-1605(96)01101-4

Rychlik, T., Szwengiel, A., Bednarek, M., Arcuri, E., Montet, D., Mayo, B., Nowak, J., & Czarnecki, Z. (2017). Application of the PCR-DGGE technique to the fungal community of traditional Wielkopolska fried ripened curd cheese to determine its PGI authenticity. *Food Control*, *73*, 1074–1081. https://doi.org/10.1016/j.foodcont.2016.10.024

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. (2023). Diversity of Filamentous Fungi Associated with Dairy Processing Environments and Spoiled Products in Brazil. *Foods*, *12*(1). https://doi.org/10.3390/foods12010153

Teixeira, C. G., Fusieger, A., Martins, E., Freitas, R. D., Vakarelova, M., Nero, L. A., & Carvalho, A. F. D. (2021). Biodiversity and technological features of *Weissella* isolates obtained from Brazilian artisanal cheese-producing regions. *LWT*, *147*. https://doi.org/10.1016/j.lwt.2021.111474

Teixeira, C. G., Belguesmia, Y., Rodrigues, R. S., Lucau-Danila, A., Nero, L. A., Carvalho, A. F., & Drider, D. (2023). Assessment of biosecurity traits and in situ antibacterial activities of *Weissella cibaria* strains isolated from Campos das Vertentes, Minas Gerais, Brazil focused for a potential probiotic and food application. *ResearchSquare.* https://doi.org/10.21203/rs.3.rs-2422626/v1

United Nations Environment Programme. (2021). *UNEP Food Waste Index - Report 2021*. United Nations Environment Programme. https://www.unep.org/resources/report/unep-food-waste-index-report-2021

Varsha, K. K., Devendra, L., Shilpa, G., Priya, S., Pandey, A., & Nampoothiri, K. M. (2015). 2,4-Di-tert-butyl phenol as the antifungal, antioxidant bioactive purified from a newly isolated Lactococcus sp. *International Journal of Food Microbiology*, *211*, 44–50. https://doi.org/10.1016/j.ijfoodmicro.2015.06.025

Varsha, K. K., Priya, S., Devendra, L., & Nampoothiri, K. M. (2014). Control of spoilage fungi by protective lactic acid bacteria displaying probiotic properties. *Applied Biochemistry and Biotechnology*, *172*(7), 3402–3413. https://doi.org/10.1007/s12010-014-0779-4

Venegas-Ortega, M. G., Flores-Gallegos, A. C., Martínez-Hernández, J. L., Aguilar, C. N., & Nevárez-Moorillón, G. v. (2019). Production of Bioactive Peptides from Lactic Acid Bacteria: A Sustainable Approach for Healthier Foods. In *Comprehensive Reviews in Food Science and Food Safety*. Blackwell Publishing Inc. https://doi.org/10.1111/1541-4337.12455

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). https://doi.org/10.3390/foods11020195

CHAPTER V.

GENERAL CONCLUSIONS AND FUTURE STUDY

General comments and conclusions

The overall objective of this thesis was to study the biodiversity of the spoilage filamentous fungi in the dairy industry and increase the comprehension of the LAB antifugal activity by *in vitro* and *in situ* tests.

109 filamentous fungi were isolated from spoilage dairy products or their processing environment totalizying 16 different fungi genera were found (*Penicillium*, *Cladosporium*, *Nigrospora*, *Riopa*, *Aspergillus*, *Hipoxylon*, *Fusarium*, *Montagnula*, *Clonostachys*, *Phaeosphaeria*, *Rhinocladiella*, *Coniochaeta*, *Trichoderma*, *Paecilomyces*, *Didymella* and *Bipolaris*). *Penicillium* and *Cladosporium* were found both in spoiled products and dairy environment, indicating a possible cross contamination. With this, we can emphasize that it is always important to review the hygiene and sanitation protocols in the industry in order to avoid the spread of fungi in dairy products.

For dairy production environments, the most frequent genera were *Cladosporium*, *Penicillium*, *Aspergillus*, and *Nigrospora*. Four species (*Hypoxylon griseobrunneum*, *Rhinocladiella similis*, *Coniochaeta rosae*, and *Paecilomyces maximus*) were identified for the first time in dairy products orin dairy production environment. Thus, the understanding of fungal biodiversity in dairy products and environment can help in the choice and dosage of sanitizers used in the environment, thereby, the best composition/dosage can be applied according to the specificity of each dairy industry.

