Universidade Estadual de Londrina

JULIANA MAREZE

INHIBITORY POTENTIAL OF LACTIPLANTIBACILLUS PLANTARUM TO BIOCONTROL SPOILAGE AND MYCOTOXIGENIC PENICILLIUM IN CHEESE

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Thesis presented to the Postgraduation Program in Animal Science at State University of Londrina to obtain the degree of Doctor of Science.

Advisor: Vanerli Beloti (UEL, Brazil)
Co-advisor: Teresa María López-Díaz (ULe, Spain)

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M326 Mareze, Juliana.
Inhibitory Potential of *Lactiplantibacillus plantarum* as Biocontrol of Spoilageand Mycotoxigenic *Penicillium* in Cheese / Juliana Mareze. - Londrina, 2021.
168 f.

Orientador: Vanerli Beloti.
Coorientador: Teresa María López-Díaz.
Tese (Doutorado em Ciência Animal) - Universidade Estadual de Londrina, Centro de Ciências Agrárias, Programa de Pós-Graduação em Ciência Animal, 2021.
Inclui bibliografia.

1. Lactobacilli - Tese. 2. Antifungal activity - Tese. 3. Biocontrol - Tese. 4. Penicillium - Tese. I. Beloti, Vanerli. II. López-Díaz, Teresa María. III. Universidade Estadual de Londrina. Centro de Ciências Agrárias. Programa de Pós-Graduação em Ciência Animal. IV. Título.

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Londrina, June 2nd, 2021.

FINANCING

We thank the Coordination of Improvement of Higher-Level Personnel (CAPES, Brazil) for the funding provided to the doctoral student through a grant that covered the development period of the Doctoral Thesis and also the Sandwich Doctorate financed by PDSE-CAPES 88881.188409/2018-01.

The experiments carried out in the Department of Food Hygiene and Food Technology (University of León, Spain) in this Doctoral Thesis have been financed by FEDER 2014-2020 (Project RTA2015- 00018-C03-03 of the National Institute of Agricultural and Food Research and Technology, INIA, Spain).

ACKNOWLEDGMENTS

I thank Post Graduate Program in Animal Science of the State University of Londrina (UEL, Paraná, Brazil) and Coordination of Improvement of Higher-Level Personnel (CAPES, Brazil) for providing me the research fellowship to carry out this study.

With gratitude and pleasure, I thank my co-adviser Teresa María López-Díaz for allowing me to work with her research group at the University of Léon (Unileon, Castilla y Léon, Spain) and for allowing me to participate in others projects. Thank you for trusting me to do my sandwich doctoral internship in Spain. In addition to giving me the freedom to carry out the project and assist me in research, she took me to see a little piece of Spain that enchanted me. I will always be grateful.

I thank all the Department of Hygiene and Food Technology-University of León staff who gave me support during my stay at the university. I also thank Jesús Ángel Santos Buelga and José María Rodríguez Calleja for their contributions. To technicians Aurelia and Rosa who were always ready to help me. To Ángel and Laura for their help with the experiments and Domingo Fernández (Institute of Food Science and Technology-University of León) for the HPLC analyzes carried out. I will always remain grateful to them.

This work could not have been accomplished without the assistance of Juliana Ramos Pereira. I am deeply indebted to you for helping me during my work. I extend my thanks to Egon Henrique Horst, for his help, support and motivation. He is always giving me advice and helping me focus on various possibilities to reach my goal.

I am deeply indebted to my adviser Vanerli Beloti, for always being kind and encouraging during my work. Her suggestions helped me to overcome the hard times of my research for which I will stay thanks to her.

I acknowledge the help received from Animal Products Inspection-LIPOA at State University Londrina. Thank you for being my second home for eight years. During this time, I learned many things and met many people for whom I will be always grateful. Felipe Nael Seixas, Ronaldo Tamanini, Edson Antonio Rios thank you for all the support to finish it.

I thank the Laboratory of Veterinary Mycology and Laboratory of Medical Mycology and Oral Microbiology at State University of Londrina for the technical support.

My inseparable friend, Natalia Gonzaga, I am immensely grateful for our friendship and her support on the countless nights and weekends of work during those years. This journey would not have been easy without you.

Special thanks to my friends Tarini, Carmem, Nathalia, Jessica, Natani, Camila, Francine, Emmanuelle, Andressa, Leiluana and Hanna for always believed in me.

Finally, to my loving, caring, and encouraging mother, father, and sister: my deepest gratitude. It’s their faith and support that gives me the strength and inspiration to pursue my dreams.

"Without data you're just another person with an opinion."

W. Edwards Deming

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RESUMO

O queijo é um excelente substrato para o crescimento de fungos e seu potencial deteriorante é uma grande preocupação para a indústria de laticínios. O emprego de aditivos alimentares é uma das alternativas utilizadas para controlar a contaminação por fungos e evitar a deterioração do produto. O uso de conservantes naturais tem se tornado popular devido à preocupação com os efeitos nocivos dos conservantes químicos. As bactérias ácido lácticas são utilizadas como culturas iniciadoras na fabricação de queijos e, devido ao seu efeito inibitório contra outros microrganismos, seu uso como agente biocontrolador pode ser uma alternativa para controlar fungos deteriorantes e micotoxigênicos em queijo. Esta tese explora a possibilidade do uso de cepas de Lactobacilos para inibir o crescimento de fungos em queijo. Com o objetivo de avaliar o potencial antifúngico de *Lactobacillus*, foram selecionadas 32 cepas previamente isoladas do Queijo Serrano Artesanal e testadas contra *P. nordicum*, *P. commune*, *P. expansum* e *P. roqueforti*. Do total dos *Lactobacillu*s avaliados, duas cepas (L119 e L49) apresentaram alta atividade antifúngica contra todos *Penicillium* spp. testados. Essas duas cepas identificadas como *L. plantarum* foram selecionadas para caracterizar o composto responsável pela atividade antimicrobiana. Nossos resultados sugerem que a capacidade inibitória não está ligada somente à produção de ácidos, uma vez que a neutralização das substâncias produzidas em meio de cultura manteve inibição frente aos patógenos e fungos filamentosos testados, indicando que outras substâncias possam estar envolvidas. *L. plantarum*-L119 e o L49 mostraram capacidade de inibir *E. coli*, *S. enterica*, *S. aureus* e *L. monocytogenes* por meio das técnicas *spot-on-lawn* e turbidimétricas, além de apresentarem características fermentativas e tecnológicas interessantes, conforme avaliação realizada com o API50CH e APIZYM, respectivamente. Dentre os principais fungos deteriorantes e micotoxigênicos, foram isoladas de amostras de queijo, 17 cepas do gênero *Penicillium* e identificadas por caracterização morfológica, análise de extrólitos e sequenciamento de DNA. Do total de cepas avaliadas, nove foram identificadas como *P. commune*. Também foi analisada a capacidade de produção do ácido ciclopiazônico (CPA) pelas cepas de *P. commune* isoladas neste estudo, e os resultados mostram que algumas cepas são boas produtoras enquanto outras produzem baixas quantidades de CPA, com interesse para a indústria de queijos. Ao confirmar que *P. commune* é a principal espécie deteriorante e micotoxigênica encontrada em queijos, estudamos o efeito de duas cepas de *L. plantarum* (L119 e L49) sobre seu crescimento e produção de CPA *in vitro* e também em queijo. L119 e L49 demonstraram boa capacidade de conter o crescimento dos fungos filamentosos e produção de micotoxinas *in vitro*. A capacidade antifúngica do L119 se estendeu à inibição da produção de CPA em fatias de queijo. Esses resultados mostraram que *L. plantarum*-L119 é um bom candidato para uso como bioconservante na indústria de laticínios, apresentando característica tecnológica, capacidade de inibir bactérias patogênicas, fungos filamentosos e suas respectivas micotoxinas em queijo. Análises adicionais devem ser realizadas para explorar sua segurança e seus efeitos organolépticos em produtos lácteos. A perspectiva futura é utilizar as BAL antifúngicas em alimentos para controlar o crescimento de fungos deteriorantes e micotoxigênicos.

Palavras-chave: Bioconservante, atividade antimicrobiana, queijo, micotoxina, bolores deteriorantes, *Lactobacillus* (*Lactiplantibacillus*) *plantarum*, *Penicillium*.

MAREZE, Juliana. **Inhibitory Potential of *Lactiplantibacillus plantarum* as Biocontrol of Spoilage and Mycotoxigenic *Penicillium* in Cheese**. 2021. 150. Thesis (Doctor's Degree in Animal Science) – Universidade Estadual de Londrina, Londrina, 2021.

ABSTRACT

Cheese is an excellent substrate for mold growth and its deterioration is a critical concern for the dairy industry. To control mold contamination and avoid spoilage of dairy products it is necessary to use some food additives as an alternative to minimize the impact of those microorganisms. Nonetheless, the use of natural preservatives has become more popular giving the concern about harmful effects of chemical preservatives. Since lactic acid bacteria have been safely used as starter cultures in cheese-making and owing their inhibitory effect against other microorganisms, their use as biocontrol agents can be an alternative to control spoilage and mycotoxigenic molds in cheese. This thesis explores the possibility use of *Lactobacilli* to inhibit fungal growth in cheese. In order to evaluate the antifungal potential of *Lactobacilli*, we selected 32 strains previously isolated from Brazilian Artisanal Serrano Cheese and screened against *P. nordicum*, *P. commune*, *P. expansum* and *P. roqueforti* through overlay method. From the total *Lactobacilli* evaluated, two strains (L119 and L49) showed high antifungal activity against all the *Penicillium* spp. tested. These two strains identified as *L. plantarum* were selected to determine the antifungal and antibacterial activity through impedance technique and the compound responsible for these antimicrobial activities. Although it was not possible to identify the antimicrobial compound, our results suggests that the inhibitory potential was not only linked to the production of acids, since the neutralization of the substances produced in culture medium maintained the inhibition against the pathogens and filamentous fungi tested, indicating that other substances may be involved. The L119 and L49 showed ability to inhibit *Escherichia coli*, *Salmonella enterica*, *Staphylococcus aureus* and *Listeria monocytogenes* through spot-on-lawn and turbidimetric techniques, and also technological characteristics evaluated in API50CH and APIZYM. Giving the main spoilage and mycotoxigenic mold found on cheeses, we isolated 17 strains of *Penicillium* genus and identified them through morphological characterization, extrolite analysis and DNA sequencing. From the total strains evaluated, nine were identified as *P. commune*. Regarding their ability to produce the cyclopiazonic acid (CPA), we also analised the production of CPA by the *P. commune* strains isolated in this study. Our results show that some *P. commune* strains are good producers of CPA while others produce low quantities, arousing interest for its use in the cheese industry. Confirming that *P. commune* is the main spoilage and mycotoxigenic species found in cheeses we studied the effect of the two *L. plantarum* (L119 and L49) on their growth and mycotoxin production *in vitro* and also in cheese slices. L119 and L49 demonstrated good ability to affect their growth and mycotoxin production *in vitro*. The antifungal ability of L119 extended to inhibit CPA production on cheese slices. Those results showed that *L. plantarum* L119 is a good candidate to use as biopreservative agent in dairy industry giving its technological characteristic and ability to inhibit pathogenic bacteria, molds and their respective mycotoxin on cheese. Further analyses should be carried out to explore their safety and organoleptic effects on dairy products. The future perspective is to use these antifungal lactic acid bacteria in food or feed products to decrease the growth of spoilage and mycotoxigenic fungi.

Key words: Biopreservative, antimicrobial activity, cheese, mycotoxin, spoilage molds, *Lactobacillus* (*Lactiplantibacillus) plantarum, Penicillium*.

GENERAL INTRODUCTION

There were many definitions of food safety but the most common among consumers is “food that causes no harm to consumer”. In the context of food microbiology, it can mean a food without pathogenic microorganism and/or their hazard metabolites.

Giving the difficulty to eliminate all kind of microorganisms that are present in the environment and consequently in food, the use of some food additives as an alternative to minimize the impact of spoilage or pathogenic microorganisms are necessary. Chemical preservatives are among the most used means for maintaining food quality by inhibiting microbes (Angiolillo et al., 2014). The growing importance given to healthy eating reflects an increased awareness about the harmful effects they may have on human health. Natural preservatives have become more popular due to its minimal impact on the nutritional and sensory properties of food (Jones et al., 2011).

Biological preservation is an example of natural preservative. In a scientific way, it consists in the use of antagonistic microorganisms or their metabolic products to inhibit or destroy undesired microorganisms in foods, in other words it implies in extend the shelf-life of food and improve food safety using natural microflora (Collins et al., 2019).

Spoilage microorganisms are responsible for significant food losses, due to an inadequate shape or appearance, decrease in nutritional value, and potential food poisoning. Microorganisms such as fungi or bacteria can strongly affect the taste and appearance of food for human consumption. However, fungi are found in a wide variety of ecological niches and tolerate extreme conditions which make them the major spoilage group of concern. Furthermore, some fungi are producers of many different secondary metabolites that are toxic (Frisvad et al., 2007).

Lactic acid bacteria have a long tradition in food fermentation and are also safe to consume. Owing to their inhibitory effect against other microorganisms as a result of competition for nutrients and/or the production antagonistic compounds, they have a major potential for use in biopreservation (Wu and Li, 2018). Since lactic acid bacteria have been safely used as starter cultures in cheese-making, their use as biocontrol agents should be another alternative to control spoilage and mycotoxigenic molds in cheese.

This thesis explores the possibility of using *Lactobacilli* to inhibit fungal growth. The future perspective is to use these antifungal lactic acid bacteria in food or feed products to decrease the growth of spoilage and mycotoxigenic fungi.

Chapter I.

LITERATURE REVIEW

1. LITERATURE REVIEW

1.1. MICROBIOLOGY OF RAW MILK

Milk and dairy products are a storehouse of a wide variety of microbes. Due to its high nutritional composition, near neutral pH and high-water activity, milk provides an ideal environment for the growth of various microorganisms. Therefore, raw milk may contain several kinds of microorganisms with variable characteristics (Perin et al., 2019; Singhal et al., 2020).

The number and diversity of microorganisms are directly influenced by several factors like sanitary conditions of the dairy herd, environmental hygiene, milking equipment used, and factors related to the raw milk handling, storage, cooling, and processing (Murphy, 2016; Moatsou and Moschopoulou, 2014). The sources of microbial contamination are multiple and diverse. Most of the bacterial and fungal species found in milk usually originate from the dairy farm or dairy processing plant (Angelidis, 2015; Vacheyrou et al., 2011). The raw milk microbiota may be composed of spoilage, pathogenic and beneficial bacteria.

The microbial count is a useful method to measure the quality of milk where high counts and groups or species of microorganisms found in milk demonstrate inadequate hygienic practices of obtaining, preserving, or processing. The hygiene indicators are constituted of aerobic mesophilic, psychrotrophic, and coliforms groups (Montel et al., 2014; Perin et al., 2017). Besides the contamination by spoilage microorganisms, milk can carry several pathogens, thus affecting the health of consumers, especially when they ingest raw milk or dairy products manufactured with raw milk (Perin et al., 2019; Singhal et al., 2020).

Other spoilage microorganisms of great importance are yeasts and molds. They are nonpathogenic but molds are mycotoxigenic and are the main cause of spoilage in dairy products (Moatsou and Moschopoulou, 2014). They are common contaminants in food and also the major group responsible for food losses during storage of crops and food products (Pitt and Hocking, 2009).

Among bacteria, they can be spoilage or pathogenic with mesophilic, psychrophilic, or thermophilic behavior and even with significant technological importance (Perin et al, 2017). These contaminating microorganisms with technological importance such as lactic acid bacteria (LAB) are essential for the production of dairy products and are generally required as a dominant population in milk, prior to pasteurization (Coppola et al., 2008).

Before the adoption of pasteurization, milk was responsible for the transmission of milk-borne diseases such as brucellosis and tuberculosis. Nowadays, besides these two zoonotic diseases other pathogens are responsible for the higher frequency of outbreaks related to the consumption of raw milk or other contaminated products (Singhal et al., 2020). Another concern is the presence of mycotoxigenic fungi. Although molds are not considered foodborne pathogens, they can produce secondary metabolites (mycotoxins) with toxicological effects, being the natural toxicants of most concern to public health (Bosco and Mollea, 2012).

Pasteurization is aimed to destroy all pathogenic and spoilage microorganisms that affect the quality and safety of milk, and because of that, the desirable microbiota of LAB are also eliminated, which causes a negative impact on food fermentations. Traditional raw-milk cheeses are highly valued for their characteristics since the microbiota presents in raw milk play an important role in flavor development (Kongo, 2013). Many recent studies, focused on the biotechnology of LAB, brought benefits to the dairy industry making it possible to re-introducing them in food. Thus, they have been extensively studied for their economic importance in food industries.

1.2. Spoilage and Pathogenic Microorganisms In Raw Milk

Raw milk and raw milk products can be a major source of foodborne pathogens of human health concern which can lead to many illnesses. The presence of foodborne pathogens in milk is due to excretion from the udder of an infected animal, such as *Mycobacterium bovis* and *Brucella abortus*, and direct contact with contaminated sources in the dairy farm environment allowing contamination by microorganisms through equipment, floor, soil, water, and feces, such as *Staphylococcus aureus*, *Campylobacter* spp., *Salmonella* spp., *Listeria monocytogenes*, *Escherichia coli* (Perin et al., 2019).

To avoid outbreaks and illness caused by pathogenic microorganisms in milk, the pasteurization conditions was designed to reduces the presence of those microorganisms to a level it cannot constitute health hazard. Also, certification programs which included culling of infected animals, the installation of refrigerated bulk tanks for milk collection on farms contributed to the reduction of such diseases (Sarkar, 2015).

Pasteurization has been demonstrated to be an adequate treatment to control any undesirable microbe combined with the application of hygienic practices. So, the incidence of pathogens in pasteurized milk and food-borne outbreaks are associated to inadequate pasteurization or post-pasteurization contamination (Jooste et al., 2014).

Regarding pathogenic bacteria in raw milk, the presence of spoilage microorganisms especially in dairy products such as cheeses, yogurts, and butter constitutes a serious problem for the dairy industry. They are responsible for promoting discoloration, poor appearance, and off-flavors and wastage of the dairy products (Ledenbach and Marshall, 2009). Food waste and losses became a significant issue for the economy and human health. It is estimated that fungal deterioration is responsible for 5 to 10% of the world's food production lost (Pitt and Hocking, 2009).

At the same time mold is required to growth on the surface of some cheeses for ripening, some of them are generally not desirable because of both visual and organoleptic defects it may result (Awasti and Anand, 2020). Besides, some species also produce mycotoxins (Bosco and Mollea, 2012). Effective hygiene is important to control spoilage microorganisms in the food industry and rigorous cleaning procedures are required to prevent their growth and its consequences (Fernandes, 2009). Furthermore, microbial spoilage is the greatest barrier to extending the shelf life of milk and other dairy products, because of this the adequate sanitization during critical stages of milk production and processing is indispensable to control bacterial and fungal growth (Lu and Wang, 2017).

1.2.1. *Escherichia coli*

Escherichia coli (*E. coli*) is a gram-negative, rod-shaped, facultative anaerobic bacterium. Its primary habitat is the gastrointestinal tract of humans and animals. It is considered the best indicator of fecal contamination for water and food products because its presence suggests that other organisms of fecal origin, including pathogens, may be present. Due to this characteristic, it is considered as an indicator microorganism in food safety and hygiene.

Pathogenic *E. coli* are responsible for the main clinical syndromes like enteric and diarrheal diseases, urinary tract infections, and sepsis/meningitis. (Jooste et al., 2014; Stein and Chirila, 2014). In food safety, the most important *E. coli* are those that cause diarrheal diseases.

Enteric *E. coli* are part of the natural flora of many animals. They belong to a pathogroup termed diarrheagenic *E. coli*, which is divided into different pathotypes based on their virulence properties and clinical manifestations. The pathotypes include: enterotoxigenic *E. coli* (ETEC), enteroinvasive *E. coli*. (EIEC), enteropathogenic *E. coli* (EPEC), enteroaggregative *E. coli* (EAEC), enterohemorrhagic *E. coli* (EHEC) and Shiga toxin-producing *E. coli* (STEC). EHEC and STEC are the most studied pathogenic *E. coli*

in farm environments and also related to outbreaks caused by the consumption of contaminated dairy products, although other types have been reported (Caprioli et al., 2014).

Milk can be contaminated with feces, usually, during milking, it can be a vehicle for *E. coli* and therefore considered an important source of contamination (Fernandes, 2009). In addition to fecal contamination, other factors such as wet udders and udder infection are the main sources of *E. coli* in bulk milk (Singhal, 2020).

Due to its tolerance to low pH, *E. coli* can survive in acidic foods and fermented dairy foods for long periods. So, the cheese-making process does not inactivate *E. coli*, and several outbreaks are associated with the consumption of cheese and other dairy products (Jooste et al., 2014).

1.2.2. *Staphylococcus aureus*

Staphylococcal food poising is one of the most common foodborne diseases worldwide with high occurrence. Is often associated with the ingestion of manually handled foods that contain heat-stable staphylococcal enterotoxins. The production of this toxin can be prevented by keeping the raw milk refrigerated until the milk can be effectively pasteurized (Fernandes, 2009).

The bovine mammary gland can be a significant source of enterotoxigenic strains of *Staphylococcus aureus*, being the main cause of mastitis in dairy cows. There is evidence of bulk tank milk as a potential source of enterotoxigenic *S. aureus* in milk and its products and the consumption of raw milk generally increases the chances of food poisoning (Jooste et al., 2014).

Its presence in milk can also occur during the handling of raw milk or dairy products involving food handlers since humans are nasal carriers of *S. aureus* or carry the microorganism as part of the normal skin microflora (Jooste et al., 2014; Perin et al., 2019).

Since sanitary conditions of the animals are not adequate or manipulation allows the contamination by *S. aureus*, the presence of some *S. aureus* strains leads to the production of enterotoxins that can induce a clinical problem in humans characterized by vomiting and dehydration (Perin et al., 2019; Singhal, 2020).

S. aureus can tolerate salt and moderate acidity and can multiply during cheese manufacture and ripening in soft cheeses. From the perspective of food safety and human health, cheese is a food in which *S. aureus* is prone to trigger important public health

concerns because it may survive in cheeses for long periods and the enterotoxin produced as well (Fernandes, 2009).

1.2.3. *Salmonella spp.*

Foodborne infections caused by *Salmonella* sp. are often associated with eating meat, poultry, eggs, milk, and dairy products without heat treatment (Jackson et al., 2013). They may be present in cheese if the pasteurization process does not occur properly and if acid production during manufacture is slow. In cheese made from raw milk, they may survive through the cheese-making process and be present in the final product (Fernandes, 2009).

Raw milk can be a source of *Salmonella* however, it is not able to survive the process. Their presence indicates that the process has not been carried out effectively, or that post-pasteurization contamination has occurred. Inadequate pasteurization and post-process contamination have occasionally resulted in outbreaks (Jooste et al., 2014). For this reason, unpasteurized milk remains a public health hazard.

Salmonella is not particularly tolerant of heat, refrigeration, or salt (Jooste et al., 2014). It is very important that effective precautions and monitoring procedures, based on HACCP principles, are necessary to prevent its presence on processed food (Fernandes, 2009).

1.2.4. *Listeria spp.*

Listeria species are widely distributed in the environment, being the *Listeria monocytogenes* species the most common source of foodborne illness. It has emerged in recent decades as a serious foodborne pathogen that can cause abortion and death in pregnant women, meningitis, encephalitis, and septicemia in newborn infants and the immunocompromised (Jooste et al., 2014).

The most important property of *L. monocytogenes* is its ability to grow at refrigeration temperatures which means that it can lead to foodborne listeriosis even if the food is stored at the proper temperature. In addition to psychrotrophic characteristic, has the ability to form biofilms, which are structures organized adhered to the surfaces of equipment and milking utensils (Fernandes, 2009).

L. monocytogenes contamination of dairy products typically occurs from the processing facility environment, where strains are well adapted to the dairy factory. The most likely sources in raw bulk-tank milk are environmental with the presence of

humidity and organic matter. The pathogen is of great concern to the dairy industry since milk and dairy products have been the transmission vehicles commonly associated with outbreaks linked to raw milk cheeses and raw milk consumption (Singhal, 2020). In terms of prevention or control procedures, to prevent the presence of *L. monocytogenes* in raw milk is the application of good sanitation based on strict hygiene on milking practices.

1.2.5. Fungi

Apart from bacteria, molds and yeasts can also be the source of spoilage in dairy products. They are present in air, water, and soil and are regularly found on surfaces, equipment, and ingredients. The spoilage by these microorganisms is associated with their potential to grow at refrigeration temperatures, acidic environment, low water activity, high salt concentrations, and in atmospheres of low oxygen tension. Some molds have an advantage for their resistance to some chemical preservatives (Garnier, 2017a; Sorhaug, 2011).

The fungal contamination in dairy foods can occur from dairy farms to dairy processing units, being the stable and milking parlor environments the most important sources. Therefore, milk and its products require attention to avoid contamination during milking, transportation, storage, processing, and distribution (Awasti and Anand, 2020).

Mold spoilage is often due to airborne fungi because their spores are easily dispersed into the dairy plant air. The most common genera associated with spoilage of dairy products are the yeasts *Candida* spp., *Debaryomyces, Kluyveromyces, Yarrowia, Galactomyces,* and *Saccharomyces* spp. and the molds *Penicillium* spp., *Aspergillus* spp., *Mucor* spp. and *Cladosporium* spp. (Garnier, 2017a; Lu and Wang, 2017).

The presence of molds in dairy products results in undesirable growth (colony or thallus), such as the “toad skin” or the “cat hair” caused by *Galactomyces geotrichum* and *Mucor* spp., respectively, and also via fungal metabolism resulting in the production of off-odors and/or off-flavors, gas production or texture alteration (Garnier et al., 2017b; Ledenbach and Marshall, 2009). Besides, spoilage species such as *Aspergillus* and *Penicillium* spp. are able to produce mycotoxins (Pitt and Hocking, 2009).

Yeasts are the most important contaminants causing spoilage of yogurt and fermented milks, whose low pH offers a selective medium for their development. They release alcohol and carbon dioxide contributing to off-flavors and gas production as the main defects on products. Some strains may additionally produce sulfides, which give the cheese an “egg” odor (Lu and Wang, 2017; Ledenbach and Marshall, 2009).

Yeasts and molds are not heat-resistant and for that reason, they should be killed after pasteurization, except for a few fungal species. Therefore, its presence during manufacturing or in the final product generally occurs after milk heat-treatment (Sorhäug, 2011). Strategies that reduce or prevent fungal contamination such as refrigeration and chemical preservatives combined with methods that avoid contamination and recontamination such as air filtration, Hazard Analysis and Critical Control Points (HACCP), good hygienic and manufacturing practices play an important role in maintaining the quality of the product (Awasti and Anand, 2020).

1.3. LACTIC ACID BACTERIA

The real application of LAB and its importance began in the 1850s and its health effect was popularized by Elie Metchnikoff who won the Nobel Prize for his works in the "longevity theory". His theory was about the large number of lactic acid bacteria in yogurt which played an important role in controlling the growth of harmful bacteria, prolonging the life of the residents in the Balkan island (Chen and Wang, 2018).

Many recent studies in food science have focused on the beneficial effects of LAB. The benefits do not include only enhancement of nutritional composition and flavor of fermented foods, but also improving host immune system by metabolites with health-promoting properties, inhibition of pathogens and spoilage microorganisms, reduction of food allergy, treating health disorders, and controlling inflammatory bowel diseases. These advantages should be taken to promote the use of a diet rich in LAB which dairy products are the major and commonest source of them (Masood et al., 2011; Ameen and Caruso, 2017; Zhang and Narbad, 2018)

The term Lactic Acid Bacteria comprehends a heterogeneous group of microorganisms whose main characteristic is the production of lactic acid as their major end product through fermentation of carbohydrates. They are classified into different genera based on cell morphology, sugar fermentation, growth at different temperatures, ability to grow at high salt concentrations, and acid tolerance (Holzapfel and Wood, 2014).

They are naturally found in many nutrient-dense foods like milk, meat, and vegetables. They are characterized by being Gram-positive, catalase-negative, non-spore-forming, anaerobic, air tolerant, acid-tolerant, with strictly fermentative metabolism. They can be classified as mesophilic or thermophilic, with optimal growth temperatures between 30 and 37 ℃ and 45 to 50℃, respectively (von Wright and Axelsson, 2019).

The genera included are *Lactobacillus*, *Lactococcus*, *Bifidobacterium*, *Carnobacterium*, *Enterococcus*, *Leuconostoc*, *Oenococcus*, *Pediococcus*, *Streptococcus*, *Aerococcus*, *Tetragenococcus*, *Vagococcus*, and *Weissella*. The *Lactobacillus*, *Lactococcus*, *Leuconostoc* and *Pediococcus* genera are of greatest interest to industry (Chen and Wang 2018; Holzapfel and Wood, 2014; Liu et al., 2014). Among them, *Lactobacilli* belongs to a large group and are also dominant in food fermentations and applied as probiotic cultures (Ibrahim and Ouwehand, 2019).

The fermentation pathways can differentiate them into homo or heterofermentative. In the first case, only lactic acid is generated by glycolysis fermentation and in the second case, in addition to lactic acid, significant amounts of CO_2 and ethanol or acetate are produced. The genera *Lactococcus*, *Pediococcus*, *Enterococcus*, and *Streptococcus* are characterized by being homofermentative while the genera *Leuconostoc* and *Weissela* have heterofermentative characteristics. The genus *Lactobacillus* has some species with homo and some with heterofermentative characteristics (von Wright and Axelsson, 2019).

Besides lactic acid, LAB produce other acids such as acetic and propionic, which is usually not favorable to the multiplication and survival of spoilage and pathogenic microorganisms. They are also producers of hydrogen peroxide, diacetyl, acetaldehyde, carbon dioxide, short-chain fatty acids, antifungal peptides, low molecular weight (reuterine, reuterocycline, and pyroglutamic acid), bacteriocins, and bacteriocin-like substances. The production of these substances in food results in the antimicrobial activity of LABs (Chen and Hang, 2019; Collins et al., 2019). These antimicrobial compounds have gained interest to use as biopreservative agents in the food industry.

Lactic acid bacteria are widely used by industries, not only for acid production but also for their ability to inhibit undesirable microorganisms. The *L. plantarum* can be pointed out the mainly species used in the dairy industry because of its ability to control spoilage and pathogenic microorganisms and also promoting health benefits (Ibrahim and Ouwehand, 2019). Due to their strong competition for nutrients, fermentative metabolism, and the production of inhibitory metabolites they also contribute to the preservation and also to stability and flavor of products (Chen and Hang, 2019).

The sensory properties are established by glycolytic, proteolytic, and lipolytic enzymes that transform nutrients into a diversity of compounds, modifying flavor, aroma and texture of fermented foods. They also preserve the nutritional value of raw food,

providing a product with proteins, essential amino acids, essential fatty acids, and vitamins (Binda and Ouwehand, 2019).

In the food industry, LAB are widely used in the production of fermented dairy, meat, vegetable, wine, and sourdough. Their use in the dairy industry is related to starter cultures, defined as strains with the ability to initiate fast acidification in the raw material giving the advantage to provide controlled and fast fermentation. They play a major role in the onset of cheese fermentation, developing acidity, favoring coagulation, and promoting ripening (Sudhakaran and Minj, 2020). The most used genus in the formulation of a starter culture is *Lactococcus* spp. for its great acidification capacity (Cogan et al., 2007).

There is also another important group that has attracted attention due to their contribution to the quality and characteristics of the final products: the non-starter lactic acid bacteria (NSLAB). NSLAB is a secondary microbiota that spontaneously develops in a variety of foods, mainly constituted by heterofermentative LAB including *Lactobacillus*, *Leuconostoc*, *Enterococcus*, *Pediococcus*, and thermophilic lactic acid bacteria. Part of this population consists mainly of mesophilic facultative and obligate hetero-fermentative *Lactobacilli* (Gobbetti et al., 2015). They do not contribute to acid production during manufacturing, but generally have an effect during maturation and may also contribute to the microbial stability of the final product by inhibiting the development of spoilage and pathogenic microorganisms (Cotter et al., 2005; Perin et al., 2015; Picon et al., 2015; Sheehan et al., 2008).

Artisanal cheeses are typically characterized by following traditional cheese-making techniques, breed, and diet of dairy animals, produced from raw milk with the addition of rennet and fermented by autochthonous microbiota, without any addition of starter culture (Settanni and Moschetti, 2010). This autochthonous microbiota provides important knowledge regarding its safety and geographic origin distinction (Perin et al., 2017).

Within the microbial community of artisanal raw milk cheeses, LAB have a crucial role in cheese making and ripening (Montel et al., 2014; Morandi et al., 2011). The NSLAB microbiota in addition to other LABs, yeasts, and/or fungi are generally specific to a certain variety of cheese, resulting in a product regionally distinct (Settanni and Moschetti, 2010).

Due to their biotechnological and health-related applications, there has been a growing interest in exploring the autochthonous microbiota of cheeses, especially those

with protected designations of origin. As an example of that is the study conducted by Seixas (2014) regarding the evaluation of the lactic microbiota present in the Brazilian Artisanal Serrano cheese. In his study the main genera present was the *Lactobacilli*, followed by *Lactococci* and *Leuconostoc.*

The Brazilian Artisanal Serrano cheese can be described as a semihard product with strong flavor, made with raw cow milk and fermented by the natural microbiota. It is covered by a yellow rind and in general, is consumed after 15-30 days of ripening. The cheese is produced in the south plateau region of Brazil with high acceptance by the local consumers, and also economically important because it is traditionally done by family farms which represents their source of income (Seixas, 2014).

The wide variety and applications of the LAB raise the need to research new strains to improve their use in the food industry (Quigley, 2013).

1.3.1. The Genus *Lactobacilli*

The genus *Lactobacilli* was first described in 1901 and until then the genus comprised more than 261 species. Most of them were classified taxonomically based on the shared features in common, including optimal growth temperature, sugar utilization, and spectrum of metabolites produced. They are extremely diverse at phenotypic, ecological, and genotypic levels (Zheng et al., 2020). Because of that, in 2020, 15 scientists studied the whole genome sequences of each *Lactobacilli* species, and based on the physiological and metabolic properties they divided the genus into groups of closely related species reclassifying it in 25 genera (Table I-1).

Table I-1. Reclassification of the genus *Lactobacillus* into 25 genera based on polyphasic approach as suggested by Zheng et al. (2020).

Reclassification of the genus *Lactobacilli*	
Lactobacillus (*L. delbrueckii* group and *Paralactobacillus*)	*Lapidilactobacillus*
Acetilactobacillus	*Latilactobacillus*
Agrilactobacillus	*Lentilactobacillus*
Amylolactobacillus	*Levilactobacillus*
Apilactobacillus	*Ligilactobacillus*
Bombilactobacillus	*Limosilactobacillus*
Companilactobacillus, Dellaglioa	*Liquorilactobacillus*
Fructilactobacillus	*Loigolactobacilus*
Furfurilactobacillus	*Paucilactobacillus*
Holzapfelia	*Schleiferilactobacillus*

Lacticaseibacillus	*Secundilactobacillus*
Lactiplantibacillus	

Source: Zheng et al., 2020.

The *Lactiplantibacillus plantarum* group comprises 6 species and subspecies: *L. plantarum subsp. plantarum, L. plantarum subsp. argentoratensis, L. paraplantarum, L. pentosus, L. fabifermentans* and *L. xiangfangensis* (Holzapfel and Wood, 2014).

The species *L. plantarum* is widely used in the food and feed industries, being easily found on various commercial probiotic, inoculants, and starter cultures products. Moreover, studies have demonstrated the beneficial use of *L. plantarum* not only for organoleptic purposes but also for inhibiting spoilage and pathogenic microorganisms (Arena et al., 2016; Bukhari et al., 2020; Dallagnol et al., 2019; Hu et al., 2019).

1.4. LACTIC ACID BACTERIA AS BIOPRESERVATIVES

The use of chemical preservatives in food has increasingly concerned consumers, who are demanding and looking for natural and healthy foods. Because of that, the food industry is interested in new strategies that involve the application of natural antimicrobial microorganisms in food to naturally preserve the product from spoilage microorganisms. In this context, with the advance in technology, it is possible to select, identify and improve LAB strains for different applicability (Sadiq et al., 2019).

Since lactic acid bacteria occurs naturally in the environment and raw foods (e.g., milk, meat, vegetables, and cereals) it is an old tradition to use them in food processing (Chen and Wang, 2018). Thousands of years ago, fermentation was a common activity used by Sumerians to preserve foods for a long period (Liu et al., 2014). At present, it stills the most economical method used in food preservation. Due to the increase in food production, the development of new technologies to improve quality, safe and nutritious foods has been a challenge. The fermentation process is a cheap, widely accessible method in which lactic acid bacteria is involved (Kongo, 2013).

The nutrient content presents in many raw foods makes an ideal environment for microbial growth, particularly spoilage and pathogenic/toxigenic microorganisms. The negative effects caused by them make the food unfit for human consumption, representing a major concern for the industry (Rahman, 2007). The fermentation process mainly results in metabolic compounds produced by certain microorganisms which help in the reduction

of food spoilage and pathogenic microorganisms and also in produce certain types of flavors (Rahman, 2020).

Lactic acid bacteria are considered harmless and also known to promote human and animal health benefits (probiotics). Thus, they are qualified as GRAS status ("Generally Recognized As Safe") regulated by the Food and Drug Administration in United States and included in the QPS ("Qualified Presumption of Safety") by the European Union (EFSA, 2012; FDA, 2012).

As an alternative instead of using chemical preservatives, the use of natural microbiota permitted the food industry to improve food safety maintaining its quality through the biopreservation method. Biopreservation or biocontrol refers to extended shelf-life and enhanced safety of foods by the natural or added microbiota or its products to inhibit the growth of undesirable microorganisms in food (Schnürer and Magnusson, 2005). Table I-2 shows the commercially available products based on LAB preservative cultures.

Table I-2. Some commercial LAB preservative cultures.

Preservative culture	**Field of application**	**Producer**
FreshQ	Fermented dairy products	Chr.Hansen (Denmark)
SafePro ImPorous	Meat products	Chr.Hansen (Denmark)
BactoFerm	Meat starter culture	Chr.Hansen (Denmark)
HOLDBAC	Fermented food and beverages	Dupont (USA)
LyoFast	Fermented milk products	Sacco (Italy)
FeedTech 3000	Silage	DeLaval (Sweden)
Dairy Safe	Cheese manufacture	CSK (Netherlands)

Adapted from Varsha and Nampoothiri, 2016.

Researches in food science have brought alternatives to enhance the shelf-life of foods using autochthonous microbiota and/or their antimicrobials compounds. This biotechnology consists in select the natural microbiota and improves these useful microorganisms and their products, as well as their technical application in food quality. The use of autochthonous starter cultures has been studied for a variety of fermented foods and is gaining popularity due to its safety approach in many different kinds of food and feed products (Sheehan et al., 2008). In this context, with the advance in technology, it is possible to select, identify and improve LAB strains for different applicability.

Lactic acid bacteria are usually used in fermented products and their beneficial and functional importance is associated mainly with their metabolism. Regarding the metabolites produced by them, some have antimicrobial properties. So, their application in food to naturally preserve the product from spoilage microorganisms is a good strategy as a biopreservative agent (Sadiq, 2019).

Cheese production is one of the oldest examples of food preservation. The combined methods such as the presence of lactic acid bacteria to transform milk into a semi-solid mass (low pH), the addition of dry salt or dipping in brine and finally let ripened for a short or long period (reduction of the presence of water in cheese) contributes inhibiting spoilage microorganisms. However, the environmental conditions during cheeses production: high humidity, low temperatures, and air circulation play an important role in mold growth (Stark, 2007; Fernandez et al., 2017).

A topic that should be more discussed in researches regarding the use of LAB as biopreservatives is the gap between *in vitro* and *in situ* antimicrobial inhibition, the impact on organoleptic properties, safety evaluation and cell viability of antifungal strains may have on the products. It is desirable that the antifungal LAB to be used as a biocontrol agents in the food industry must meet technological properties, health promotion, and food-borne illness prevention (Levya Salas et al., 2017).

1.5. Antifungal Lactic Acid Bacteria

One of the main problems associated with food and, particularly, cheese spoilage are fungi, it causes deterioration in food and feed systems and also some of them are able to produce toxic compounds for humans and animals which is a concern for public health. In addition, they cause losses in the quality of foodstuffs, resulting in an economic problem (Pitt and Hocking, 2009). Mold spoilage can be prevented using physical and chemical methods, but the use of chemical preservatives in food is not pleasing consumers.

Due to their economic importance in preserving food, the use of LAB is an interesting alternative method instead of those normally applied in food. They play an important role as biopreservatives since they have a long history in fermented foods. Besides, some studies revealed that LAB is capable to produce antifungal compounds (Crowley et al., 2013; Dalié et al., 2010; Schnürer and Magnusson, 2005).

Even some of them are not limited to inhibiting fungal growth, but also reducing or inactivating mycotoxins (Dalié et al., 2010). These compounds have recently been

exploited in a variety of fermented and nonfermented foods (Cheong et al., 2014; Delavenne et al., 2013; Gerez et al., 2009; Russo et al., 2017; Sathe et al., 2017). Examples of such compounds are organic acids (lactic acid, acetic acid, propionic acid, butyric acid, formic acid, phenyllactic acid), phenolic compounds, hydroxy-fatty acids, esters, hydrogen peroxide, reuterin, carbon dioxide, and proteinaceous compounds (Pawlowska et al., 2012). (Table I-3).

Table I-3. Summary of the identified antifungal compounds produced by lactic acid bacteria (LAB) isolated from different food matrices.

LAB isolate	**Source**	**Antifungal compound(s) isolated and identified**	**Reference(s)**
L. plantarum	Grass silage	3-PLA	Prema et al., 2010
L. plantarum AF1	Kimchi	Cyclo (LeueLeu) and d-dodecalactone	Yang and Chang, 2010; Yang et al., 2011
L. plantarum LB1	Raw wheat germ	Lactic acid, PLA and formic acid	Rizzello et al., 2011
L. rossiae LB5			
L. amylovorus DSM 19280	Cereal environment	Lactic acid, acetic acid, salicylic acid, D-glucuronic acid, cytidine, 20-deoxycytidine, sodium decanoate, p-coumaric acid, 3-phenylpropanoic acid, (E)-2-methylcinnamic acid, 3-PLA, 3-(4 hydroxyphenyl) lactic acid, cyclo(L-Pro-L-Pro), cyclo(L-Leu-L-Pro), cyclo (L-Try-L-Pro), cyclo (L-Met-L-Pro) and cyclo (L-His-L-Pro)	Ryn et al., 2011
L. plantarum VE56	Fermented cassava	2-hydroxy-4methylpentanoic acid	Ndagano et al., 2011
W. paramesenteroides LC11			
L. plantarum IMAU10014	Koumiss	3-PLA; benzeneacetic acid and 2 propenyl ester	Wang, Shen, et al., 2012
L. casei AST18,	Unknown	Cyclo-(Leu-Pro), 2,6-diphenyl-piperidine, 5,10-diethoxy-2,3,7,8-tetrahydro-1H and 6Hdipyrrolo[1,2-a;10,20-d] pyrazine	Li et al., 2012
L. amylovorus FST2.1	Cereal environment, cheese,	DL-r-hydroxyphenyllactic acid, 1,2-dihydroxybenzene, 4-hydroxybenzoic acid, vanillic acid, (S)-(-)-2-	Brosnan et al., 2012
L. arizonensis R13			
L. plantarum FST 1.7			
L. reuteri R2			

W. cibaria PS2	malted, barley	hydroxyisocaproic acid, 3-(4-hydroxy-3-methoxy-3-methoxyphenyl) propanoic acid, p-coumaric acid, azelaic acid, PLA, benzoic acid, hydrocinnamic acid,3-hydroxydecanoic acid, DL-b-hydroxylauric acid, decanoic acid, 2-hydroxydodecanoic acid, DL-b-hydroxymyrstric acid, salicylic acid,hydrocinnamic acid D9, 1,2 edihydroxybenzene and 3-(4-hydroxy-3-methoxyphenyl) propanoic acid	
L. reuteri ee1p	Porcine	(S)-(-)-2-hydroxyisocapric acid, hydrocinnamic acid, phenyllactic acid, decanoic acid,azealic acid,4-hydroxybenzoic acid, p-coumaric acid,vanillic acid, DL-b-hydroxyphenyllactic acid and 3-hydroxydecanoic acid	Guo et al., 2012
L. hammesii DSM 16381	French wheat sourdough	Mono-hydroxy C18:1 fatty acid	Black et al., 2013
L. plantarum IS10	Tempeh	Peptides	Muhialdin et al., 2016
L. plantarum CECT749	Pickled cabbage	Peptides	Luz et al., 2017
L. plantarum M5MA1	Chicha	Phenyllactic acid, 3-Propanoic acid, 2-Deoxycytidine, Cyclo (L-His-L Pro), Cyclo (L-Tyr-L Pro), Phenylpyruvic acid, Caffeic acid derivative,Quercetinpentoside ,Quinic acid derivative, caffeoylhexose-deoxyhexoside and 3,5-Di-O-caffeoylquinic acid	Yépez et al., 2017
Lactobacillus sp. RM	Fermented milk (Rayeb milk)	6-octadecenoic acid methyl ester, hexadecanoic acid methyl ester, phenol, 2,4-bis(1,1-dimethylethyl), (Z)-7-Hexadecenal, pentadecane (4.1%), Dotriacontane and 2-methyldecane	Shehata et al., 2019
L. plantarum EM	Kimchi	3-hydroxy-5-dodecenoic acid	Mun et al., 2019

Adapted from Crowley et al. (2013).