Regarding the antifungal activity of LAB, among the 52 isolates of LAB tested in the *in vitro* test, five strains of LAB, W. *confusa* (W5, W8), *W. paramesenteroides* (W9), *W. cibaria* (W25) and *L. plantarum* (Q4C3) had the greatest antifungal effect against the target fungi tested in suspensions of undiluted , 4-fold and 16-fold concentrations. These cultures also were selected for in situ testing on a simulated cheese matrix. In general, the five selected

strains had a positive antifungal effect against the fungal target inoculated in the cheese matrix, but when combined with a starter culture the antifungal effect was not fully effective. Thus, *in vitro* antifungal activity can be better understood; the compounds produced can be identified and quantified. The antifungal activity in cheese does exist, what needs to be better understood is how one can lessen the effect of the starter culture on this. Thus, it would be important to evaluate even the cultures that had low antifungal activity in the *in vitro* test in combination with the starter culture to verify what kind of interaction can occur.

General recommendations for future study

Many research groups (as presented in our Chapter II Review– Table 1) are currently studying the potential of bacterial cultures for their antimicrobial effect, mainly related to the production of bacteriocins and characterization of these compounds. But in relation to antifungal compounds in the food matrix, it is still something recent and studies have intensified in recent years. I believe that the results of this thesis can be useful to inspire other groups and also for companies to expand research into the antifungal activity of LABs. I still believe that many studies are still needed to characterize the complex interactions between fungal bacteria and the food matrix. With the knowledge obtained so far, I can affirm that I still have many doubts and that, in a way, my critical thinking is already improved. Thus, I believe that several experimental works can be considered and explored in the future. The following are recommendations for further potential study:

Identification and quantification of antifungal compounds in vitro and the in situ

In our study, we were able to verify a strong antifungal activity of LAB both in the *In vitro* and *In situ* tests. We can indeed conclude that there was an antifungal activity in the cheese matrix by the LAB. But we cannot prove what kind of antifungal compound was produced and its quantity. Some recent studies, as we present in our published review article, have sought to identify the compounds involved in the antifungal action, as well as the interactions between fungal bacteria and the matrix and also synergistic action between the compounds, because, some times, an antifungal compound can present a antifungal action even in small proportions and/or an synergic action.

Challenge tests with different dairy products

The centesimal composition of the food is also a major factor that can influence the antifungal activity of LAB. The complexity of the matrix influences the bacterial adsorption process as well all the characteristics related to its intrinsic factors (pH, Aw, oxido-reduction potential, natural microrganisms, among others). Thus, it is interesting that the antifungal test is performed in other other varieties of cheese and/or other dairy products in general.

Evaluation of shelflife of products with the LABs and compare with the commercial cultures

A demand from the dairy industries is, in fact, in addition to providing bioprotection to their products, it is to increase their shelf life. Adjunct culture strains may be able to retard the growth of pathogenic and spoilage microorganisms for long periods. In order to verify the ability of LAB to increase the shelf life of dairy products, this would also be another test to be performed.

Performed the antifungal assay with the LABs in combination (Mixes)

The synergistic effect of mixed cultures is also a possibility to be explored, considering that in addition to providing bioprotection related to filamentous fungi, it can also present on other microorganisms such as pathogenic ones. In addition, the use of mixed cultures can help in the antifungal action against a wider spectrum of filamentous fungi, considering that some can acquire resistance to certain types of acids as discussed in our IV Chapter.

Evaluation of technological and safety aspects, impact on the processing and sensory properties of products.

The strains studied for *in vitro* and *in situ* antifungal activity can be subjected to several complementary tests before proceeding to their potential application as a

bioprotective. Therefore, we strongly recommend evaluating the safety of the strains, as well as tests related to their technological potential. Some strains that we used in the study, mainly those of the genus *Weissella*, already have characterizations, as described by Teixeira et al. (2021). Another important point, especially before starting a test on a pilot scale, we recommend checking the sensory properties on the processing with culture to be tested.