Although many studies examined the antifungal compounds produced by LAB, there are few studies that report the isolation and purification of these compounds and also the difficulty of such process (Li et al., 2012; Magnusson and Schnurer, 2001; Niku-Paavol et al., 1999; Yang and Chang, 2010). Another important point is that the antifungal compounds produced under laboratory conditions may differ from those produced in food matrices (Crowley et a., 2013).

The selection of specific strains of LAB with antifungal properties and use them as biopreservative agents would help the reduction or inhibition of molds and therefore improves the shelf-life of fermented products, being an advantage for the dairy industry. There are several studies about LAB playing important role in the field of antifungal activity and most of them report the *L. plantarum* as the most studied species (Table I-4).

Table I-4. Antifungal activity spectrum of *L. plantarum* strains.

Spectrum of *L. plantarum*	**Reference**
Genus *Aspergillus*	
A. candidus	Coloretti et al., 2007
A. carbonarius	Djossou et al., 2011
A. flavus	Lavermicocca et al., 2000; Yang and Chang, 2010; Ryu et al., 2014; Sangmanee and Hongpattarakere, 2014; Muhialdin et al., 2016; Russo et al., 2016
A. fumigatus, A. nidulans	Ström et al., 2002; Sjögren et al., 2003; Ryu et al., 2014
A. niger	Lavermicocca et al., 2000; Dal Bello et al., 2007; Gupta and Srivastava, 2014; Yasmin et al., 2015; Russo et al., 2016
A. ochraceus	Ryu et al., 2014
A. parasiticus	Sangmanee and Hongpattarakere, 2014; Saladino et al., 2016
A. petrakii	Ryu et al., 2014
A. versicolor	Cheong et al., 2014
Genus Cladosporium	
Cladosporium spp.	Russo et al., 2016
C. gossypiicola	Ryu et al., 2014
C. herbarum	Cheong et al., 2014
Genus *Fusarium*	
F. avenaceum	Niku-Paavola et al., 1999
F. culmorum	Dal Bello et al., 2007; Russo et al., 2016
F. graminearum	Dal Bello et al., 2007
F. oxysporum	Dal Bello et al., 2007
F. proliferatum	Deepthi et al., 2016
F. sporotrichoides	Gupta and Srivastava, 2014
Genus *Penicillium*	
Penicillium spp.	Yasmin et al., 2015
P. chrysogenum	Gupta and Srivastava, 2014; Russo et al., 2016
P. commune	Ström et al., 2002; Sjögren et al., 2003; Cheong et al., 2014

P. corylophilum	Lavermicocca et al., 2000
P. expansum	Russo et al., 2016; Saladino et al., 2016
P. nordicum	Schillinger and Villarreal, 2010; Guimarães et al., 2018
P. roqueforti	Lavermicocca et al., 2000; Sjögren et al., 2003; Ryu et al., 2014; Muhialdin et al., 2016; Russo et al., 2016
P. solitum	Cheong et al., 2014
Other Genus	
Rhizopus stolonifer	Gupta and Srivastava, 2014
Endomyces fibuliger	Lavermicocca et al., 2000
Eurotium repens	Lavermicocca et al., 2000
E. rubrum	Lavermicocca et al., 2000
Mucor spp.	Ström et al., 2002
Mucor racemosus	Yasmin et al., 2015
Monilia sitophila	Lavermicocca et al., 2000

Adapted from Dinev et al. (2018).

The most-reported antifungal activity of LAB is generally associated with the production of organic acids. Many organic acids are resulted from carbohydrates fermentation as end products of LAB metabolism. Primarily, lactic and acetic acids are the main obtained products. Researches about the organic acids produced by LAB reports the lactic, acetic, formic, propionic, butyric, phenyllactic and hydroxyphenyllactic acids as the main with the antifungal ability (Table 3). The antimicrobial effects of these acids are attributed to the reduction of pH resulting in an acidic environment and metabolic inhibition, thus, creating a protection from unwanted microorganisms (Ameen and Caruso, 2017; Crowley et al., 2013).

Lactic acid alone, despite being produced in high quantities, is not very effective against spoilage fungi compared to acetic acid. Although, acetic acid in the presence of lactic acid increases its minimal inhibitory concentration (MIC) (Dagna et al., 2015). Antifungal inhibitory mechanisms are assumed to be related to a synergy of low-molecular-mass compounds (Schwenninger et al., 2011). Several methods have been developed to purify and identify antifungal metabolites and these compounds are generally produced at very low levels. So, it is believed that the antifungal activity of LAB is a combined effect of several interrelated factors (Ndagano et al., 2011; Laitila et al.,2002).

The most active LAB with antifungal activity belongs to the *Lactobacilli* group. They are usually more resistant to lowest pH than other LAB, being a competitive advantage, as they continue to grow during fermentation when the pH is too low for other LAB. Another fact is the heterofermentative metabolism, which results in a variety

production of organic acids that apparently play a role as antifungal compounds (Ameen and Caruso, 2017).

Antifungal LAB are also supposed to decrease mycotoxin production (Dalié et al. 2010). There are reports about LAB efficiently removing mycotoxins from the media (Fazeli et al. 2009; El-Nezami et al. 2002; Fuchs et al. 2008; Gratz et al. 2004; Niderkorn et al. 2006). This binding interaction is a potential biological strategy that could be used to reduce mycotoxin availability in food (Dalié et al. 2010).

1.6. Mycotoxigenic And Spoilage Fungi Of Cheese

As mentioned before, filamentous fungi are one of the major food spoilage agents. They are considered ubiquitous because most of them are present in a range of habitats like soil, vegetation, air, indoor environments, and also food products (Pitt and Hocking, 2009). Since they have great versatility for growing in different substrates and conditions, are able to contaminate and spoil processed foods being responsible for technological problems and economical loss (Snyder and Worobo, 2018). In addition, fungal growth on foodstuffs may also produce mycotoxins, which are known to be potentially dangerous for human health (Ledenbach and Marshall, 2009; Pitt and Hocking, 2009).

The main genera involved in spoilage dairy product are *Penicillium*, *Cladosporium*, *Mucor*, *Aspergillus*, and *Geotrichum* (Ledenbach and Marshall, 2009; Pitt and Hocking, 2009). Those belonging to the genus *Penicillium*, are the main agents responsible for the alteration in ripened cheeses, causing, in addition to product deterioration, public health concern for being potentially toxigenic (Dalié et al., 2010).

Some *Penicillium* species have positive impacts in the food industry, they are considered to be the secondary microbiota involved in the fermentation process which mainly provides quality attributes like texture and flavor (Meena et al., 2018). They are particularly important for mold-ripened cheeses especially those artisanal known by the originality of their flavor and other characteristics. In addition, their useful enzymes required for the processing of feed and food products contribute to the organoleptic properties.

Among the *Penicillium* species, *P. camemberti*, *P. roqueforti*, and *P. nalgiovense* are the most common filamentous fungi used as ripening cultures of white cheeses, blue cheeses, and mold-fermented meat sausages, respectively (Meena et al., 2018; Visagie et al., 2014). Similarly, novel candidates for fermenting foods are *Penicillium caseifulvum*

and *Penicillium solitum*, as they are nontoxic and have been found food products (Larsen et al., 2002).

But fungi also may have a negative impact on cheese production. Cheese is an excellent substrate for mold growth and its deterioration is a critical concern for the dairy industry. The microbial community and level of contamination directly affect cheese quality and safety. The resulting defects include discoloration (visible surface growth of mold) and off-flavors, which represents economic losses, negative impact on sensory properties and public health by their production of mycotoxins (Stark, 2007; Dalié et al., 2010; Batt and Tortorelo, 2014).

The presence of certain molds on the surface of cheese does not automatically imply that mycotoxins are present and even the absence of visible molds imply that it is mycotoxin-free (Hymery et al., 2014; Pleadin et al., 2019). Although no direct evidence of toxicity to humans has been reported for the consumption of mycotoxin contaminated cheeses, there is a recommendation to discard a portion of at least 2.5 cm from mold contaminated jams, yogurt, soft cheese, and bread (USDA, 2013).

The incidence of molds in cheese is mainly attributable to *Penicillium* genera, although it can be contaminated by a wide variety of molds. Species such as *P. solitum*, *P. verrucosum*, *P. brevicompactum*, *P. commune*, *P. palitans*; *P. crustosum* and *P. chrysogenum* were commonly associated with cheese. The diversity and number of these microorganisms depend on the sources of contamination mainly from the environment (Hymery et al., 2014; Mounier et al., 2006).

Penicillium isolates generally grows as blue/green colonies, sometimes white, are composed of branched conidiophores (*monoverticillate* to *quaterverticillate*) and flask-shaped phialides. They are aerobic, able to grow in a wide range of environments, some are psychrotolerant but almost all of them are mesophilic with optimum temperature around 25 ℃, and characterized by producing high number spores that are easily disseminated by air (Ledenbach and Marshall, 2009; Pitt and Hocking, 2009). A variety of factors including pH, water activity (a_w), storage conditions, and the presence of other microbes dictate their ability to grow on food (Rouse et al., 2007).

Identifying *Penicillium* genus at the species level is a very complex task, as most species have similar properties. As an example, more than 1000 names were introduced in the genus but, to date, 354 are accepted species (Visagie et al., 2014). According to Frisvad and Samson (2004), Frisvad et al. (2007) and Pitt and Hocking (2009) there are 15 species of *Penicillium* subgenus *Penicillium* associated with cheese deterioration

(Table I-5). Most of these species are mycotoxigenic with some of them produced in cheese.

Frisvad and Samson (2004) proposed a polyphasic approach to identify species of *Penicillium* subgenus *Penicillium* based on morphological, chemical, and molecular analysis. Conventional identification is based on macroscopic and microscopic observation of morphology, growth rates, presence of pigment and exudate on growth media, and mycotoxin production (Pitt, 1979). Molecular identification was also proposed as a tool of an accurate phylogenetic reference system given its advantage over conventional phenotypic methods (Perrone and Susca, 2017).

Table I-5. Main species of *Penicillium* (subgenus *Penicillium*) associated to cheese spoilage and mycotoxins associated to this food.

Section *Brevicompacta*	**Section *Roquefortorum***	**Section *Chrysogena***	**Section *Penicillium***	**Section *Fasciculata***
Series *Olsonii*	**Series *Roqueforti***	**Series *Chrysogena***	**Series *Expansa***	**Series *Viridicata***
P. brevicompactum[a]	*P. roqueforti*[abch]	*P. chrysogenum*[b]	*P. expansum*[bdi]	*P. viridicatum*[e]
		P. nalgiovense		
				Series *Verrucosa*
				P. nordicum[f]
				P. verrucosum[fi]
				Series *Camemberti*
				P. solitum
				P. discolor
				P. echinulatum
				P. commune[g]
				P. palitans[cg]
				P. crustosum[b]
				P.atramentosum[b]

[a]Mycophenolic acid, [b]Roquefortine C, [c]Isofumigaclavine, [d]Patulin, [e]Penicillic acid, [f]Ochratoxin A, [g]Cyclopiazonic acid, [h]PR toxin, [i]Citrinin.

Source: Ramos-Pereira et al., 2019.

1.6.1. Chemical Preservatives to Control Spoilage Fungi on Cheese

Controlling the contamination of spoilage molds in dairy foods is not an easy task, but it can be achieved through several approaches including cleaning and sanitation of

the processing facilities, control of air quality, and use of protective cultures, preservatives and packaging strategies (Martin et al., 2020).

Good Manufacturing Practices (GMPs) is an important strategy that reduces the entry of spoilage molds during the processing of cheeses but once introduced into the product, it won't work to inhibit mold growth (Buehler et al., 2019). In this case, other alternatives are required to control them such as the use of protective cultures (biopreservation), packaging strategies, or even the use of chemical preservatives (Martin et al., 2020). Preservation methods using chemical substances, such as sorbate and benzoate, have for long been used as reliable to control spoilage microorganisms. However, the demand for fewer chemical additives has increased the interest in replacing them by natural preservatives (Garnier et al., 2017; Ledenbach and Marshall, 2009).

Preservatives are defined as chemicals that extend the shelf-life of foods by controlling spoilage microorganism's growth, consequently preventing organoleptic changes caused by them. They are generally used to inhibit mold and yeast growth but are also effective against many bacteria. Chemical preservatives, such as sorbic, benzoic, and propionic acid as well as their salts are well known for their antifungal activity. The most popular mold inhibitors used on cheeses are sorbates and natamycin (Davidson et al., 2002), allowed in the European Union (Reglament 1333/2008).

Natamycin is an antifungal compound produced by *Streptomyces natalensis* during fermentation. It is a polyene macrolide antibiotic and is active against nearly all molds and yeasts, but has no effect on bacteria or viruses. The mold inhibition concentration of natamycin ranges from 0.5 to 25 μg mL^{-1}. In general, natamycin acts as binding to ergosterol and other sterol groups of the fungal cell membrane. This causes inhibition of ergosterol biosynthesis and perturbs the permeability of the cell membrane resulting in leakage of essential ions and small peptides, thereby causing cell lysis (Davidson et al., 2002). The commercially available products containing natamycin, i.e., film coating, include polyvinyl alcohol (PVA), a synthetic polymer whose safety use has been discussed (EFSA., 2005).

Sorbic acid and its potassium, calcium, or sodium salts are known as sorbates. They are recognized as an effective inhibitor of fungi and certain bacteria in food. Sorbic acid is an unsaturated monocarboxylic fatty acid, which is slightly soluble in water. It is generally used at concentrations of 0.2-0.3% in cheese. Certain mold species are resistant to sorbic acid and also able to decarboxylate it into 1,3-pentadiene, causing kerosene or hydrocarbon off-flavor in cheese (Davidson et al., 2002). *Penicillium roqueforti* is an

example of a species that are resistant and able to degrade acid sorbic (Ledenbach and Marshall, 2009).

The preservatives to show complete antimicrobial effectiveness depend on some factors such as antimicrobial spectrum, chemical and physical properties, concentration, pH, a_w, and storage temperature of food. Also, some researchers demonstrated that minimal concentrations of these chemical preservatives may inhibit mold growth but on the other hand, they also stimulate mycotoxin production (Medina et al., 2007; Kogkaki et al., 2016; Yigit et al., 2007; Fodil et al., 2018).

Choosing a preservative to be used in food requires attention not only for what was mentioned above but also for subjects as the type and microorganism load on food, cost, and its effect on the quality and organoleptic properties of food. Therefore, there is a significant interest to develop natural preservatives to enhance or replace those chemical preservatives.

1.6.2. Mycotoxins

Through their metabolic pathways, molds produce various chemical compounds, some in the primary metabolism and others on the secondary one. The primary metabolism provides essential substances for mold survival while the secondary metabolism provides substances not essential to their growth, but rather synthesized in response to environmental challenges (Pleadin et al., 2019).

Mycotoxins are secondary metabolites with low molecular weight and very diverse chemical structures produced by filamentous fungi (Magan and Aldred, 2007; Fox and Howlett, 2008). They are not directly essential for fungal reproduction and growth but are capable of causing biochemical, physiological, and pathological changes in vertebrates, plants, and other microorganisms (Magan and Aldred, 2007; Fox and Howlett, 2008).

Mycotoxin biosynthesis may change under different mold strains, specific microclimatic circumstances, the composition of the food matrix, temperature, water activity (a_w), pH, oxygen concentration, microbial interactions, presence of chemical preservatives, degree of its physical damage, and the presence of mold spores. The most important and well-known mycotoxins in food and feed are produced by *Aspergillus*, *Fusarium* and *Penicillium* genera (Kokkonen et al., 2005; Magan and Aldred 2007; Pleadin et al., 2019; Sengun, 2008).

Mycotoxins are found in cheese primarily as a result of either indirect contamination, resulting from the manufacture of cheese from animals that have ingested contaminated feed, or directly resulting from mold growth on cheese (Pleadin et al., 2019). Once mycotoxins are present in food, decontamination may be almost impossible to achieve and some of them are known to be resistant to industrial processing (Kabak, 2010). The most common mycotoxins isolated from spoiled cheeses are mycophenolic acid, roquefortine C, PR toxin, isofumigaclavine A, penitrem, patulin, penicillic acid, ochratoxin A and cyclopiazonic acid (Sengun, 2008).

Cyclopiazonic acid (CPA) is a potent mycotoxin that causes necrosis, renal and hepatic disorder, ability to be immunotoxic and mutagenic which were reported in several animal species (Ostry et al., 2018). Although no direct evidence of toxicity has been described in humans, some studies have reported its involvement in animal disease (Jand et al., 2005; Perrone and Susca, 2017) and its presence in cheeses has been reported (Le Bars, 1979; Ansari and Häubl, 2016; Zambonin et al., 2001). CPA can be produced by *Aspergillus* and *Penicillium* species, being *P. commune* and *P. palitans* the major producers of CPA within *Penicillium* genus (Moretti and Susca, 2017). In addition, the dominant *Penicillium* species responsible for spoilage of ripened cheeses is *P. commune* (Frisvad and Samson, 2004; Pitt and Hocking, 2009; Ramos-Pereira et al., 2019).

As for ochratoxin A (OTA), major producers are found among *Aspergillus* and *Penicillium* genus. OTA is a potent carcinogenic, nephrotoxic, teratogenic and immunosuppressive mycotoxin, and it is listed as a probable human carcinogen (Class 2B) (Frisvad et al., 2007; Moretti and Susca, 2017; IARC, 1993). Within the *Penicillium* genus, *P. nordicum* is the main OTA producer found in dry-cured meat and cheeses while *P. verrucosum* is the major producer in stored cereals (Frisvad et al., 2007). These OTA producers are not commonly associated as spoilage molds of cheese. However, they have recently been found in this dairy product (Ramos-Pereira et al., 2019) and also OTA has been detected in Gorgonzola and Roquefort cheeses at levels of 0.02 μg kg^{-1} (Dall'Asta et al., 2008). Nevertheless, OTA levels found were very low and the origin of the contamination was not clear, its presence in such products opens a new issue for risk assessment and quality control.

Patulin is produced by several species of *Aspergillus*, *Penicillium*, and *Byssochlamys.* It is a highly toxic mycotoxin to animal and plant cells, but the toxicity for humans has not been conclusively demonstrated. There have been reports about patulin affecting renal cells, damaging the DNA, and causing oxidative stress response (ROS) of

mammalian cells (Pitt and Hocking, 2009). However, it is still classified as a Group 3 by the International Agency for Research on Cancer (IARC) on the basis that cytotoxicity in animals does not constitute adequate evidence of human toxicity (IARC, 1993). *Penicillium expansum* is the most important source of patulin and is the major species causing spoilage of apples and pears. Therefore, patulin is the most common mycotoxin in pear and apple-derived products but it is also a contaminant of other fruits, such as grapes, oranges, and peaches. In addition, *Penicillium griseofulvum* is another very efficient producer of patulin (Moretti and Susca, 2017).

Researchers have developed accurate methods to detect and quantify toxigenic molds to avoid the presence of mycotoxins in the final product. Among the molecular methods, polymerase chain reaction (PCR) is an alternative technique that replaces microbiological and chemical methods in the detection and identification of some toxigenic molds (Paterson, 2006). Besides, some studies were conducted to precisely detect mycotoxigenic molds based on the expression of gene clusters that might be related to mycotoxin synthesis. Molecular methods to detect and quantify cyclopiazonic acid (CPA), patulin (PAT), and ochratoxin A (OTA) producing molds have been designed (Bogs et al., 2006; Geisen et al., 2004; Karolewiez and Geisen, 2005; Luque et al., 2011; Rodríguez et al., 2012). However, the absence or presence of these genes is useful only for detecting potentially mycotoxigenic molds but not for their identification in food.

Cheese contamination by spoilage molds and mycotoxin producers is one of the main problems faced by the cheese industries. Controlling the entry and growth of molds in these products in a safe and healthy way is of importance to reduce the consequences caused by them, so this topic is a frequent aim among researchers.

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Chapter II.

IDENTIFICATION AND CHARACTERIZATION OF *LACTOBACILLI* ISOLATED FROM BRAZILIAN ARTISANAL CHEESE WITH ANTIFUNGAL AND ANTIBACTERIAL ACTIVITY AGAINST SOME *PENICILLIUM* SPP.

1. INTRODUCTION

Recent studies in food science have focused on the beneficial effects of lactic acid bacteria (LAB), which not only include enhancement of flavor, texture, and nutritional value of fermented foods, but also prevention of the growth of pathogens and spoilage microorganisms due to production of antimicrobial compounds (Ameen and Caruso, 2017; Chen and Narbad, 2018). The use of LAB in the food industry to extend the shelf life is a new branch of food science, mainly because of the metabolites produced during the fermentation process (Mozzi et al., 2010).

Fermented dairy products, like cheeses, provide great source of nutrients to the development of spoilage and pathogenic microorganisms. Besides, the humidity, temperature and the long period used for ripening, make a suitable habitat for filamentous fungi proliferation (Pitt and Hocking, 2009). Some of these spoilage molds should be highlighted, as they can also produce mycotoxins known to be potentially dangerous for public health (Ledenbach and Marshall, 2009; Pitt and Hocking, 2009).

Those belonging to the genus *Penicillium*, are the main agents responsible for the alteration in ripened cheeses (Dalié et al., 2010; Frisvad and Samson, 2004; Frisvad et al., 2007; Pitt and Hocking, 2009). Notably, *Penicillium* spp. is the most prevalent genus causing spoilage on hard, semi-hard and semi-soft cheeses from different countries (Lund et al., 1995; Ramos-Pereira et al., 2019).

Although the presence of pathogenic bacteria is a major problem for the health of consumers, the main microorganisms responsible for food losses and economic problems are fungi (Snyder and Worobo, 2018). Since they are present in the environment, it is difficult to eliminate them. Preservation methods using chemical substances, such as sorbate and benzoate, have for long been used as reliable to control spoilage microorganisms. However, the demand for fewer chemical additives has increased the interest in replacing them with natural preservatives (Garnier et al., 2017; Ledenbach and Marshall, 2009).

Traditional raw-milk cheeses are highly valued for their characteristics since the microbiota presents in raw milk play an important role in flavor development and it is basically constituted by LAB (Kongo, 2013). The wide variety and applications of the autochthonous LAB in artisanal cheeses raise the need to research new strains to improve their use in the food industry (Quigley, 2013). For this reason, this chapter has aimed to select a *Lactobacilli* strain isolated from Brazilian Artisanal Serrano cheese with the

ability to inhibit toxigenic and spoilage *Penicillium* sp. for future use as a biopreservative agent in dairy products.

2. OBJETIVES

- Evaluate the inhibitory activity of *Lactobacilli* previously isolated from a Brazilian cheese against spoilage and toxigenic *Penicillium* strains (*Penicillium nordicum*-M32; *Penicillium commune*-M35 and *Penicillium roqueforti*) isolated from cheese and *Penicillium expansum* isolated from a spoiled orange.
- Identify *Lactobacilli* strains with antifungal activity at the species level through polymerase chain reaction (PCR) using16S rRNA as the target gene.
- Analyze the antifungal activity of the selected *Lactobacilli* isolates, against two selected *Penicillium* spp. and identify the nature of the antifungal activity with impedimetric measurements.
- Evaluate the antagonistic activity of the selected *Lactobacilli* against pathogenic bacteria that can be transmitted by dairy products (*Escherichia coli*, *Staphylococcus aureus*; *Salmonella enterica* and *Listeria monocytogenes*) and identify the antibacterial activity with automated turbidometry.

3. MATERIALS AND METHODS

3.1. EVALUATION OF THE ANTIFUNGAL ACTIVITY OF *LACTOBACILLI* STRAINS AGAINST *PENICILLIUM* SPP.

3.1.1. *Lactobacill*i Strains

The antifungal activity was carried out with 32 *Lactobacilli* that have been previously isolated from Brazilian Artisanal Serrano Catarinense cheese (Brazil, Seixas, 2014) and stored at -80 ℃ in MRS broth (Oxoid, Basingstoke, UK) supplemented with 20% of glycerol. For the experiments, *Lactobacilli* strains were incubated on MRS broth (De Man Rogosa and Sharpe Agar, OXOID) at 30 ℃ for 48 h.

3.1.2. *Penicillium* spp. Strains

Three strains of toxigenic *Penicillium* spp. (*Penicillium expansum*; *Penicillium commune* and *Penicillium nordicum*) were selected to evaluate the amplitude of antagonistic activity. *Penicillium roqueforti* was also selected to evaluate if there is inhibition against a mold with technological interest and not a mycotoxin-producer.

P. commune-M35 and *P. nordicum*-M32 were strains isolated from Spanish semi-hard cheeses (produced in Castilla and León) at the University of Léon (Department of Food Hygiene and Food Technology, T.M. López) and identified by Ramos-Pereira et al. (2019) (available at the Spanish Type Culture Collection, *P. nordicum*-M32 CECT 20939 and *P. commune*-M35 CECT 20940). *P. expansum* and *P. roqueforti* were provided by the Laboratory of Medical Mycology and Oral Microbiology of the Biological Sciences Center of State University of Londrina. The molds cultures were plated on malt extract agar at 25 ℃ for 5/7 days.

3.1.3. Screening of the Antifungal Activity

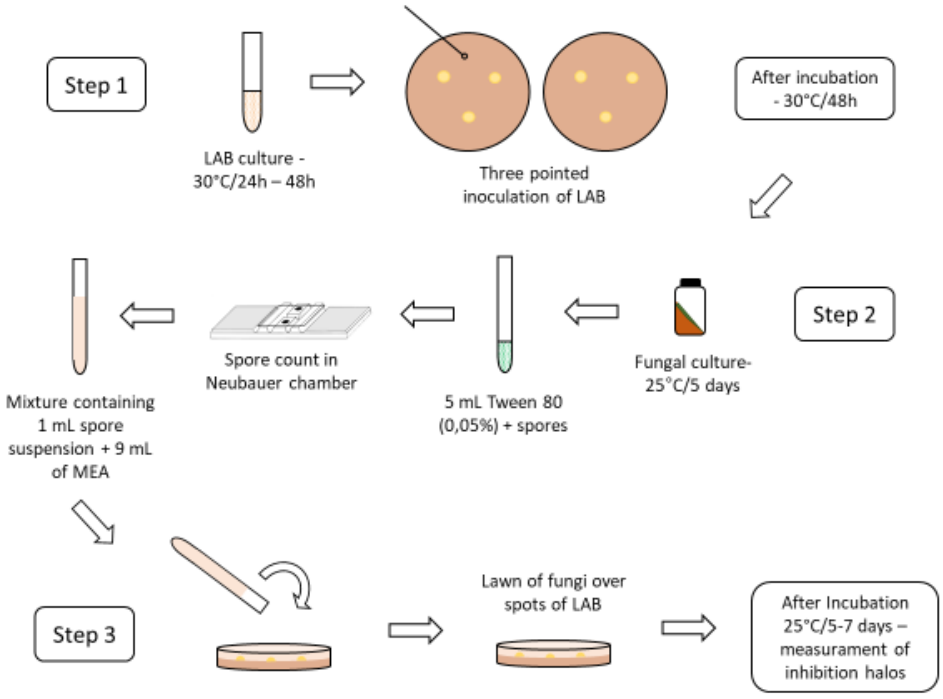

Figure II-1. Schematic protocol to evaluate antifungal activity of lactic acid bacteria (LAB). Step 1, three-pointed inoculation of LAB on MRS agar. Step 2, spores suspension preparation. Step 3, overlay method.

The antifungal activity of *Lactobacilli* strains assay was carried out using the overlay method according to Magnusson and Schnürer (2001) modified. From a fresh culture of *Lactobacilli* strains (48 hours), 1 µL was three-point inoculated on MRS agar plates in duplicate and incubated at 30 ℃ for 48 hours. After that, 1 mL of the spore suspension at 10^4 spores mL^{-1} was transferred into 9 mL of liquefied MEA maintained at 45 ℃ and overlayed on MRS plate containing the LAB, with subsequent incubation at 25 ℃ for 5-7 days.

The spore suspension was prepared from 5-7 days culture at 25 °C in MEA medium (Malt Extract Agar, OXOID) in a slant agar bottle by adding 5 mL of a sterile 0.05% Tween 80 solution. The spore counts were made in a Neubauer chamber and the dilutions in peptone water until containing approximately 10^4 spores mL^{-1}.

After 7 days of incubation at 25 °C, the antifungal activity was discriminated based on the diameter of the halos surrounding the *Lactobacilli* spots. The results were determined using the geometric mean of the inhibition halo (mm) obtained and were classified as strong inhibition (+++, halo >8.77), medium inhibition (++, halo between 5.86 and 8.77), low (+, halo <5.86) and without inhibition (-). Those *Lactobacilli* strains that produced strong inhibition for all mold strains were selected for further analysis.

3.2. Identification And Characterization Of *Lactobacilli*

3.2.1. Molecular Identification

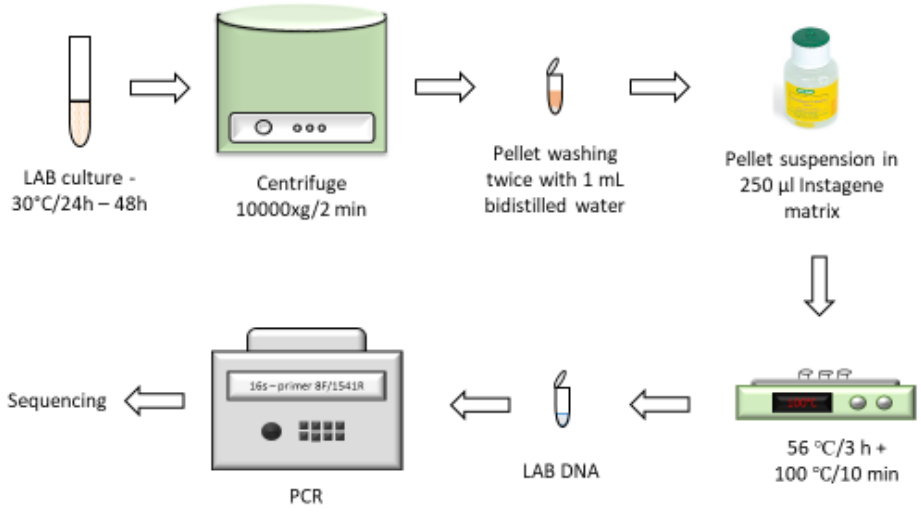

Figure II-2. Schematic representation of DNA extraction and amplification of Lactobacilli using InstaGene matrix.

The *Lactobacilli* (n = 32) were identified by amplification and sequencing of the 16S rRNA gene. They were recovered in MRS broth and streaked for purity on MRS agar plates at 30 °C. For DNA extraction, cells from 1 mL of a 24-h culture were centrifuged at 10,000 g for 2 minutes, discarding the supernatant. The obtained pellet was washed twice with 1 mL of sterile double-distilled water and suspended in 250 µL of Instagene Matrix reagent (Bio-Rad, Hercules, CA, USA). The obtained bacterial suspension was treated, according to the manufacturer's instructions, at 56 °C for 15 minutes and 100 °C for 8 minutes and, finally, centrifugation at 10,000 g for 2 minutes (Figure II-2).

Following DNA extraction, 16S rRNA gene was amplified according to the protocol described by De Garnica et al. (2014), using 2.5 µL of template DNA and 1 µL of 8F (AGA GTT TGA TCC TGG CTC AG) and 1541R (AAG GAG GTG ATC CAG CCG CA) primers (Baker et al., 2003). The reaction was confirmed by agarose gel electrophoresis. The amplified fragments were purified by NucleoSpin Gel and Clean-up PCR kit (Macherey-Nagel, Germany) and were sequenced on a MegaBACE 500 sequencer (GE Healthcare Life Sciences, United Kingdom) using the same primers used for the amplification. The quality of the sequences obtained were visually analyzed with Chromas Lite 2.01 software (http://technelysium.com.au/) and aligned with the ContigExpress module of the Vector NTI program (Thermo Fisher Scientific). The consensus sequences obtained from the alignment of the two strands were compared with the sequences available in the GenBank 16SRNA sequences database using BLAST platform ("Basic Local Alignment Search Tool", http://blast.ncbi.nlm.nih.gov/Blast. CGI).

For the differentiation of *L. plantarum*, *L. pentosus* and *L. paraplantarum* a single reverse primer (5'-TCG GGA TTA CCA AAC ATC AC-3') and three forward primers planF (5'-CCG TTT ATG CGG AAC ACC TA-3'), pentF (5'-CAG TGG CGC GGT TGA TAT C-3'), and paraF (5'-GTC ACA GGC ATT ACG AAA AC-3') were used (Torriani, 2001). The expected sizes of the amplicons were 318 bp for *L. plantarum*, 218 bp for *L. pentosus*, and 107 bp for *L. paraplantarum*. PCR were performed on a thermocycler (AerisTM Thermal Cycler, Esco® Micro Pte, Singapore) with initial denaturation at 94 ℃ for 3 min, 30 cycles of denaturation at 94 ℃ (30 s), annealing at 56 ℃ (10 s), and elongation at 72 ℃ (30 s), and final extension at 72 ℃ for 5 min. The PCR products were visualized on a 1.5% agarose gel in 1 X TAE buffer at constant voltage of 90 V for 1 hour. The gels were stained with an ethidium bromide solution of 0.2 mg mL^{-1} for 20 min before visualization. A 1kb molecular weight marker (Ladder Kasvi) was used to determine the weight of the PCR products.

3.2.2. Phenotypic Characteristics

According to the results obtained from antifungal activity and molecular identification of *Lactobacilli*, two strains (L119 and L49) that presented the best results against all the tested molds were selected. Both strains were analyzed for their sugar fermentation profiles and enzymatic activity using miniaturized galleries.

3.2.2.1. *Carbohydrate Fermentation Profile*

The two strains (L119 and L49) were grown on MRS agar at 30 ℃ for 48 h. Five to ten colonies were suspended in API 50 CH medium (API systems, BioMérieux España S.A.), using sterile Pasteur pipettes, homogenized suspensions of the cells in the medium, with subsequent vortex mixing, were transferred into each of the 50 wells on the API 50 CH strips. All wells were overlaid with sterile paraffin oil (Merck) to perform anaerobiosis, as recommended by the manufacturer, and incubated at 30 ℃ for 48 h. Changes in color from violet to yellow were represented by a positive sign (+), while a negative sign (-) represented any change. The biochemical profile was obtained using the Apiweb software by comparison with the manufacturer's database (BioMérieux España, S.A).

3.2.2.2. *Enzymatic Profile*

The L119 and L49 strains were grown on MRS agar at 30 ℃ for 48 h. Five to ten colonies were resuspended in 2 mL of sterile distilled water to produce a dense suspension (5 of McFarland standard). Sixty-five microliters of the cell suspension were inoculated to each of the 19 wells of the APIZYM gallery (BioMérieux España, S.A) containing dehydrated chromogenic enzyme substrates and the strips were incubated for 4 hours at 37 ℃. A humid atmosphere was maintained during the incubation by dispensing approximately 5 mL of sterile water into the incubation tray provided with the enzyme kit, as described by the manufacturer.

After incubation, one drop of the zym-A and zym-B reagent was added. The activity was measured by comparing the degree of color developed after 5 minutes with the color table provided by the manufacturer. A value ranging from zero (no activity) to five (maximum activity) is assigned, corresponding to the color developed.

3.3. Determination of the Antifungal Activity of L49 and L119 strains Through Impedance Technique

The two *Lactobacilli* that presented the best results in item 3.1.3 were selected to determine the antifungal activity against *P. commune*-M35 and *P. nordicum*-M32. Mold strains were cultivated on MEA at 25 ℃ for 7 days. Spores suspension were diluted with 0.05% Tween 80 and spore densities were determined microscopically by Neubauer chamber.

The BacTrac equipment (mini Trac 4000 Sy-Lab) was used to check growth inhibitory activity of L119 and L49 and determine its nature. The methodology used was an adaptation of Gerez et al. (2013) and Laitila et al. (2002). First, the vials were filled with 1 mL of mold suspension at 1×10^4 spores mL^{-1} concentration in 5 mL of the cell-free supernatant (CFS) of L119 and L49. As a positive control for mold growth, 5 mL of TSB (Triptic soy broth, OXOID) + 1% YE (Yeast extract, OXOID) was used.

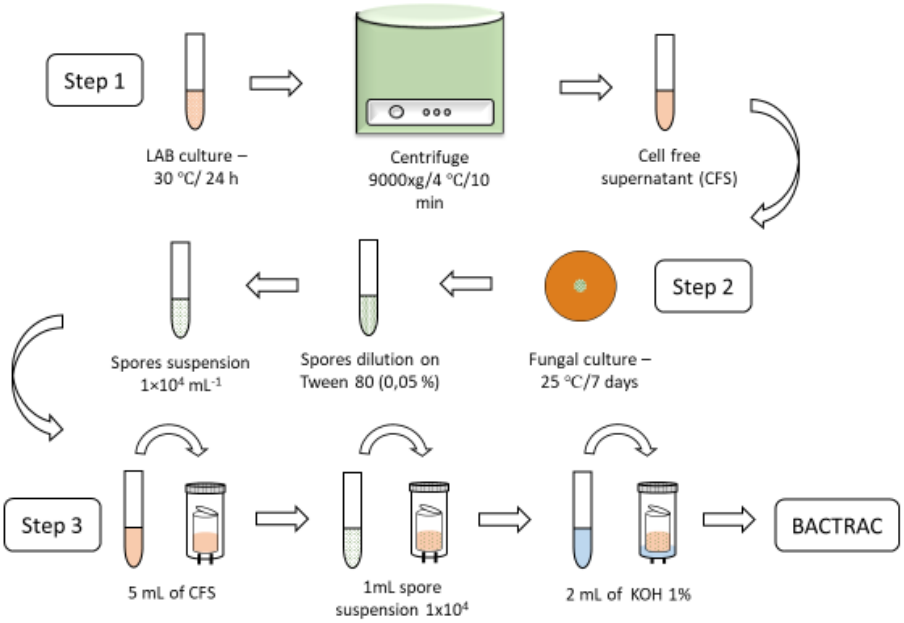

Figure II-3. BacTrac protocol of fungal growth by impedance technique. Step 1, preparation of cell-free supernatant (CFS) of lactic acid bacteria (LAB). Step 2, preparation of spore fungal suspension. Step 3, samples preparation on BacTrac machine.

The CFS were obtained from *Lactobacilli* growth at 30 °C for 24 h in TSB + 1% YE broth, centrifuged at 9,000×g for 10 min at 4 °C, and filtered to obtain the sterile CFS. In order to characterize the antifungal compound, the CFS were processed in two different ways: (a) supernatant without treatment (b) neutralized supernatant to pH 7.0 with 1 N of sodium hydroxide (NaOH). Once this was done, 2 mL of 1% potassium hydroxide (KOH) were filled into measuring tubes to absorb the production of carbon dioxide (CO_2) by fungi and the electrodes measured the conductivity.

The chemical reaction between CO_2 and the KOH solution changes the conductivity creating a decreasing impedance measured by the parameter "%M". The detection parameter used was the M-threshold and was set at 7%. M value is the impedance expressed by the equipment, according to the amount of carbon dioxide

produced by the mold. The detection time was defined as the time taken to surpass these thresholds, as suggested by Laitila et al., (2002). Two repetitions were carried out in duplicate and the results expressed as average values. The vials were incubated at 25 ℃ for 72 h, taking automatic measurements every 15 minutes (Ramos-Pereira et al., 2019).

3.4. Antibacterial Activity of L119 and L49 Strains Against Pathogenic Bacteria

The antagonistic activity of L119 and L49 was evaluated against *Escherichia coli* CECT 4972, *Salmonella enterica* CECT 4156, *Staphylococcus aureus* CECT 5190 and *Listeria monocytogenes* (CECT, Spanish Type Culture Collection). The "spot-on-the-lawn" method with some modifications as described by Perin and Nero (2014) was used. The pathogenic strains were incubated in BHI broth (Brain Heart Infusion, OXOID) at 37 ℃ for 24 hours. A suspension of the cultures was made in peptonated water (0.1%) and used at an approximate concentration of 3×10^8 CFU mL^{-1} (1 Mc Farland standard).

Using pure cultures, a three-point inoculation was carried out from a fresh culture of LAB on MRS agar plate, in duplicate, and incubated at 30 ℃ for 24 to 48 hours. After incubation, 1 mL of the pathogenic bacteria cell suspension was added to 9 mL of semi-solid BHI agar, homogenized and overlayed on MRS agar containing the *Lactobacilli*. After solidification, the plates were incubated at 37 ℃ for 24 hours. The antagonistic activity was determined by the formation of inhibition halos surrounding the LAB culture, where well-defined halos (> 0.5 cm) were classified as total inhibition and diffuse halos (< 0.5 cm) as partial inhibition (Nero et al., 2008).

3.5. Determination of the Antibacterial Activity Of L49 and L119 Strains Through Automated Turbidometry

The nature of antagonistic activity was evaluated using an adaptation of the techniques described by Gerez et al. (2013) and Gourama and Bullerman (1997) using an automated turbidimetry equipment (Bioscreen, Labsystems, Finland).

Cell-free supernatants of *Lactobacilli* strains (30 ℃ for 24 h in MRS broth) were obtained by centrifugation at 9,000 g for 10 min at 4 ℃ and filtered (nylon filters, 0.22 μm pore size, Pall Life Sciences Corporation). In order to characterize the antibacterial compound, the CFS were processed in three different ways: (a) supernatant without treatment (b) neutralized supernatant (NCFS) to pH 6.5 with 1 N of sodium hydroxide (NaOH) to evaluate if the antibacterial compound was an organic acidic and (c) heated

for 10 min at 100 ℃, to analyze if it was a proteinaceus and/or heat-stable compound (Gerez et al., 2013).

The assays were performed in a sterile Bioscreen plate containing 190 µL of CFS (a) with 10 µL of each pathogenic bacteria suspension (3×10^8 CFU mL^{-1}). All the assays were made in quadruplicate. A negative control (duplicate), was performed containing only BHI broth, and positive controls with 10 µL of bacteria suspension in 190 µL BHI broth were also included in the experiment. The optical density (O.D) was recorded every 30 minutes using the 600 nm filter over 18 hours (Gerez et al., 2013).

4. RESULTS

4.1. Evaluation of the Antifungal Activity *of Lactobacilli* Strains Against *Penicillium* spp.

P. commune-M35 was the most resistant mold strain since 66% of the tested *Lactobacilli* were unable to inhibit its growth while *P. nordicum*-M32 was the most inhibited with only 3% of the tested *Lactobacilli* that could not inhibit its growth (Fig. II-4 and II-5).

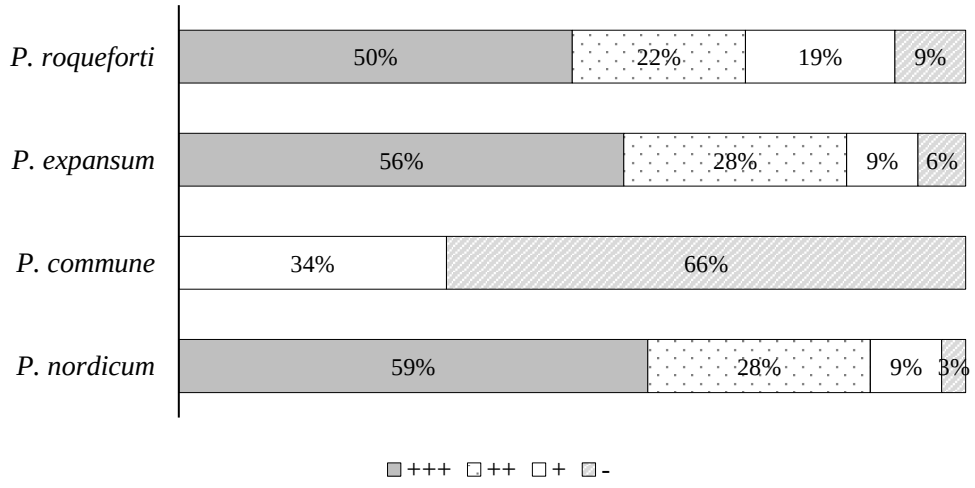

Figure II-4. Screening of *Lactobacilli* with antifungal activity (+++, halo >8.77 mm; ++, halo between 5.86 and 8.77 mm; +, halo <5.86 mm and without inhibition (-)).

Most of the *Lactobacilli* analyzed exhibited medium to strong inhibition against all the *Penicillium* spp. tested. Contrastingly *P. commune* was the only species that was inhibited by 34% of the *Lactobacilli* and characterized as low inhibition (Figure II-4 and Table II-1). *P. nordicum* was strongly inhibited by 59% of the *Lactobacilli* tested followed by *P. expasum* and *P. roqueforti* (56% and 50%, respectively) (Figure II-4).

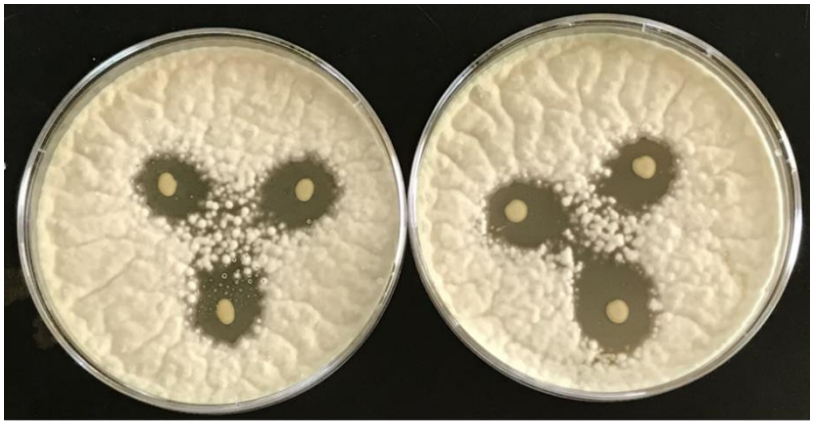

Figure II-5. Inhibition halos of L119 against *Penicillium nordicum*-M32 CECT 20939 (Spanish Type Culture Collection) classified as strong inhibition (+++, 19.3 mm) through overlay method.