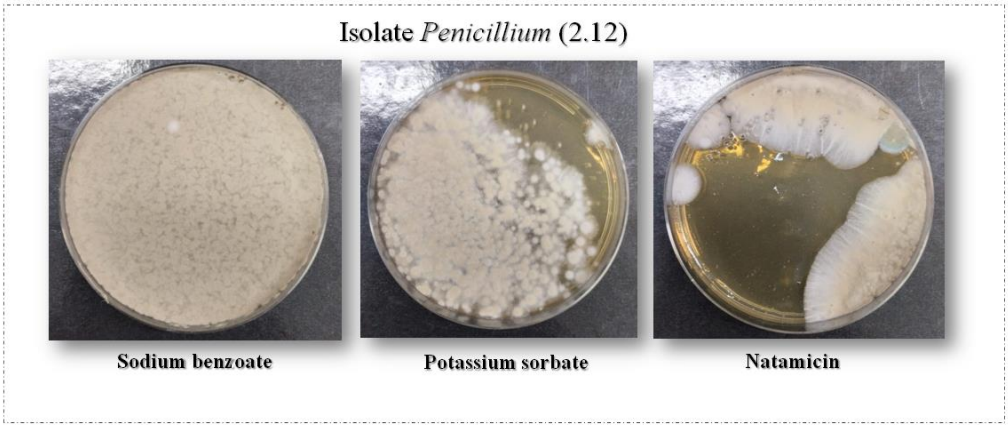
Performed the antifungal assay in pilot scale

Once the bioprotective potential of a strain has been verified and proven in both *in vitro* and *in situ* tests, the last step is to carry out the tests on a pilot scale. This stage is carried out as one of the last phases of the strategy to identify a bioprotective culture. This step is performed last because it involves a greater amount of resources; where it aims evaluate the potential that the culture will in fact meet the needs of the market. The pilot test tries to mimic the conditions of a large-scale industrial production, but is carried out on smaller-scale equipment (size and energy demand), also with the aim of reducing analysis costs and preventing the industrial processing plant from being paralyzed, even temporarily, to carry out the test. In these cases, the bioprotective culture can be added during product processing or inoculated during the final stages of processing, depending on the food product.

Inhibitory effect of LABs against others filamentous fungal

Different filamentous fungi can present different mechanisms of resistance to antifungal compounds, *Aspergillus*, for example, has its cellular structure with melanin that confers a certain resistance to this fungal. And as stress response mechanisms, studies have already been characterizing *Aspergillus* species with resistance to acids. In our research group, we recently carried out an experimental *In vitro* test (to be published) with some fungi

from our collection that are already showing a certain resistance to chemical preservatives in the maximum concentration allowed by brazilian legislation (RDC Nº 4, DE 15 DE JANEIRO DE 2007), such as sodium benzoate, potassium sorbate (1000 mg/kg) and natamicin (5 mg/kg for cheese on the surface, cannot be detected at a depth of 2 mm and must be absent on the inside of cheese) (See figure below).



Thus, considering the importance of these fungi and the possibility of resistance to acids and mainly to the usual chemical preservatives, we strongly recommend that tests be carried out on various types of fungi and especially on those that are already present in the dairy products and your environment.

CHAPTER VI. PHD

OUTPUT

Published papers

- **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.

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.

- **Fungos deterioradores em leite e derivados**: **Alternativas para a redução do uso de conservantes químicos**

Manuscript published in *Revista Indústria de Laticínios*

Authors: Luana Virgínia Souza, Evandro Martins, Raiane Rodrigues da Silva, Antônio Fernandes de Carvalho

- **Characterization of silver nanoparticles and evaluation of their antimicrobial effect on *Salmonella***

Manuscript published in *Research Society and Development*

- DOI: 10.33448/rsd-v9i9.6435.

Abstract

Currently, the success of nanotechnology affects several areas of science, medicine, technology and, especially, the food industry. Silver nanoparticles (Ag-NPs) stand out, with their antimicrobial effect. *Salmonella* is a cause of foodborne diseases and there are reports of resistant serotypes. The use of Ag-NPs is an alternative for bacterial control in foods. The aim of this study was synthesize, characterize and verify the antimicrobial activity of Ag-NPs on serotypes of *Salmonella*. The size of Ag-NPs was estimated and it was possible to detect two populations of 4.7 ± 0.09 and 35.7 ± 2.12. The zeta potential was -33.7 ± 11.8 mV indicating good dispersion stability.Ag-NP antimicrobial activity was determined from minimum inhibitory concentration (MIC).The lowest MIC found was 4.7 $\mu g \cdot mL^{-1}$ for *Salmonella* Enteritidis and the highest was 27.7 $\mu g \cdot mL^{-1}$ for *Salmonella* Infantis 1 isolate.The use of Ag-NPs is promising with respect to antimicrobial activity, however, improvements in synthesis methods should be explored in order to make commercial use viable.