P. roqueforti, *P. expansum*, and *P. nordicum*-M32 were not inhibited for just a few *Lactobacilli* and the maximum inhibition halo was 20.0, 20.5, and 20.0 mm respectively (Table II-1). In contrast, the maximum inhibition halo for *P. commune* was evidenced by L49 and L119 with 5.1 and 5.3 mm of diameter, respectively. Thirty-one *Lactobacilli* presented inhibition against at least one mold species and only one strain (L326) did not present any inhibition.

According to the classification of the inhibition halos, 11 strains presented strong inhibition against *P. nordicum*, *P. expansum* and *P. roqueforti.* From these strains only 2 (L119 and L49) presented the highest diameter for *P. commune* while 3 strains (L114, L570 and L628) did not presented inhibition against *P. commune* but for the other *Penicillium* strains they presented strong inhibition as it shown in Table II-1.

Table II-1. Inhibition halos (mm) and antifungal activity of *Lactobacilli* isolated from Brazilian Artisanal Serrano Catarinense Cheese (n=32).

Species	Strain	Antifungal Activity							
		P. nordicum		*P. commune*		*P. expansum*		*P. roqueforti*	
Lactiplantibacillus plantarum	49	20.0	+++	5.1	+	17.5	+++	17.5	+++
	53	16.0	+++	1.9	+	19.0	+++	16.0	+++
	99	13.3	+++	2.0	+	17.0	+++	17.0	+++
	119	19.3	+++	5.3	+	16.5	+++	20.0	+++
	126	12.7	+++	-	-	7.5	++	8.5	++
	171	12.0	+++	0.8	+	12.0	+++	11.0	++
	224	12.8	+++	1.9	+	11.0	+++	17.0	+++
	227	12.7	+++	2.6	+	9.5	+++	13.0	+++
	275	7.5	++	-	-	9.0	+++	10.0	++
	298	8.7	++	-	-	7.0	++	10.5	++
	391	10.7	+++	-	-	9.5	+++	5.5	+

	407	10.7	+++	-	-	10.5	+++	7.0	++
	414	11.0	+++	-	-	9.5	+++	4.0	+
	509	6.0	++	-	-	10.0	+++	3.5	+
	510	9.3	+++	-	-	7.5	++	4.5	+
	558	6.7	++	-	-	8.5	++	9.5	+++
	570	10.0	+++	-	-	10.5	+++	10.0	+++
	577	14.0	+++	2.4	+	9.5	+++	9.0	+++
	628	10.7	+++	-	-	9.5	+++	9.0	+++
	648	7.3	++	-	-	4.0	+	7.0	++
	659	14.0	+++	1.0	+	7.0	++	8.0	++
	714	10.0	+++	-	-	7.5	++	5.5	+
	720	7.3	++	-	-	8.0	++	7.0	++
	732	8.7	++	-	-	8.5	++	7.0	++
	744	14.7	+++	0.9	+	10.0	+++	11.0	+++
Lactobacillus sp.	114	13.3	+++	-	-	20.5	+++	15.0	+++
	320	4.6	+	0.4	+	10.0	+++	10.5	++
	517	6.0	++	-	-	5.0	+	7.0	++
Limosilactobacillus fermentum	326	-	-	-	-	-	-	-	-
Lacticaseibacillus paracasei	21	4.0	+	-	-	1.5	+	-	-
Lacticaseibacillus casei	16	7.3	++	-	-	-	-	-	-
Lacticaseibacillus rhamnosus	347	4.0	+	-	-	7.5	++	5.5	+

*Strong inhibition (+++, halo >8.77 mm), medium inhibition (++, halo between 5.86 and 8.77 mm), low (+, halo <5.86 mm) and without inhibition (-). The classification was determined using geometric mean of the inhibition halo (mm) ± standard deviation (7.31 mm ± 1.46 mm).

In Figure II-6, box plot graphics are shown with the mean values, standard deviation (S.D.), and outliers of the inhibition halos measured against all the *Penicillium* spp. tested.

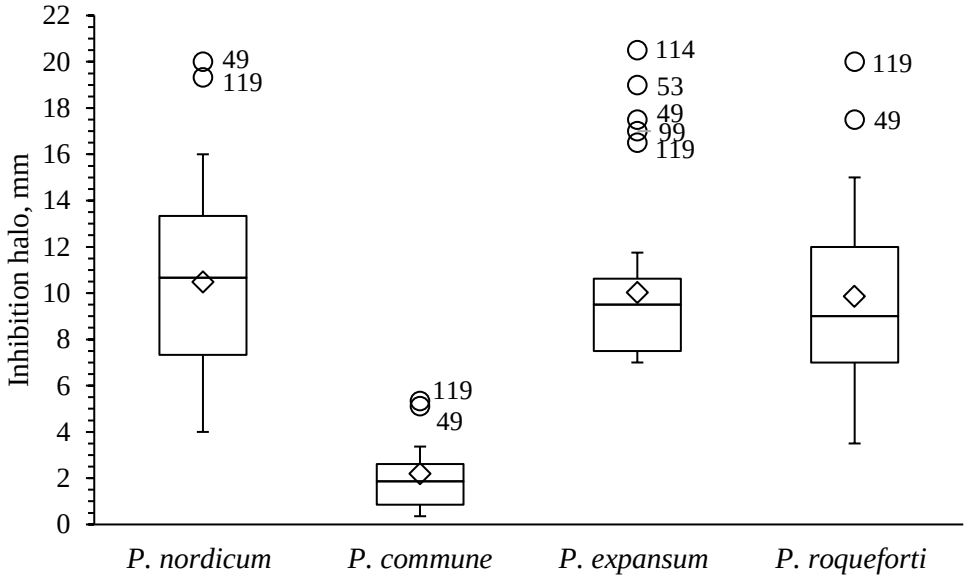

Figure II-6. Box plot of the inhibition halos of the *Lactobacilli* isolates with a highest antifungal activity (L49, L53, L99, L114 and L119).

According to the classification of the inhibition halos (Table II-1), none of the *Lactobacilli* presented strong inhibition for *P. commune*. However, it is important to note that only two *Lactobacilli* exhibited a wide spectrum of antifungal activity for all the *Penicillium* spp. strains. Therefore, these two isolates, *L. plantarum*-L119 and L49, were selected for further investigations, since they were able to inhibit the four molds tested (Figure II-6).

4.2. MOLECULAR IDENTIFICATION

Complete sequences of 16S rRNA were obtained from the thirty-two strains of *Lactobacilli*. For the identification at the species level was adopted similar percentages greater than 99%. All the species identified in this study were described according to the new classification suggested by Zheng et al. (2020).

As it is shown in Table II-1, the results of the molecular identification indicate the description and the inhibition classification against the *Penicillium* spp. tested. Twenty-five strains of the 32 *Lactobacilli* were identified as *Lactiplantibacillus plantarum*, three were not able to be identified at species level and the others were identified as *Limosilactobacillus fermentum*, *Lacticaseibacillus paracasei*, *Lacticaseibacillus casei* and *Lacticaseibacillus rhamnosus*. The major species that exhibited inhibition against all the *Penicillium spp.* tested was *L. plantarum* and the only strain that did not presented inhibition was *L. fermentum*.

Based on the results obtained in 4.1 item, the two *L. plantarum*-L119 and L49 were selected for further analysis. The final identification of the two selected strains using the *recA* gene as proposed by Torriani et al. (2001) confirmed their identity.

4.3. PHENOTYPIC CHARACTERISTICS

The two *L. plantarum*-L119 and L49 were selected to establish their phenotypic identification (Figure II-7). Regarding the sugar fermentation profiles (AP50CH; Figure II-7), L119 was identified as *L. plantarum* according to the program identification Apiweb™ (Biomerieux, España, ES), while L49 did not present a reliable result, being the identification between *L. brevis* and *L. plantarum*. From the 49 sugars analyzed in the gallery, L119 was capable to react with 20 sugars while L49 reacted with 21 sugars and the difference between them were: D-ribose, Methyl-D-mannopyranoside, and D-Melibiose (Figure II-7 and Table II-2).

Figure II-7.Results of sugar fermentation test of L119 and L49 using AP50CH after 48 hours of incubation at 36°C. Yellow indicates positive; Blue indicates negative; Black indicates positive for esculin.

Table II-2. Fermentation profiles of *L. plantarum* L119 and L49 (AP50CH, Biomerieux España, S.A).

Carbohydrate		Strain		Carbohydrate		Strain	
		L119	L49			L119	L49
0	Control	-	-	25	Esculine	+	+
1	Glycerol	-	-	26	Salicine	+	+
2	Erythritol	-	-	27	D-Celiobiose	+	+
3	D-Arabinose	-	-	28	D-Maltose	+	+
4	L-Arabinose	-	-	29	D-Lactose	+	+
5	D-Ribose	-	+	30	D-Melibiose	-	+
6	D-Xylose	-	-	31	D-Saccharose	+	+
7	L-Xylose	-	-	32	D-Trehalose	+	+
8	D-Adonitol	-	-	33	Inuline	-	-
9	Methyl-D-xylopyraniside	-	-	34	D-Melezitose	+	+
10	D-Galactose	+	+	35	D-Raffinose	-	-
11	D-Glucose	+	+	36	Amidon	-	-
12	D-Fructose	+	+	37	Glycogen	-	-
13	D-Mannose	+	+	38	Xylitol	-	-
14	L-Sorbose	-	-	39	Gentiobiose	+	+
15	L-Rhamnose	-	-	40	D-Turanose	-	-
16	Dulcitol	-	-	41	D-Lyxose	-	-
17	Inositol	-	-	42	D-Tagatose	-	-
18	D-Mannitol	+	+	43	D-Fucose	-	-
19	D-Sorbitol	-	-	44	L-Fucose	-	-
20	Methyl-D-mannopyranoside	+	-	45	D-Arabitol	+	+

21	Methyl-D-glucopyranoside	-	-	46	L-Arabitol	-	-
22	N-acetylglucosamine	+	+	47	Potasssium gluconate	+	+
23	Amygdaline	+	+	48	2-Ceto-gluconate	-	-
24	Arbutine	+	+	49	5-Ceto-gluconate	-	-

To compare the biochemical properties of the isolated strains, the enzyme activities of the selected strains were tested using API ZYM system on a semi-quantitative scale from 0 to 5 (Figure II-8).

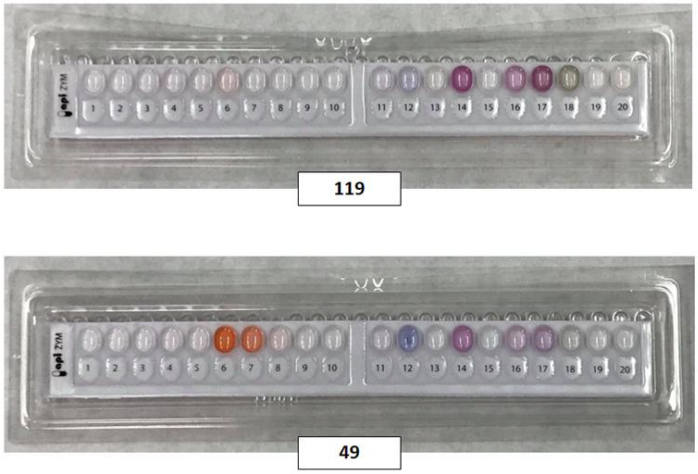

Figure II-8. Results of enzyme activity of *L. plantarum*-L119 and L49 using API ZYM. The resulting colors were recorded as intensities (0 to 5) using a color code supplied by the manufacturers.

The results showed that of the 19 enzymatic activities analyzed, the strains were positive for 6 enzymes and negative for 11 (Table II-3). Therefore, both strains presented very similar enzyme profiles, the only differences found between them were the presence of valine arylamidase and α-galactosidase for L49 and the presence of n-acetyl-β-glucosaminidase for L119. The L49 displayed a high leucine arylamidase, valine arylamidase and L119 displayed a high β-glucosidase and β-galactosidase activity.

Table II-3. Enzymatic profiles of L119 and L49 (APIZYM).

Enzyme		**Strain**		**Enzyme**		**Strain**	
		L119	**L49**			**L119**	**L49**
1	Control	0	0	11	Acid phosphatase	1	1
2	Alkaline phosphatase	0	0	12	Naphthol-AS-BI-phosphohydrolase	2	3
3	Esterase (C4)	0	0	13	α-Galactosidase	0	1
4	Esterase lipase (C8)	0	0	14	β-Galactosidase	5	3
5	Lipase (C14)	0	0	15	β-Glucuronidase	0	0
6	Leucine arylamidase	1	5	16	α-Glucosidase	3	2
7	Valine arylamidase	0	4	17	β-Glucosidase	4	2

8	Cystine arylamidase	0	0	18	n-Acetyl-β-glucosaminidase	3	0
9	Trypsin	0	0	19	α-Mannosidase	0	0
10	α-Chymotrypsin	0	0	20	α-Fucosidase	0	0

4.4. Determination of the Antifungal Activity of L49 and L119 Through The Impedance Technique

In addition to the overlay method, an impedimetric method was used to verify the antagonistic activity of the cell free supernatant (CFS) of the two *L. plantarum* strains (L49 and L119) against *P. commune*-M35 and *P. nordicum*-M32. As seen from the results presented in Figure II-9, the growth curves of *P. commune*-M35 and *P. nordicum*-M32 obtained from BacTrac measurements, demonstrate the change in impedance over 72 hours of incubation. In control samples, both *Penicillium* spp. grew well and intensive production of carbon dioxide was recorded after 33-h, reaching the maximum value in 72 hours of incubation, while in the presence of both *L. plantarum* CFS caused a significant delay in the metabolic activity.

Impedance values of *P. commune*-M35 reached the threshold level (7%) during the 52-h incubation when L119 CFS were used and 54-h in the presence of L49 CFS. While in the presence of L119 and L49 CFS, the impedance value of *P. nordicum*-M32 reached the threshold level in 60-h incubation.

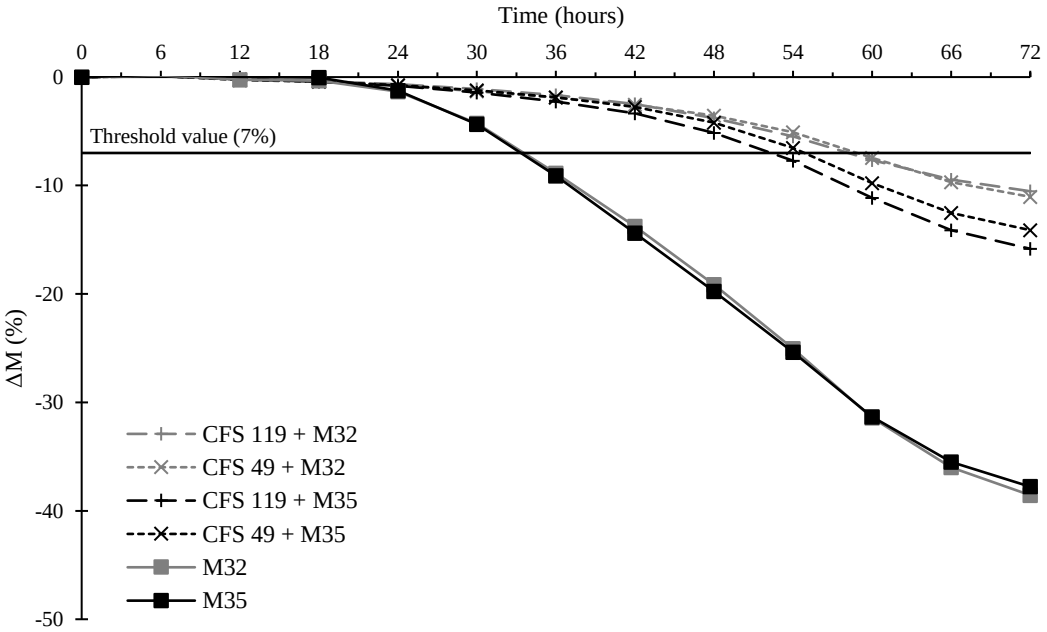

Figure II-9. Indirect impedance results of the cell-free supernatant (CFS) of *Lactobacilli* against *P. nordicum*-M32 and *P. commune*-M35 fungi.

It was also possible to evaluate the action of the compounds produced by both *L. plantarum* on the growth of *Penicillium* spp. As it is shown in Figure II-10, in the presence of L119 and L49 neutralized CFS (NCFS) with NaOH, the detection time of *P. commune*-M35 was 42 and 54 hours, respectively. The presence of L119 and L49 NCFS still caused a delay in *P. commune*-M35 growth (39 h), being more evidenced by L49 (54 h). Meanwhile, the presence of L119 and L49 NCFS showed the same inhibition for *P. nordicum*-M32 growth (65 h) compared to the control (39 h). Even with the neutralization, both CFS delayed the detection time of *P. nordicum*-M32 growth.

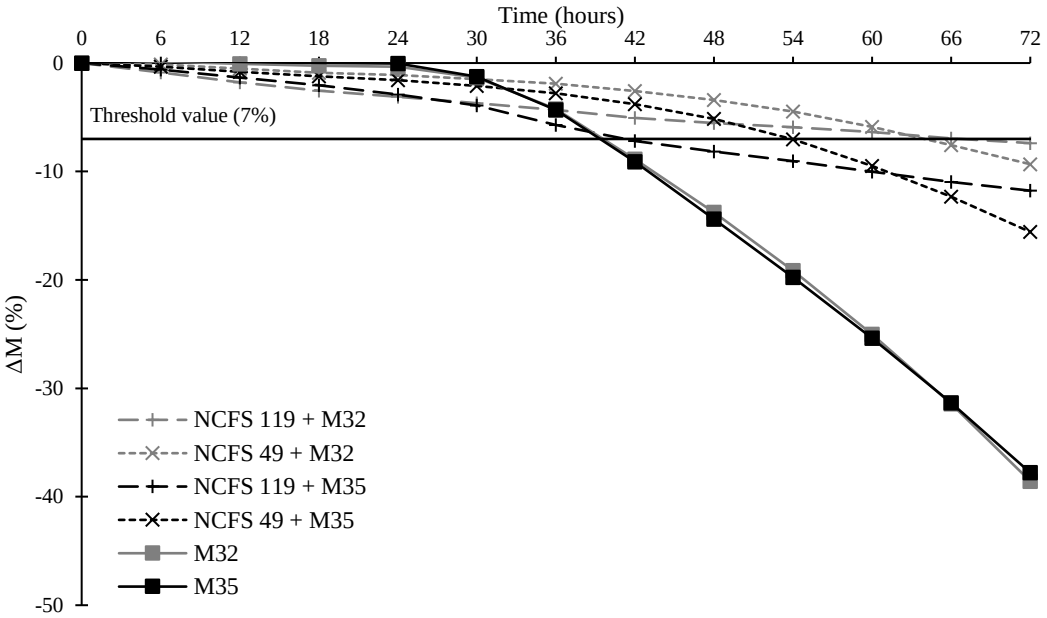

Figure II-10. Indirect impedance results of *Lactobacilli* neutralized cell-free supernatant (NCFS) at pH 7.0 against *P. nordicum*-M32 and *P. commune*-M35 fungi.

4.5. Antibacterial Activity of the *L. plantarum* Strains Against Pathogenic Bacteria

Figure II-11 shows an example of the inhibition zone obtained by *L. plantarum*-L119. Both L119 and L49 performed well-defined inhibition halos against the four pathogenic bacteria commonly found in milk (*Escherichia coli*, *Salmonella enterica*, *Staphylococcus aureus,* and *Listeria monocytogenes*).

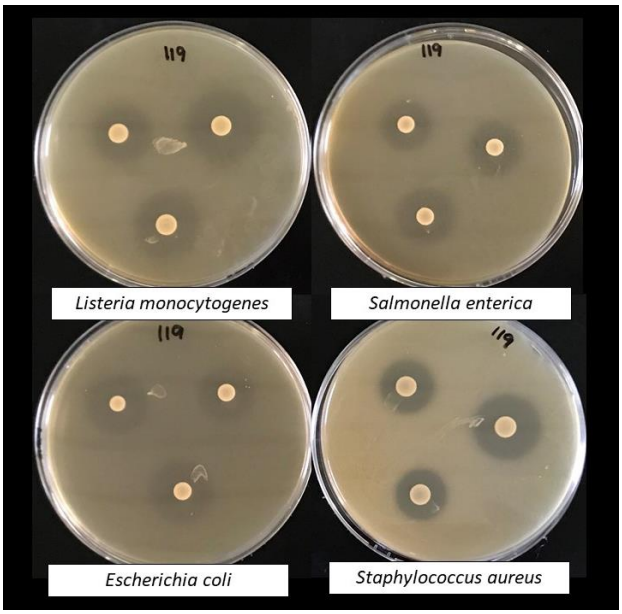

Figure II-11.Inhibition halos of *L. plantarum*-L119 against *Listeria monocytogenes*, *Salmonella enterica* CECT 4156, *Escherichia coli* CECT 4972 and *Staphylococcus aureus* CECT 5190 (CECT, Spanish Type Culture Collection).

4.6. Determination of the Antibacterial Activity of L49 and L119 Strains Through Automated Turbidometry

As shown in Figure II-12, the CFS of both *L. plantarum*-L49 and L119 have decreased more than 70% the growth of all pathogens (*Escherichia coli*, *Salmonella enterica*, *Staphylococcus aureus*, and *Listeria monocytogenes*) after 18 hours of incubation.

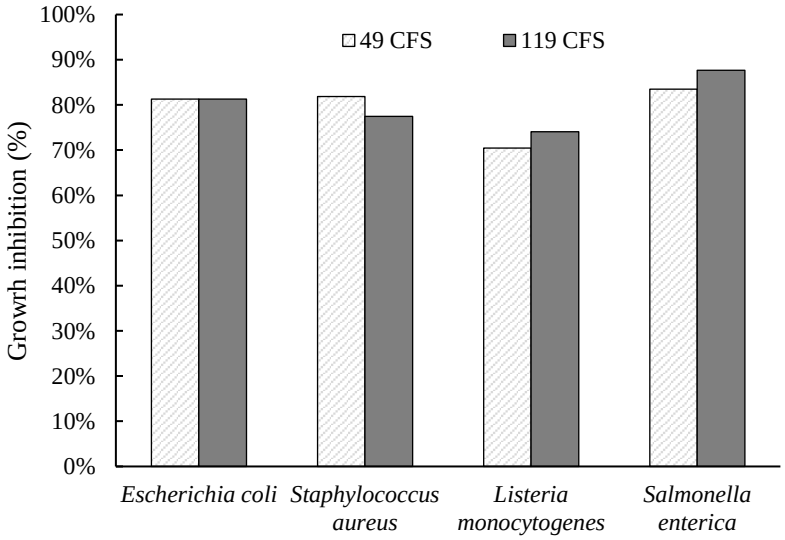

Figure II-12. Growth inhibition (%) of *Escherichia coli* CECT 4972, *Staphylococcus aureus* CECT 5190, *Listeria monocytogenes* and *Salmonella enterica* CECT 4156 (CECT, Spanish Type Culture Collection) by cell-free supernatant (CFS) of *L. plantarum*-L119 and L49 measured after 18 hours of incubation. Values are means of four replicates.