Conference abstracts

Silva, R.R.; Honorato, J.A.; Fusieger, A.; Teixeira, C.G.; Silva, S.R.J.; Pena, M.L.; Souza, L.V.; Simoncello, B.A.; Andretta, M.; Carvalho, A.F. (2021). Influence of culture media on the inactivation of *Bacillus* spp. in processed cheese: *in vitro* evaluation. In: *31º Congresso Brasileiro de Microbiologia*. On-line.

Teixeira, C.G.; Silva, R.R.; Nascimento, L.G; Souza, L.V.; Martins, E.; Carvalho, A.F. (2020). Influência do encapsulamento de nisina sobre a multiplicação de *Listeria monocytogenes* ATCC 7644 em leite. In: *65º Congresso Internacional de Gastronomia*. On-line.

Souza, L.V.; Silva, R.R.; Nascimento, L.G; Teixeira, C.G.; Martins, E.; Carvalho, A.F. (2020). Queijo Minas Frescal adicionado de Nisina e avaliação do seu efeito inibitório sobre *Listeria monocytogenes* ATCC 7644. In: *65º Congresso Internacional de Gastronomia*. On-line.

Certificates Extension Service (Companies)

The present document has been faithfully translated from Portuguese into English
Enedina Maria de A. Sacramento
Translator - UFV - 3848-X

CERTIFICATE

We certify that LUANA VIRGINIA SOUZA participated in the provision of the extension service "IP AND EP COMPOSITION ANALYSIS OF PRATO AND DAMBO CHEESES DURING RIPENING", registered in the Extension Activities Registration System, RAEX, under No. PRS-376/2022, from 06/10/2022 to 7/10/2022, as a Collaborator. Total workload: 1200 minutes.

Viçosa, October 27, 2022.

The present document has been faithfully translated from Portuguese into English
Enedina Maria de A. Sacramento
Translator - UFV - 3848-X

CERTIFICATE

We certify that LUANA VIRGINIA SOUZA participated in the provision of the extension service "ANALYSIS OF CHEESE RIPENING", registered in the Extension Activities Registration System, RAEX, under No. PRS-244/2020, from 04/01/2020 to 12/01/2020, as a Collaborator. Total workload: 960 minutes.

Viçosa, October 27, 2022.

UFV Universidade Federal de Viçosa

PRÓ-REITORIA DE ENSINO

Certificate

Luana Virgínia Souza

Student of the Doctorate in Food Science and Technology course, carried out Monitoring activities, Scholarship holder, Level II - Special Demands, at the Food Technology Department, during the Remote Special Period 2 (PER 2), from March 5th to September 21st May 2021, with assignments established by the Regulation in force at this University, accounting for a total of 128 (one hundred and twenty-eight) hours.

Summary: TAL414 - Microbiology of Milk and Derivatives 6(2-4) II. MBI100* or MBI101*. TAL484 - Principles of Food Preservation 4(2-2) II. MBI130 and TAL472*.

Viçosa, 07 de julho de 2021.

João Carlos Pereira da Silva

UFV Universidade Federal de Viçosa

PRÓ-REITORIA DE ENSINO

Certificate

Luana Virgínia Souza

Student of the Doctorate course in Food Science and Technology, carried out activities of Monitoring, Scholarship holder, for Remote Teaching, at the Department of Food Technology, during the PER - Special Period Remote, from August 31 to December 18, 2020, with duties established by the current Regulation at this University, accounting for a total of 135 (one hundred and thirty-five) hours.

Summary: TAL 440 - Processing of Products of Animal Origin 5(3-2) I and II. MBI 100.

Viçosa, 07 de julho de 2021.

João Carlos Pereira da Silva
Pró-Reitor de Ensino - PRE

APPENDICE

Appendix 1. Main keywords of the thesis.