S. enterica was the strain most affected by L119 CFS and L49 CFS 49 (88 and 83%, respectively), and *L. monocytogenes* the less by L49 CFS and L119 CFS (70 and 74%, respectively). The same occurs when the supernatant was submitted to heat treatment, deducing that the possible compound responsible for the inhibition is heat stable (Figure II-13). Instead, when the CFS were subjected to neutralization with NaOH the antagonistic activity decreased between 50% to 20% (Figure II-14).

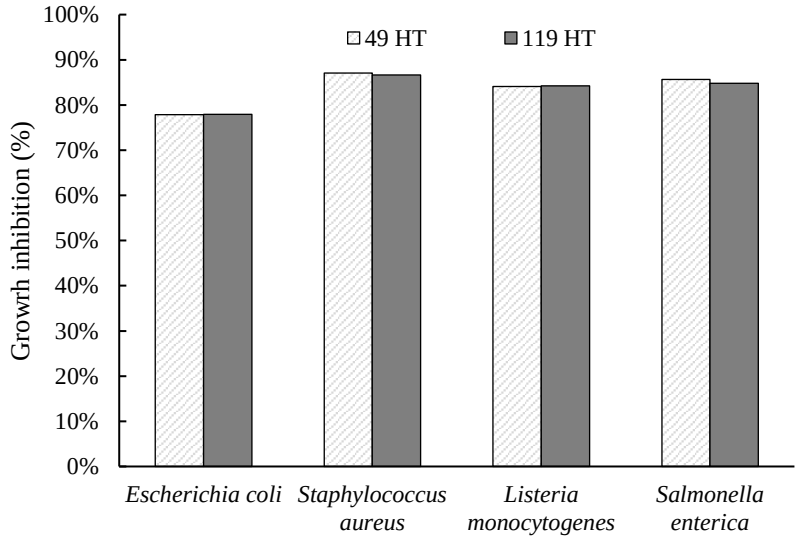

Figure II-13. Growth inhibition (%) of *Escherichia coli* CECT 4972, *Staphylococcus aureus* CECT 5190, *Listeria monocytogenes* and *Salmonella enterica* CECT 4156 (CECT, Spanish Type Culture Collection) by cell-free supernatant (CFS) of *L. plantarum*-L119 and L49 following heat treatment (HT) measured after 18 hours of incubation. Values are means of four replicates.

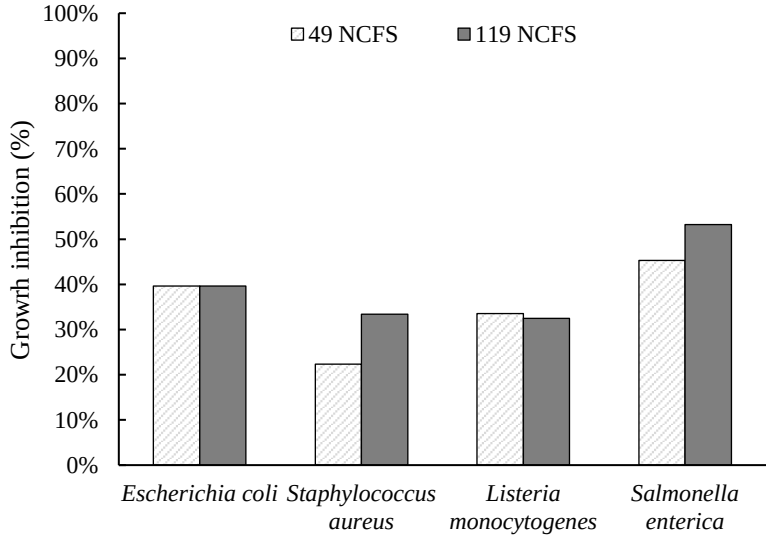

Figure II-14.Growth inhibition (%) of *Escherichia coli* CECT 4972, *Staphylococcus aureus* CECT 5190, *Listeria monocytogenes* and *Salmonella enterica* CECT 4156 (CECT, Spanish Type Culture Collection) by neutralized cell-free supernatant (NCFS) of *L. plantarum*-L119 and L49 measured after 18 hours of incubation. Values are means of four replicates.

The results below show the growth curves (turbidimetric technique of each pathogenic bacteria) in the presence of both *L. plantarum*-L119 and L49 CFS, NCFS and CFS submitted to heat treatment (HT) over 18 hours of incubation (Figures II-15, 16, 17 and 18), showing different degrees of inhibition.

As it can be seen, both CFS of *L. plantarum* strains (L119 and L49) were capable of inhibiting the growth of all pathogenic bacteria tested. In contrast, the NCFS showed a reduction of this inhibition compared to the growth curve of pathogenic bacteria, indicating that the acid production may be an active factor in the inhibition, but it is not the only one. Possibly, other compounds may be responsible for it.

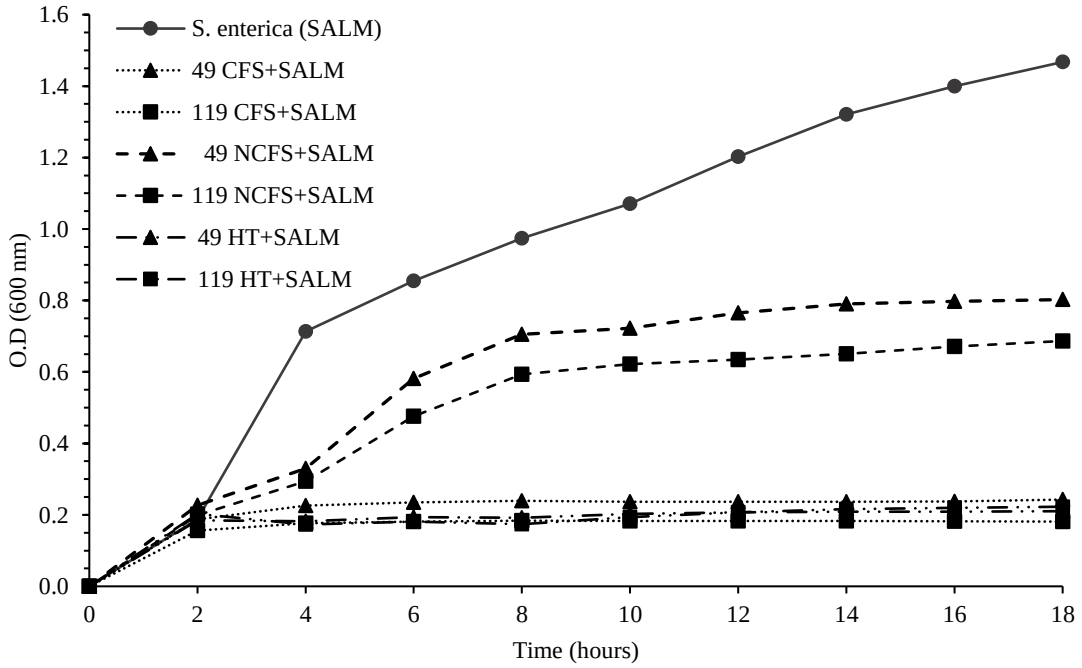

Figure II-15.Evaluation of *Salmonella enterica* CECT 4156 growth and characterization of the antagonistic compound produced by *L. plantarum*-L119 (■) and L49 (▲) by a turbidimetric technique. SALM, *S. enterica*; CFS, cell free supernatant; NCFS, neutralized cell free supernatant; HT, heat treatment.

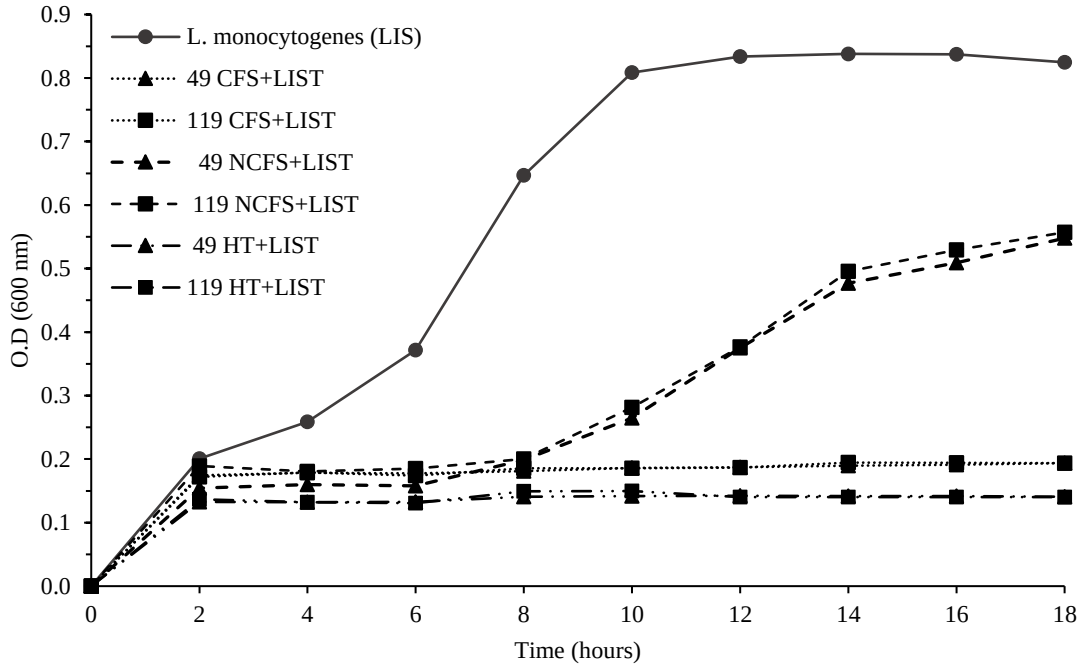

Figure II-16.Evaluation of *Listeria monocytogenes* (●) growth and characterization of the antagonistic compound produced by *L. plantarum*-L119 (■) and L49 (▲). LIS, *L. monocytogenes*; CFS, cell free supernatant; NCFS, neutralized cell free supernatant; HT, heat treatment.

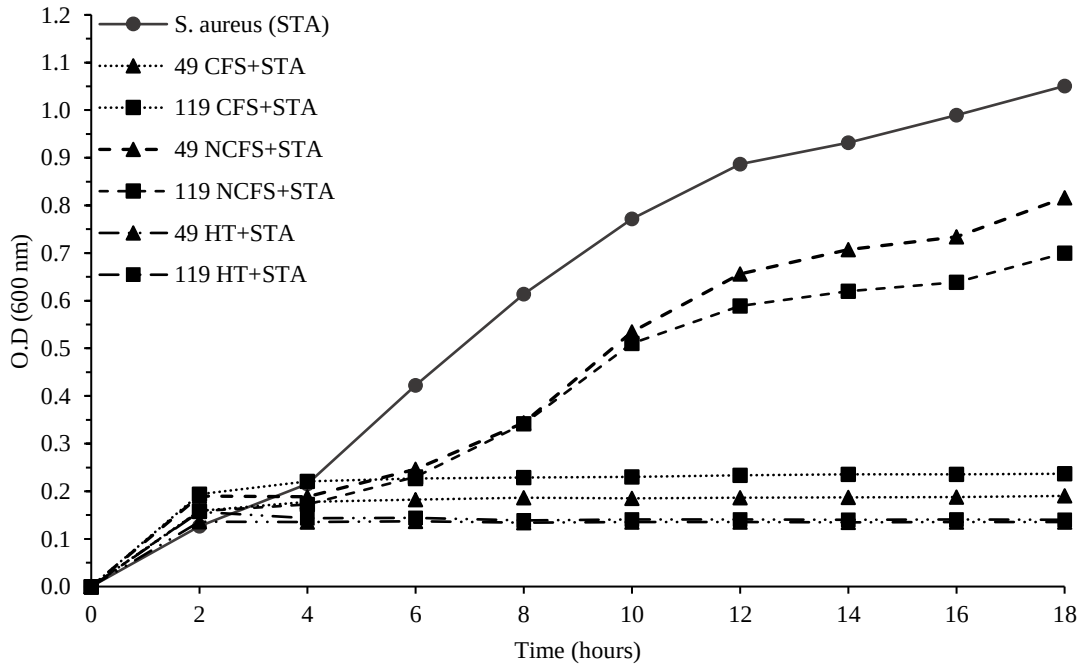

Figure II-17.Evaluation of *Staphylococcus aureus* CECT 5190 (●) growth and characterization of the antagonistic compound produced by *L. plantarum*-L119 (■) and L49 (▲). STA, *S. aureus*; CFS, cell free supernatant; NCFS, neutralized cell free supernatant; HT, heat treatment.

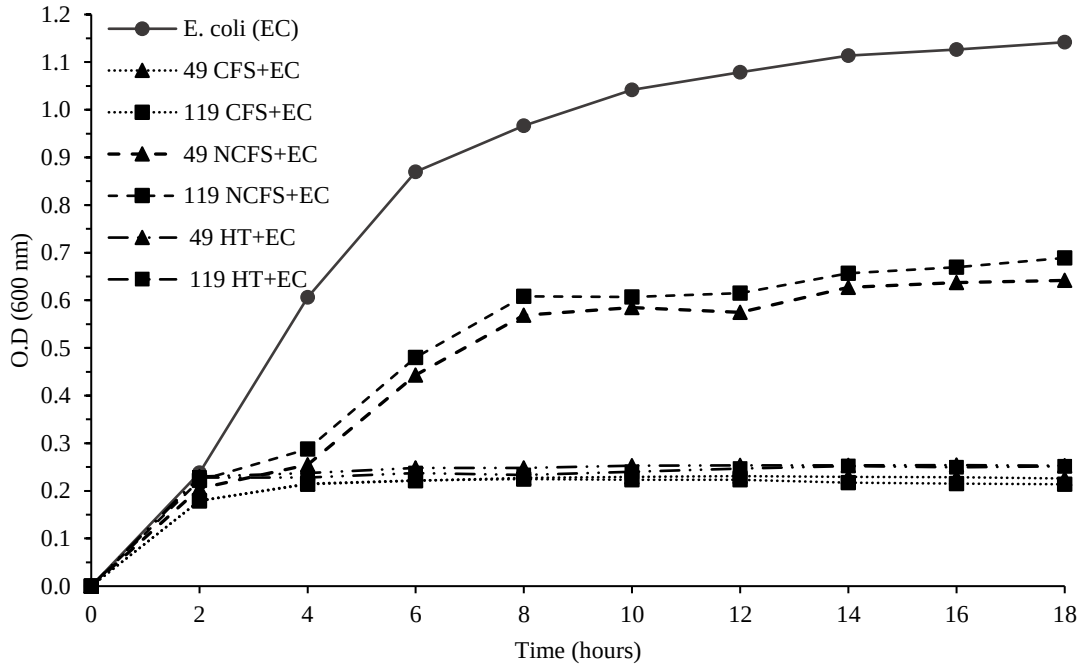

Figure II-18.Evaluation of *Escherichia coli* CECT 4972 (●) growth and characterization of the antagonistic compound produced by *L. plantarum* (L 119 (■) and L 49 (▲)). EC, E. coli; CFS, cell free supernatant; NCFS, neutral cell free supernatant; HT, heat treatment.

5. DISCUSSION

5.1. EVALUATION OF *LACTOBACILLI* WITH ANTIFUNGAL ACTIVITY AGAINST PENICILLIUM SPP. STRAINS.

The antifungal activity of *Lactobacilli* such as *L. plantarum, L. rhamnosus, L. casei* and *L. paracasei* against *Penicillium* spp. has been reported previously (Cabo et al., 2002; Delavene et al., 2013; Schillinger and Villarreal, 2010, among others). Recently, in another study at University of León (Department of Food Hygiene and Food Technology), Ramos-Pereira (2019) found *L. paracasei* as the main antifungal species isolated from milk, being active against *P. commune, P. verrucosum* and *P. nordicum*. In particular, strains of *L. plantarum* have been extensively investigated as mold controlling agents as reviewed by Sadiq et al. (2019). In agreement with Cheong et al. (2014), this study proved that *L. plantarum* is a great species with antifungal activity specially against *P. commune.*

This study reported that *P. commune* showed the highest resistance against some *Lactobacilli* strains compared to the other strains of *Penicillium* spp. This mold clearly defined whose *Lactobacilli* would be selected as the major antifungal profile since this species is commonly found as spoilage contaminant in cheeses (Kure et al., 2001; Kure et al., 2004; Pitt and Hocking, 2009; Ramos-Pereira et al., 2019).

Based on that information, two *Lactobacilli* with the highest activity against all *Penicillium* strains, especially *P. commune*, were selected, and it is in accordance to others researches. Ouiddir et al. (2019) found that *L. plantarum* clearly had the highest antifungal activity compared to other LAB tested in sour cream and sourdough bread, concluding that these strains could be good candidates for industrial application of these products. Sorrentino et al. (2013) found in their study that the best antimicrobial activity against *Penicillium* strains isolated from black truffles was due to *L. plantarum.* Izzo et al., (2020) also found satisfactory antifungal effect of *L. plantarum* against *P. expansum* and *P. brevicompactum* on fermented whey.

Therefore, our results show once again, that *L. plantarum* indeed exhibits antimicrobial activity regardless of the food source, giving a broad applicability for industries.

5.2. Molecular and Phenotypic Identification

L. plantarum was the dominant species in our study. This is one of the non-starter LAB species most frequently isolated from ripened cheeses (Rantsiou *et al*., 2008, Van Hoorde *et al*., 2008).

Identifying LAB strains at the species level is not an easy task, even with the advanced tools for molecular identification. The use of only phenotypic methods the results may be not reliable because it requires the determination of bacterial properties beyond those of the common fermentation tests (Tannock et al., 1999).

In our study it was possible to identify at species level 29 *Lactobacilli*, presenting all of them equal or more than 99% of similarity. Based on the aim of this study, the two *L. plantarum* that presented the best results (L119 and L49) were characterized phenotypically. According to McCartney, (2002) strains that generally show higher than 97% similarity of the 16S rDNA sequence are considered to be the same species, however for some species this may not be suitable (De Bruyne et al. 2009; Větrovský and Baldrian, 2013).

Dellaglio et al. (1975) demonstrated heterogeneity of the *L. plantarum* group, dividing into three groups that were classified as *L. plantarum*, *L. pentosus* and *L. paraplantarum*. Giving that information, we confirmed the identity of our strains (L119 and L49) and was established as *L. plantarum* by *recA* gene, as proposed by Torriani et al., (2001).

The phenotypic identification did not show a reliable method when used alone or have poor concordance with genomics-based tests (Boyd et al., 2005; Shehata et al., 2019), although our study demonstrated that was possible to characterize and differentiate the strain L119 from L49 based on the fermentation of sugar and enzymatic profile.

The enzymatic activity of the cultures is important in cheese production (Arora et al. 1990). In general, high aminopeptidase activities, such as the leucine- and valine-arylamidase of L49, are interesting; in contrast, high proteinase and esterase-lipase activities are not desired (usually they are linked to bitterness; Collins et al., 2003) (neither trypsin, nor esterase-lipase activities were found in our strains). Finally, a medium to high β-galactosidase and α- and β-glucosidase activities were found in our strains. This is interesting as a possible probiotic feature (particularly, regarding the lactose intolerance), and the presence of these enzymes may be beneficial for the utilization of galacto- and gluco-oligosaccharides commonly used lately as prebiotic ingredients in the dairy industry (Georgieva et al., 2009). According to these results, both strains would be suitable for cheese production.

5.3. Determination of The Antifungal Activity of L49 and L119 Strains Through Impedance Technique

The evaluation of antifungal activity of LAB is still complex to understand and the mechanism of action of these compounds are still unknown or not clearly elucidated. Zalán et al., (2010) pointed in their study that the organic acid production of *Lactobacilli* was considerably influenced by the media and the strains. Schillinger and Villarreal (2010) found that the acetic acid was the main factor involved in the inhibition of *P. nordicum* and also showed that the culture supernatants were more effective than the acidified MRS medium, indicating that besides acetic and lactic acid, other LAB metabolites contribute to the inhibition.

In our study, we verified that the two strains of *L. plantarum*-L119 and L49 did not show the same inhibition against the *Penicillium* spp. Therefore, as Figure II-10 shows, the neutralization of both CFS affected the metabolism of *P. nordicum*-M32, registering this activity with 65 hours compared to the positive control (39 hours). This result means that the compound was probably not only the organic acids.

A large number of substances with antifungal properties have already been identified (Chen and Narbad, 2018; Sadiq et al., 2019). These compounds such as phenolic acids, organic acids, fatty acids, diacetyl, bioactive peptides, carboxylic acids,

bacteriocins, hydrogen peroxide, lactones, alcohols, and reuterin are significantly influenced by growth temperature, availability of nutrients, atmosphere, pH and viscosity (Crowley et al., 2013; Dalié, 2010; Schillinger and Villarreal, 2010).

These studies are in agreement with ours results, indicating that the antifungal compounds are diversified and widely distributed among different strains of *L. plantarum*. Besides, the organic acids and other metabolic products of the LAB contribute to the fungal inhibition acting in synergism.

Regarding the identification of the possible compound responsible for the antifungal activity, a quantification of the production of phenyllactic acid (PLA), was carried out by HPLC at the University of Léon (Department of Food Hygiene and Food Technology, T.M. López, unpublished data). We found a production by our strains of *L. plantarum*-L49 and L119 of around 40 mg L^{-1} (in MRS broth/24 h/30 ℃), double than that of other antifungal strains identified by Ramos-Pereira (2019) (*L. paracasei* 25/1 and *L. casei* 51/3, unpublished data). On the other hand, in other studies carried out recently (unpublished data), we found a Minimum Inhibitory Concentration (MIC) of PLA for *P. commune*-M35 of 7.5 mg mL^{-1}. This result is higher than that found by Lavermicocca et al. (5.5 mg mL^{-1}; 2003), which means a higher resistance of *P. commune*-M35.

PLA is considered a marker of antifungal activity, being one of the major compounds occurring in the culture of *L. plantarum*, being also antibacterial (Lavermicocca et al., 2003). According to these authors, *P. commune* would have a minimum inhibitory concentration of 5 mg mL^{-1}. This means that this compound could participate in the antifungal activity of our strains against this fungus, although other compounds should contribute to this action in a synergistic manner.

5.4. Determination of the Antibacterial Activity of L49 and L119 Strains Through Automated Turbidometry

The L119 and L49 strains showed antibacterial inhibition against all indicated pathogenic bacteria. The automated turbidometry assisted to characterize the compound responsible for the growth inhibition. The thermostability at 100 ℃ for 10 min and residual activity after neutralization indicated that the overall inhibitory activity may be a result of a synergistic action between acid and a possible bacteriocin-like inhibitory substance (BLIS). The same result was obtained by Parada et al., (2007), the *L plantarum* strain isolated from Turkish dairy products showed antibacterial activity after heat treatment. Similarly, was reported by Todorov and Dicks (2005).

Rao et al., (2013) showed in their study that bacteriocins and BLISs produced by *L. plantarum* showed wide inhibition and remained active between pH range and heat-treatment. It is suggested that the heat stability of different bacteriocins of LAB may be attributed to ecological and environmental adaptation (Ponce et al., 2008).

It is well documented that *L. plantarum* has beneficial use as adjunct or protective culture for fermented foods (De Vuyst and Leroy, 2007). Studies reported by Cizeikiene et al., (2013), Digaitiene et al., (2012), Nespolo and Brandelli, (2010), Nishie et al., (2012), Ventimiglia et al., (2015) proved that *L. plantarum* producing bacteriocins or bacteriocin-like inhibitory substances (BLISs) have a broad spectrum against spoilage and pathogenic microorganisms.

In recent years, several reports have been published on the antibacterial activity of *L. plantarum* against *Salmonella sp*. (Rodríguez et al., 2012; Ołdak et al., 2017; Zhu et al., 2014) *Escherichia coli* (Dubourg et al., 2015; Kumar et al., 2016; Todorov et al., 2010; Wen et al., 2016), *Listeria sp*. (Todorov et al., 2010; Todorov et al., 2014; Venkadesan and Sumathi, 2015; Wen et al., 2016) and *Staphylococcus aureus* (Buntin and Hongpattarakere, 2014; Liu et al., 2016; Todorov et al., 2010; Venkadesan and Sumathi, 2015). The effect of L119 and L49 isolates against pathogenic bacteria is very important for the dairy industry, as these pathogens are commonly isolated from dairy products.

Our results demonstrated that *L. plantarum* strains have a broad inhibitory activity, especially those pathogens associated with outbreaks and these findings are in agreement with the cited studies. The nature of the main compound responsible for the antagonistic activity still unclear, but were demonstrated that more than one compound acts in synergism against the investigated microorganisms.

6. CONCLUSION

The *Lactobacilli* strains demonstrated great ability to inhibit *Penicillium* spp., showing the importance of these genera not only for probiotic purposes or production of fermented food but their use to biocontrol molds. *L. plantarum*-L119 and L49 were capable of inhibiting *P. commune,* known as the major species found on cheese responsible for spoilage and also a mycotoxigenic mold.

The identification of the selected strains showed that the species *L. plantarum* indeed exerts antifungal ability and also antibacterial activity against pathogenic bacteria of interest in milk and dairy products. Although it was not possible to identify the

antimicrobial compound of L119 and L49, our results suggest that there is a synergism interaction between them. Another point is that the antibacterial and antifungal compound may be not the same suggesting that the mode of action is microorganism-dependent.

These findings are of great importance since L119 and L49 have the antibacterial and antifungal ability, and also demonstrated suitability for cheese production according to the enzymatic profiles.

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Chapter III.

POLIPHASIC IDENTIFICATION OF *PENICILLIUM* IN SEMI-HARD SPANISH CHEESES, MYCOTOXIN GENES ANALYSIS AND CYCLOPIAZONIC ACID PRODUCTION BY *PENICILLIUM COMMUNE*.

1. INTRODUCTION

Mold-ripened cheese displays a considerable diversity within the mycobiota they contain, leading to specific appearance, texture, and flavor. An example is the production of blue or white coat cheeses characterized by their particular flavor, texture, and color such as the production of the well-known Roquefort and Camembert cheeses, and some fermented sausages (Visagie et al., 2014). On the other hand, they are considered contaminants in food, especially in ripened cheeses (Desmasures, 2014; Montagna et al., 2004; Ribeiro et al., 2020).

The main genera involved in spoilage dairy product are *Penicillium*, *Cladosporium*, *Mucor*, *Aspergillus,* and *Geotrichum* (Ledenbach and Marshall, 2009; Pitt and Hocking, 2009). Those belonging to the *Penicillium* genus, are the main agents responsible for the alteration in ripened cheeses, causing, in addition to product deterioration, public health concern for being potentially toxigenic (Dalié et al., 2010). The most important and well-known mycotoxins in food and feed are produced by *Aspergillus*, *Fusarium* and *Penicillium* genera (Kokkonen et al., 2005; Magan and Aldred 2007; Pleadin et al., 2019; Sengun, 2008).

The microbial community and level of contamination directly affect cheese quality and safety. Studies a long of the identification of molds in cheeses indicates potential desirable as well as those undesirable strains (mycotoxin producers) (Dugat-Bony et al., 2016; Dobson, 2017; Ramos-Pereira et al., 2019). According to Frisvad and Samson (2004), Frisvad et al. (2007), Pitt and Hocking (2009), and Houbraken et al. (2016), there are 15 species of *Penicillium* subgenus *Penicillium* belonging to the sections: *Brevicompacta*, *Roquefortorum*, *Chrysogena*, *Penicillium* and *Fasciculata*, associated with cheese spoilage. Most of these species are mycotoxigenic with some of them produced in cheese: ochratoxin A (OTA), patulin (PAT), and cyclopiazonic acid (CPA) (Hymery et al., 2014; Weidenbörner, 2013).

Penicillium is commonly found in a diverse range of habitats and some species have positive impacts on the food industry. In addition, their useful enzymes required for the processing of feed and food products contribute to the organoleptic properties. Species such as *P. solitum*, *P. verrucosum*, *P. brevicompactum*, *P. commune*, *P. palitans; P. crustosum,* and *P. chrysogenum* were commonly associated with mold-ripened cheese. The diversity and number of these microorganisms depend on the sources of contamination mainly from the environment (Hymery et al., 2014; Mounier et al., 2006).

Identifying the *Penicillium* genus at the species level is a very complex task, as most species have very similar properties. As an example, more than 1000 names were introduced in the genus but to date, 354 are accepted species (Visagie et al., 2014). Conventional identification is based on macroscopic and microscopic observation of morphology, growth rates, presence of pigment and exudate on growth media, and mycotoxin production (Pitt, 1979). Molecular identification was also proposed as a tool of an accurate phylogenetic reference system given its advantage over conventional phenotypic methods (Perrone and Susca, 2017). Frisvad and Samson (2004) proposed a polyphasic approach to identify species of *Penicillium* subgenus *Penicillium* based on morphological, chemical, and molecular analysis.

This chapter aimed to investigate the occurrence of natural *Penicillium* contaminants associated with ripened cheeses based on morphology, molecular identification, and mycotoxin production of cyclopiazonic acid (CPA), ochratoxin A (OTA), and patulin (PAT).

2. OBJECTIVES

- Identify filamentous fungi isolated from ripened cheeses based on the morphology, molecular identification and the production of three mycotoxins (CPA, OTA and PAT) following a "polyphasic" approach;
- Analyze the presence of *dmaT, otanpsPN* and *idh* genes that are responsible for the production of CPA, OTA and PAT extrolites, respectively;
- Quantify the production of CPA by *P. commune in vitro* in order to know the strain effect and verify the risk derived from its growth on the surface of the cheese.

3. MATERIALS AND METHODS

3.1. ISOLATION AND IDENTIFICATION OF *PENICILLIUM* GENUS IN SEMI-HARD CHEESES

Seven sheep cheeses samples were analyzed, of these, three were semi-hard mostly rectangular known as "pata de mulo" type with natural mold growth and produced with raw milk. The other four were also semi-hard, cylindrical known as "Castellano" type and produced with pasteurized milk. The samples were provided from different dairy industry located in the provinces of León and Zamora (Northwest of Spain).

The isolation of the strains was carried out by different techniques: direct plating that involved the use of adhesive tape to take a sample from the surface and place it on Chloramphenicol Glucose agar plates (CGA, Scharlab, Spain) and dilution plating where

10 g of cheese was homogenized in 90 mL of 2% citrate solution and then serial dilutions were made in 0.1% peptone water (Samson et al., 2010). After incubation (25 ℃/5-7 d), colonies with different morphologies per sample were selected and inoculated in Malt Extract Agar plates (MEA, Oxoid Thermo Fisher, UK) until pure cultures were obtained. Isolates were kept in 60% glycerol at -20 ℃ until further analysis.

Identification at the genus level of the selected isolates was carried out according to Samson et al. (2010) and to the macro and microscopic characteristics. Identification at the species level was carried out using a polyphasic approach (Frisvad and Samson 2004; Visagie et al. 2014) that consisted of a morphological characterization according to the keys and descriptions of Frisvad and Samson (2004), Frisvad et al. (2007), and Pitt and Hocking (2009), extrolite analysis (CPA, OTA, and PAT) and DNA barcoding (Visagie et al., 2014).

3.2. Morphologic Characterization

The isolates belonging to genus *Penicillium* were three-point inoculated in different media plates according to Frisvad and Samson (2004): Czapek Yeast Extract Agar (CYA), Yeast Sucrose Extract Agar (YES), Malt Extract Agar (MEA) and Creatine Agar (CREA). The plates were incubated for 7 days at 25 ℃ and also at 30 ℃ (CYA plates). After incubation, the following morphological characters were evaluated: colony diameter; texture; color of conidia; observe and reverse colors; existence of soluble pigment and exudate; degree of growth. In addition, acid/base production on CREA was assessed.

The microscopic slides were prepared using a strip of transparent adhesive tap pressed gently onto the surface of the isolates (from MEA plates) and placed on a drop of lactic acid (60%) as mounting fluid, and the micromorphological characters studied were: degree of branching of the conidiophores; dimension, shape and texture of stipes; ornamentation of stipes and conidia.

3.2.1. Extrolite Analysis

All the isolates were assessed to extrolite analysis (OTA, CPA and PAT) by high performance thin layer chromatography (HPTLC). The detection was carried out after incubation on YES agar plates (25 ℃/7-14 days) using the agar plug technique described by Samson et al. (2010). Aluminum plates (silica gel 60 F254; Merck, Germany) were used directly for OTA and PAT analysis. For CPA detection, the plates were previously

submerged into 10% oxalic acid in methanol for 2 minutes and heated in an oven at 110 ℃ for 2-5 minutes (Gqaleni et al., 1996).

The inoculation of the plates was carried out using a Camag Nanomat 4 (Camag, Switzerland). CPA and PAT standards were obtained from Sigma (Sigma-Aldrich Merck, Spain) and OTA from Cayman Chemical (Cayman Chemical Company, USA). The mobile phase was TEF (toluene/ethyl acetate/90% formic acid, 5:4:1) (Samson et al., 2010). After drying, plates were treated as follows: OTA (NH_3 vapors for 2 min; fluorescent blue-turquoise spots were observed under ultraviolet light) (Frisvad et al., 1989); CPA (pulverization with Ehrlich reagent; a violet-blue spot was observed after some minutes) (Gqaleni et al., 1996); PAT (pulverization with 0.5% 3-methyl-2-benzothiazolinone hydrazine, MBTH, and heating in an oven at 105 ℃/10 min; a yellow spot appears in visible light) (Frisvad et al., 1989). The detection limit was 1 μg mL^{-1}.

3.2.2. Ehrlich Test

The Ehrlich test was performed with all isolates using the filter paper method described by Lund (1995). It consists in depositing a piece of mycelium about 4 mm from CYA plate (25 ℃/7 d) on a piece of filter paper soaked with Ehrlich reagent. Before 10 minutes, in case of a positive result (by production of CPA and certain related alkaloids) a violet ring should appear, if the ring appears after 10 minutes the reaction is considered weak. Some strains produce a ring from pink to red or yellow (Frisvad and Samson, 2004).

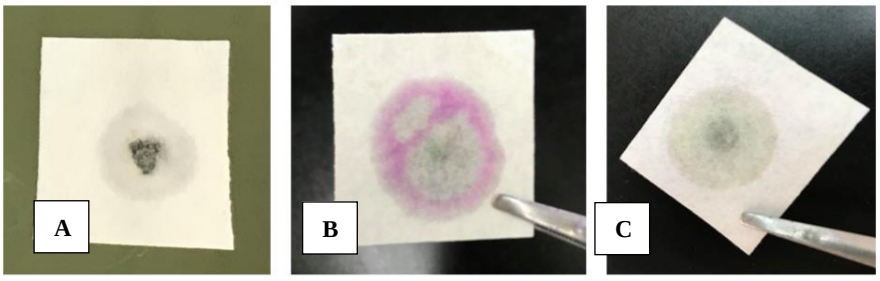

Figure III-1. Ehrlich color reaction of some *Penicillium*. A, no reaction; B, purple ring and C, yellow.

3.3. MOLECULAR IDENTIFICATION OF *PENICILLIUM* STRAINS

The *Penicillium* strains were cultured on slants of MEA at 25 ℃ for 7 days. The mycelium and spores were collected with 5 mL of sterile Tween 80 (0.05 %), rubbing with the help of a sterile platinum loop. Then, 2 mL of the suspension were transferred to a microtube and centrifuged at 16,000 g for 3 minutes. The pellet was suspended with

500 µL of Buffer lysis (50 mM Tris-HCL; 1 mM EDTA; 1 mM SDS, pH 8.0) and 50 mg glass beads (> 106 µm, Sigma-Aldrich) and 2 µL of proteinase K (80 µg) was added. The suspension was mixed at maximum vortex speed for 3 minutes and incubated at 56 ℃ for 3 hours. After that, it was homogenized again at maximum vortex speed for 3 minutes and centrifuged at 16,000 g/3 min to pellet the glass balls and collect the supernatant. For the purification of the supernatant, the Bacteria genomicprep Mini Spin kit (GE Health Care) was used, following the commercial protocol from step 5 (wash and dry).

For PCR amplification, according to Ciardo et al. (2007), 25 µL reactions were performed in a Personal Mastercycler (Eppendorf Iberica, Spain) and 2.5 µL of the purified DNA were used. Amplification of the β-tubulin (*BenA)* gene and, in some occasions, calmodulin (*CaM*) was performed using the primers and conditions described by Visagie et al. (2014).

The PCR products were purified by NucleoSpin Gel kit and PCR Clean-up (Macherey-Nagel, Germany). Both strands were sequenced on a MegaBACE 500 sequencer (GE Healthcare Life Sciences, UK). Strain identification was performed by BLAST comparison in a verified database for β-tubulin sequences (Visagie et al., 2014). Phylogenetic trees were constructed using the UPGMA method, with the distances estimated by Kimura 2-parameter model and a bootstrapping analysis of 1000 replications using the MEGA7 software (Kumar et al., 2016).

3.3.1. *Penicillium* spp. Mycotoxin Producing Genes

All isolates identified in 3.1. and 3.2. plus, a reference strain of *P. commune* (MP82, CBS 468.84), a reference strain of *P. verrucosum* (MP88, CECT 20766) were analyzed for the presence of genes associated to the production of CPA, OTA and PAT.

Table III-1. Sequences of PCR primers used for the detection of the enzyme dimethylallyl tryptophan synthase (*dmat*), isoepoxidon dehydrogenase (*idh*) and ochratoxin A non-ribosomal peptide syntethase (*otanps*PN) gene in mycotoxin-producing molds.

Primer	Nucleotide sequences (5'-3')	Amplicon length (bp)	Position[1]	Target gene	Mycotoxin
C-dmaTF	GAAGGTTGATGGCCAGATT	394	392	*dmaT*	Cyclopiazonic Acid
C-dmaTR	ATCATCACCAATCCAGCGAG		665		
FC2	CGATGTTGCTAGCAAAGACG	496	2297	*idh*	Patulin
IDH2R	ACCTTCAGTCGCTGTTCCTC		2774		

otanps_F	AGTCTTCGCTGGGTGCTTCC	750	4404	*otanps*PN	Ochratoxin A
otanps_R	CAGCACTTTTCCCTCCATCTATCC		5116		

[1]Positions are in accordance with the published sequences of *dmaT* gene of *Penicillium roqueforti* (GenBank accession number DQ121455); *idh* gene of *Penicillium urticae* (GenBank accession number AF006680) and *otanps*PN gene of *Penicillium nordicum* (GenBank accession number AY534879).

3.3.1.1. Cyclopiazonic Acid (CPA)

All the isolates identified in this study and the reference strain of *P. commune* (MP82, CBS 468.84) were amplified using a pair of C-dmaTF and C-dmaTR primers based on the *dmaT* gene (Rodriguéz, 2012; GenBank accession number DQ121455). Reactions were performed in a total volume of 25 µL, containing 12.5 µL MyTaq ™ Red Mix (BioLine), 1 µL of each primer and 2.5 µL of genomic DNA. The PCR was performed in a Personal Mastercycler (Eppendorf Iberica, Spain) and were amplified through the following thermal cycling program: initial denaturation at 94 ℃ for 5 min, followed by 35 cycles of denaturation at 94 ℃ for 45s, annealing at 60 ℃ for 45s and extension at 72 ℃ for 60s. A final extension of 7 min at 72 ℃ was included.

Amplification products were analyzed using 1.5% agarose gel in 1 X TAE buffer with 2 µL RedSafe™ Nucleic Acid Staining Solution (20,000x) (iNtRON Biotechnology) at constant voltage of 120V for 45 min. A 1kbp DNA molecular size marker (Biotools, Spain) was used to determine the size of the PCR products. Reference strain *P. commune*-MP82 (CBS 468.84) was used as positive control.

3.3.1.2. Patulin (PAT)

DNA from all the strains identified in this study was amplified using a pair of FC2 and IDH2R primers based on the *idh* gene (Luque, 2011; Paterson, 2007; GenBank accession number AF006680). Reactions were performed in a total volume of 25 µL, containing 12.5 µL MyTaq™ Red Mix (BioLine), 1 µL of each primer and 2.5 µL of genomic DNA. The PCR was performed in a Personal Mastercycler (Eppendorf Iberica, Spain) and were amplified through the following thermal cycling program: initial denaturation at 94 ℃ for 5 min; followed by 35 cycles of denaturation at 94 ℃ for 45s, annealing at 52 ℃ for 45s, and extension at 72 ℃ for 60 s. A final extension of 7 min at 72 ℃ was included.

Amplification products were analyzed using 1.5% agarose gel in 1X TAE buffer with 2 µL RedSafe™ Nucleic Acid Staining Solution (20,000x) (iNtRON Biotechnology)

at constant voltage of 120V for 45 min. A 1kbp DNA molecular size marker (Biotools, Spain) was used to determine the size of the PCR products.

3.3.1.3. Ochratoxin A (OTA)

All *Penicillium* strains identified in this study were investigated to the presence or not of *otapks*PN gene (Bogs, Battilani and Geisen, 2006; GeneBank accession number AY534879). A reference strain of *P. verrucosum*-MP88 (CECT 20766) was used as a positive control.

The DNA of fungal strains was subjected to PCR with a pair of otanps_F and otanps_R primers. Reactions were performed in a total volume of 25 µL, containing 12.5 µL MyTaq ™ Red Mix (BioLine), 1 µL of each primer and 2.5 µL of genomic DNA. The PCR was performed in a Personal Mastercycler (Eppendorf Iberica, Spain) and were amplified through the following thermal cycling program: initial denaturation at 94 ℃ for 5 min; followed by 35 cycles of denaturation at 94 ℃ for 45s, annealing at 60 ℃ for 45s, and extension at 72 ℃ for 60s. A final extension of 7 min at 72 ℃ was included.

Amplification products were analyzed using 1.5% agarose gel in 1 X TAE buffer with 2 µL RedSafe™ Nucleic Acid Staining Solution (20,000x) (iNtRON Biotechnology) at constant voltage of 120V for 45 min. A 1kbp DNA molecular size marker (Biotools, Spain) was used to determine the size of the PCR products.

3.4. Determination of Mycelium Growth and CPA Production *in vitro* by *P. commune* Strains

All *P. commune* strains identified in this study plus other strains previously identified by Ramos-Pereira et al. (2019), and a reference strain (MP82, CBS 468.84) were analyzed for their growth and potential to produce CPA *in vitro*. The strains were inoculated in Petri dishes (three-point) with 20 mL of Yeast Extract Sucrose (YES) agar. After incubation (7d/25 ℃), the dryness (in percentage) of the mycelium was determined in one of the colonies according to Taniwaki et al. (2006) and the value was applied to the other two colonies. The other two colonies were weighed and analyzed using 50 mL of the solvent (dichloromethane: methanol, 80:20 v:v) according to Gqaleni et al. (1996). The final extracts were resuspended in 1 mL of methanol and analyzed by HPLC. All samples were made in duplicate.

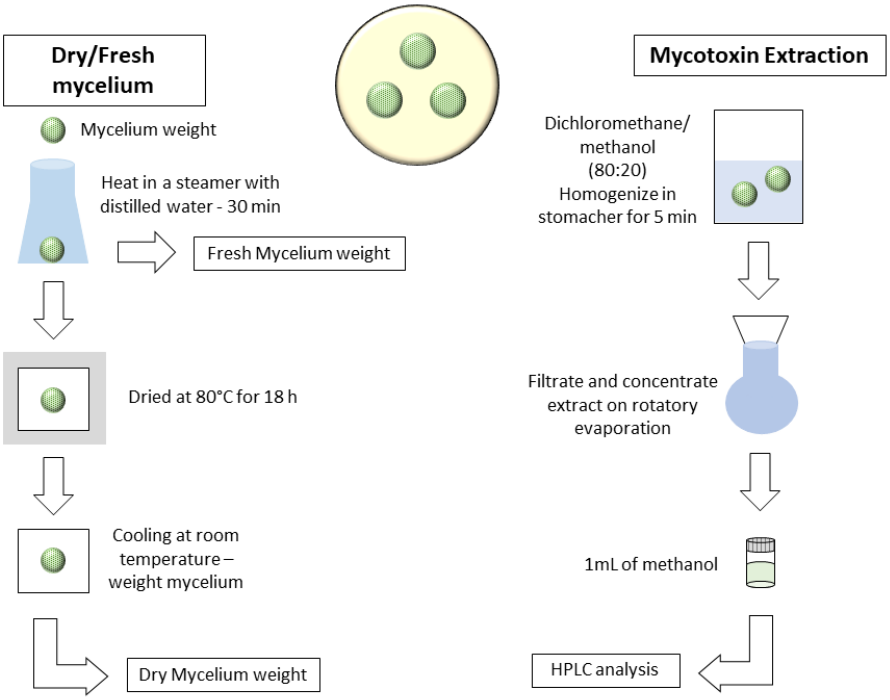

Figure III-2. Scheme of fresh and dry mycelium weight and mycotoxin extraction to quantify in HPLC analysis.

The extracts were analyzed by HPLC at the Institute of Food Science and Technology (ICTAL) of the University of León, using a Waters 2690 device with a Waters 2487 dual absorbance detector (Waters Cromatografía, S.A., Barcelona, Spain). Analyses were performed on a 150 x 4.6 mm Supelco Ultrabase 5 µm C18 column (Sigma-Aldrich, Merck Life Sciences, Madrid, Spain). The method described by Frisvad (1987) was used, using Mili-Q water as eluent A and 0.05 % trifluoroacetic acid in acetonitrile as eluent B. The conditions of the gradient employed began by applying an initial percentage of eluent B of 10 %, which was raised to 50 % in 30 min, then increased to 90 % in 10 min, was maintained for 3 min at 90 %, subsequently it was reduced again to 10 % in 6 min and, finally, it remained 1 min in these conditions (10 %). The flow used was 2 mL min^{-1} and 20 µL of each sample was injected.

The detection CPA was performed at 254 nm. Quantification of the samples was performed using an external standard, by means of a calibration curve obtained from the dilutions made from the CPA standard (Merck Life Sciences). The limit of detection, linearity (r2) and repeatability were 0.83 µg mL^{-1}, 0.995 and 90.41 %, respectively.

4. RESULTS

4.1. ISOLATION AND IDENTIFICATION OF *PENICILLIUM* GENUS IN SEMI-HARD CHEESES

A total of 17 isolates were obtained and identified as belonging to the genus *Penicillium* (Table III-2). From the total (n=7) cheeses analyzed, in only one sample (Q1) were nor found *Penicillium* strains.

Table III-2. Origin of the identified *Penicillium* spp. isolates.

Cheese sample	Type	Strain	Identification (n=17)
Q2	Semi-hard, ripened, blue coat without surface fungal culture added (mostly, rectangular, "pata de mulo")	Q2M1, Q2M2, Q2M3, Q2M4, Q2M7, Q2M11	*P. commune* (6)
		Q2M5	*P. solitum* (1)
Q3		Q3M1	*P. cvjetkovicii* (1)
		Q3M2, Q3M3, Q3M4	*P. commune* (3)
Q4	Semi-hard, ripened without fungal culture added (cylindrical, "Castellano" type)	Q4M1	*P. cyclopium* (1)
Q5		Q5M1	*P. griseofulvum* (1)
Q6		Q6M1	*P. brevicompactum* (1)
		Q6M2	*P. melanoconidium* (1)
Q7		Q7M1, Q7M2	*P. solitum* (2)

The morphological characterization of *Penicillium* strains based on observable characteristics in different media is represented in Figure III-3 and extrolite analysis in Figure III-4.

Identification at the species level was achieved through morphological characterization, extrolite analysis and DNA sequencing. From the species of *Penicillium* found in our study, *P. commune* was the most prevalent in cheese sample Q2 (6 isolates) and Q3 (3 isolates) and *P. solitum* was the second one with 2 isolates in cheese sample Q7, while only one isolate of *P. cvjetkovicii*, *P. cyclopium*, *P. griseofulvum*, *P. brevicompactum*, and *P. melanoconidium* was found on the other samples.

All the *P. commune* strains were CPA producers and formed a violet reaction in the Ehrlich test. Microscopically they showed rough-walled stipes and globose to subglobose conidia (Table III-3). *P. griseofulvum* was the only patulin producer species isolated.

Table III-3 summarizes the results of the polyphasic identification based on the main phenotypic and extrolite characteristics of *Penicillium* genus described by Frisvad and Samson (2004).

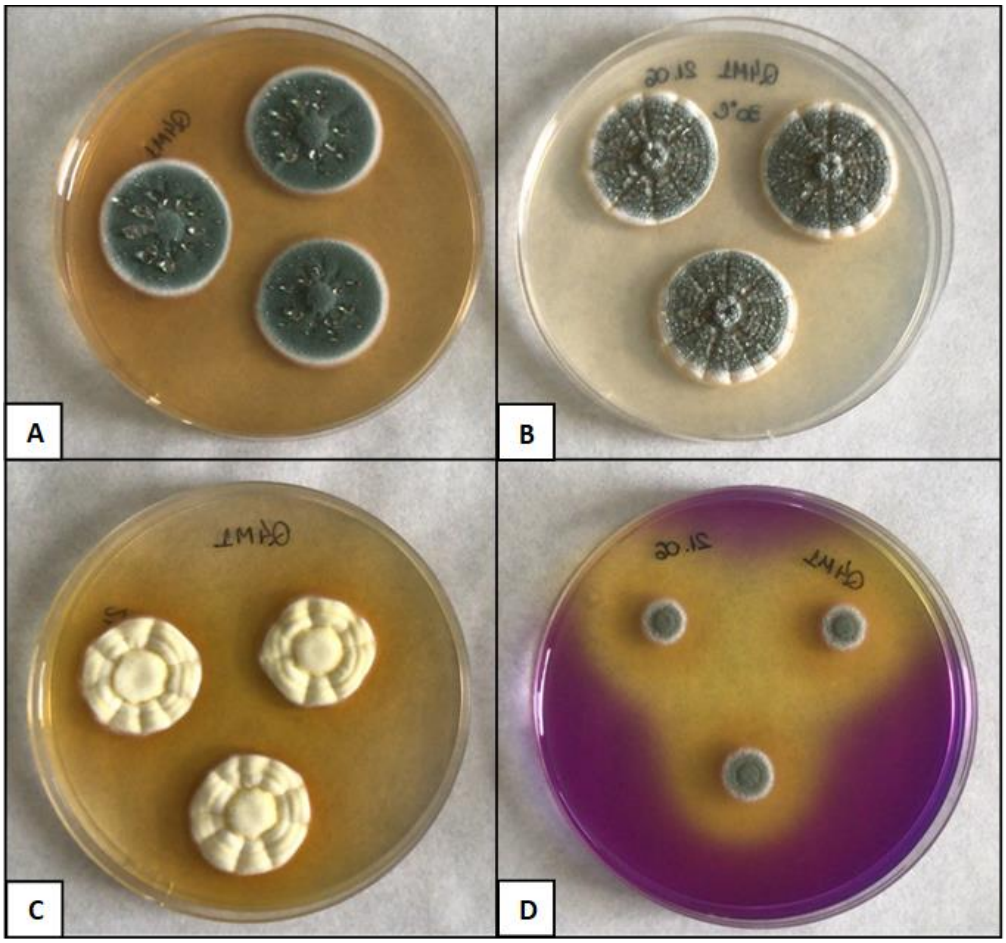

Figure III-3.Morphologic characterization of *P. cyclopium* (Q4M1) in four different media as part of the polyphasic identification according to Samson and Frisvad (2004). A, Malt Extract Agar (MEA); B, Yeast Extract Sucrose Agar (YES); C, Czapek Yeast Extract Agar (CYA) and D, Creatine Agar (CREA).

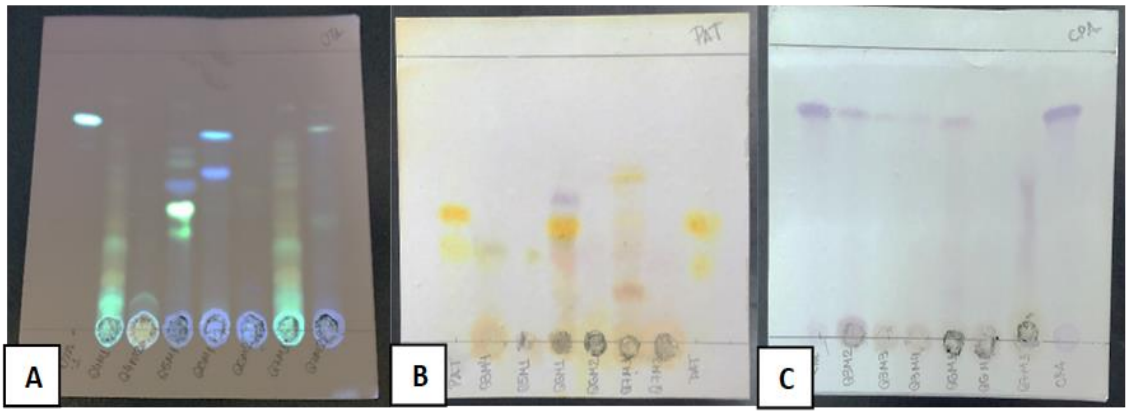

Figure III-4. High performance thin layer chromatography (HPTLC) plates to evaluate extrolite production by *Penicillium* strains. A, Ochratoxin A (OTA, fluorescent blue spots); B, Patulin (PAT; yellow spots) and C, Cyclopiazonic acid (CPA; violet-blue spots). The extrolite OTA standards are on the first line. The extrolite CPA and PAT standards are on the first and last line.

Table III-3. Phenotypic characteristics of the extrolite analysis of *Penicillium* strains isolated from cheese.

Strain	Species	CYA 30°C	CYA 25°C				YES 25°C		MEA 25°C	CREA 25°C		Microscopical characters		Mycotoxins		
		Diameter (mm)	Diameter (mm)	Conidium color	Reverse color	Ehrlich reaction	Diameter	Color reverse	Diameter	Diameter	Acid-base production	Conidia	Stipes	CPA	OTA	PAT
Q2M1*	*P. commune*	0	26	grey-green	beige	strong violet	30	yellow light brown	24	13	moderate acid	smooth globose	finely rough	+	-	-
Q2M2*	*P. commune*	0	25	grey-green exsudate incolor	pale	strong violet	34	yellow	26	15	moderate acid	smooth globose	rough walled	+	-	-
Q2M3*	*P. commune*	0	25	blue-green	beige	strong violet	33	yellow light brown	25	13	moderate acid	smooth globose	rough walled	+	-	-
Q2M4*	*P. commune*	4	29	grey-green exsudate incolor	beige	strong violet	33	yellow	24	19	weak acid	smooth subglogose	rough walled	+	-	-
Q2M5*	*P. solitum*	0	28	green	pale	no reaction	30	yellow brown center	20	14	moderate acid	smooth subglogose	rough walled	-	-	-
Q2M7*	*P. commune*	0	20	cream with some green	beige	weak violet	28	beige light brown	19	13	moderate acid	smooth globose	rough walled	+	-	-
Q2M11*	*P. commune*	0	24	cream with some green	beige	weak violet	32	beige light brown	23	14	moderate acid	smooth globose	finely rough	+	-	-
Q3M1*	*P. cvjetkovicii*	0	13	green olive	red	no reaction	17	green	12	8	moderate acid	fine roughened globose	rough walled	-	-	-
Q3M2	*P. commune*	5	24	greenish grey exsudate incolor	beige	violet	37	beige	28	20	moderate acid	smooth subglogose	rough walled	+	-	-
Q3M3	*P. commune*	6	27	greyish turquose	beige	weak violet	32	yellow	21	16	moderate acid	smooth subglogose	rough walled	+	-	-
Q3M4	*P. commune*	2	22	olive brown	yellow to orange	weak violet	34	yellow	22	22	weak acid	smooth subglogose	rough walled	+	-	-
Q4M1	*P. cyclopium*	10	30	dull green	beige to yellow exsudate incolor	strong pink	25	orange	29	23	moderate acid	smooth globose	rough walled	-	-	-
Q5M1	*P. griseofulvum*	28	28	greenish white	beige to brown	weak violet	30	yellow	25	13	none	smooth subglogose	smooth	-	-	+
Q6M1	*P. brevicompactum*	9	18	dull green	beige	no reaction	20	beige	14	10	none	smooth globose	smooth	-	-	-
Q6M2	*P. melanoconidium*	13	28	dark green exudate incolor	beige	violet	37	yellow	31	26	good acid	smooth ellipsoidal	rough	-	-	-
Q7M1	*P. discolor*	0	33	dark green	beige	weak violet	41	orange	32	17	weak acid	fine roughened subglobose	rough walled	-	-	-
Q7M2	*P. solitum*	0	28	dull green	beige	no reaction	29	yellow	24	13	moderate acid	smooth subglobose	rough walled	-	-	-

*Data published by Ramos-Pereira et al. (2019

4.2. MOLECULAR IDENTIFICATION OF *PENICILLIUM* STRAINS

Phenotyping and extrolite production according to Samson et al. (2010) and BLAST identity results of the *Penicillium* strains (n=17) are indicated in Table III-4.

Table III-4. Phenotypic, mycotoxin production and molecular identification of *Penicillium* strains isolated from cheeses.

Isolate	Identification[a]	*BenA*[b]	%[b]	Final identification
Q2M1*	*P. commune*	*P. biforme* *P. commune* *P.camemberti*	100 100 100	*P. commune*[c]
Q2M2*	*P. commune*	*P. biforme* *P. commune* *P.camemberti*	99 99 99	*P. commune*
Q2M3*	*P. commune*	*P. biforme* *P. commune* *P.camemberti*	99 99 99	*P. commune*
Q2M4*	*P. commune*	*P. biforme* *P. commune* *P.camemberti*	100 100 100	*P. commune*
Q2M5*	*P. solitum*	*P. solitum*	100	*P. solitum*
Q2M7*	*P. commune*	*P. biforme* *P. commune* *P. camemberti*	99 99 99	*P. commune*
Q2M11*	*P. commune*	*P. biforme* *P. commune* *P. camemberti*	99 99 99	*P. commune*
Q3M1*	*P. cvjetkovicii*	*P. cvjetkovicii*	100	*P. cvjetkovicii*
Q3M2	*P. commune*	*P. biforme* *P. commune* *P. camemberti*	99 99 99	*P. commune*
Q3M3	*P. commune*	*P. biforme* *P. commune* *P.camemberti*	99 99 99	*P. commune*
Q3M4	*P. commune*	*P. biforme* *P. commune* *P. camemberti*	99 99 99	*P. commune*
Q4M1	*P. cyclopium*	*P. cyclopium*[d] *P. polonicum*	99 99	*P. cyclopium*
Q5M1	*P. griseofulvum*	*P. griseofulvum*	100	*P. griseofulvum*
Q6M1	*P. brevicompactum*	*P. brevicompactum*	100	*P. brevicompactum*
Q6M2	*P. melanoconidium*	*P. melanoconidium*[d]	100	*P. melanoconidium*
Q7M1	*P. solitum*	*P. discolor* *P. solitum*	100	*P. solitum*

Q7M2	*P. solitum*	*P. solitum*	100	*P. solitum*

[a] Phenotyping and extrolite production (Samson et al., 2010)
[b] BLAST identification and percentage of identity.
[c] *P. commune* (*P. biforme*).
[d] Confirmed by CaM
*Data published by Ramos-Pereira et al. (2019).

Figure III-5 shows the results of the phylogenetic analysis based on the sequences of the *BenA* gene.

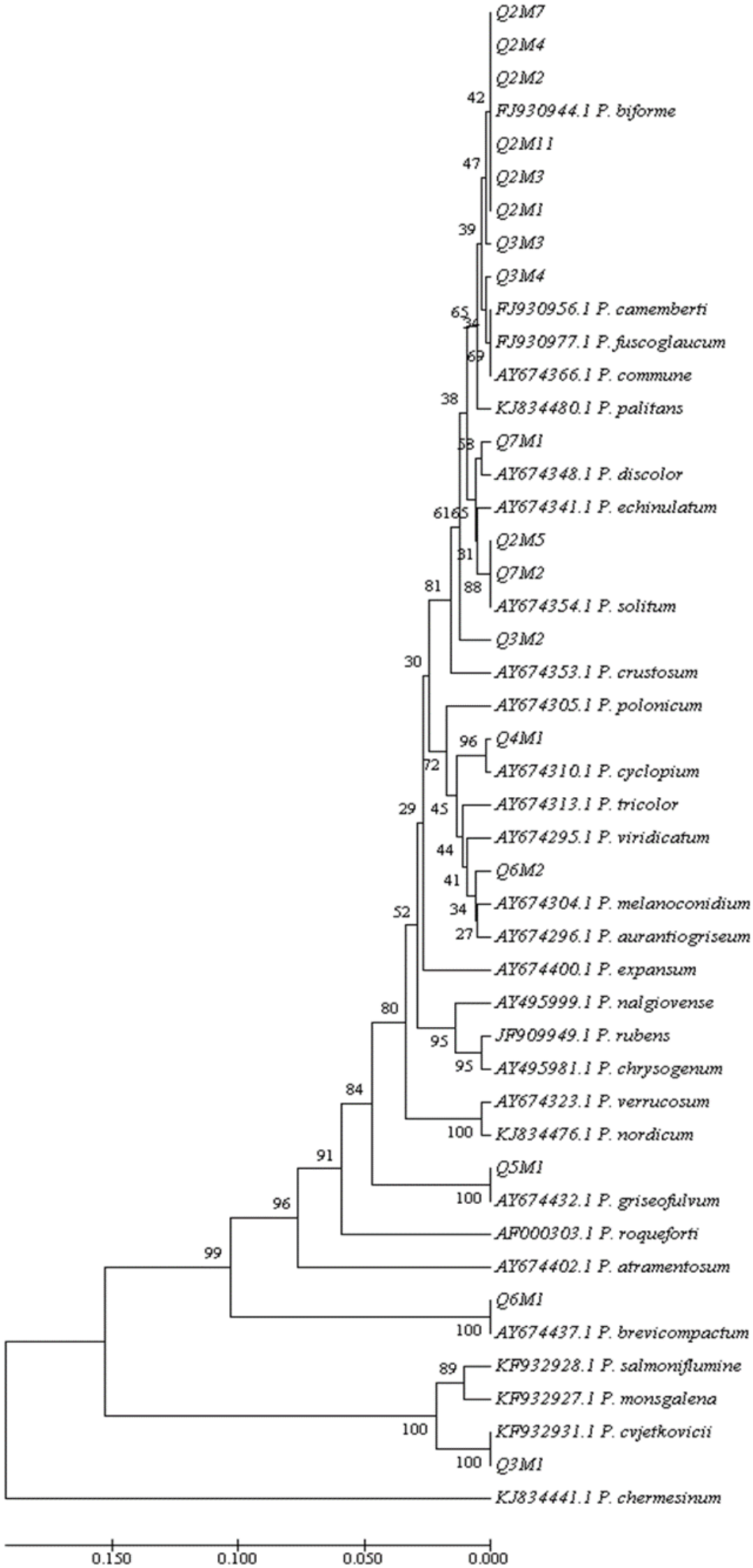

Figure III-5.UPGMA tree obtained from the phylogenetic analysis of the *BenA* sequences.

4.2.1. *Penicillium* spp. Mycotoxin-Producing Genes

PCR products in the expected sizes (bp) were obtained by using the three primer pairs FC2/IDH2 (for PAT), C-dmaTF/C-dmaTR (for CPA) and otanps_F/otanps_R (for OTA) in the 17 *Penicillium* strains isolated and identified (Table III-1). The results of the genes related to mycotoxin production are shown in Table III-5.

Table III-5. Genes related to mycotoxin production found in our study (n=17).

Isolate	Species	*dmaTp* (CPA)	*idh* (PAT)	*otan*PS (OTA)
Q6M1	*P. brevicompactum*	nd	nd	nd
Q2M1	*P. commune*	+	+	nd
Q2M2	*P. commune*	+	+	nd
Q2M3	*P. commune*	+	nd	nd
Q2M4	*P. commune*	+	+	nd
Q2M7	*P. commune*	+	+	nd
Q2M11	*P. commune*	+	+	nd
Q3M2	*P. commune*	+	+	nd
Q3M3	*P. commune*	+	+	nd
Q3M4	*P. commune*	+	+	nd
CBS. 468.84*	*P.commune*	+	nd	nd
Q4M1	*P. cyclopium*	nd	nd	nd
Q3M1	*P. cvjetkovicii*	nd	nd	nd
Q5M1	*P. griseofulvum*	nd	+	nd
Q6M2	*P. melanoconidium*	nd	+	nd
Q2M5	*P. solitum*	nd	nd	nd
Q7M1	*P. solitum*	nd	+	nd
Q7M2	*P. solitum*	nd	+	nd
MP88*	*P. verrucosum*	nd	nd	+

*Reference strains. *P. commune* MP82- CBS. 468.84; *P. verrucosum*-MP88.
Nd, not detected.

None of the 17 *Penicillium* isolates were ochratoxin A (OTA) producers on TLC analysis and so, they did not present the *otan*PS gene as expected by the given results above. Only the reference strain of *P. verrucosum*-MP88 (CECT 20766) presented the *otan*PS gene.

All the *P. commune* CPA-producing strains (Q2M1; Q2M2; Q2M3; Q2M4; Q2M7; Q2M11; Q3M2; Q3M3 and Q3M4) and the reference strain of *P. commune* (MP82, CBS 468.84) showed the presence of *dmaTp* gene (for CPA; Figure III-7 and Table III-5) giving a reliable result to use it as a complementary analysis to identify mycotoxigenic *Penicillium* strains. In none of the non-*P. commune* strains the *dmaTp* gene was found.

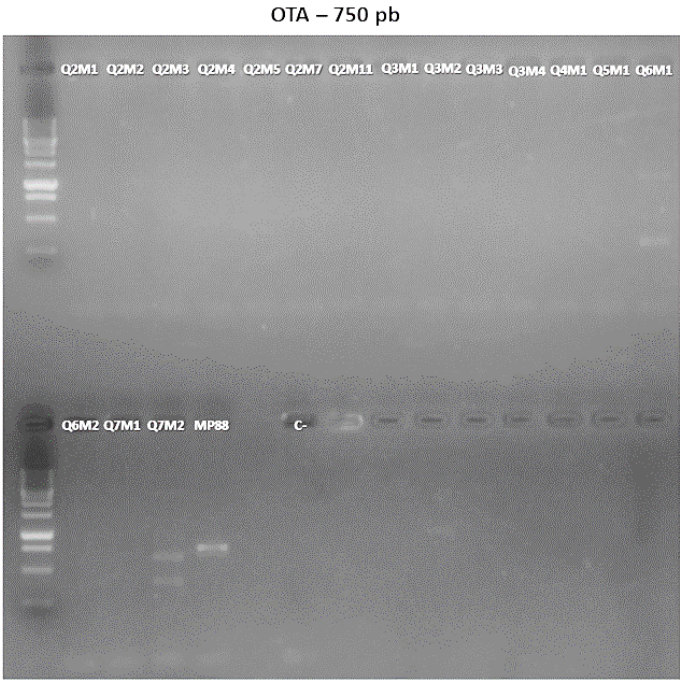

Figure III-6. Agarose gel electrophoresis of PCR products obtained with primer pairs otanps_F/otanps_R (Ochratoxin A - OTA) and genomic DNA from *P. verrucosum* reference strain (MP88). First lane with DNA molecular size marker of 1kbp. *P. commune* (lanes Q2M1, Q2M2, Q2M3, Q2M4, Q2M7, Q2M11, Q3M2, Q3M3 and Q3M4); *P. solitum* (lanes Q2M5, Q7M1 and Q7M2), *P. cvjetkovicii* (lane Q3M1); *P. cyclopium* (lane Q4M1); *P. griseofulvum* (lane Q5M1); *P. brevicompactum* (lane Q6M1) and *P. polonicum* (Q6M2); C-, negative control using water.

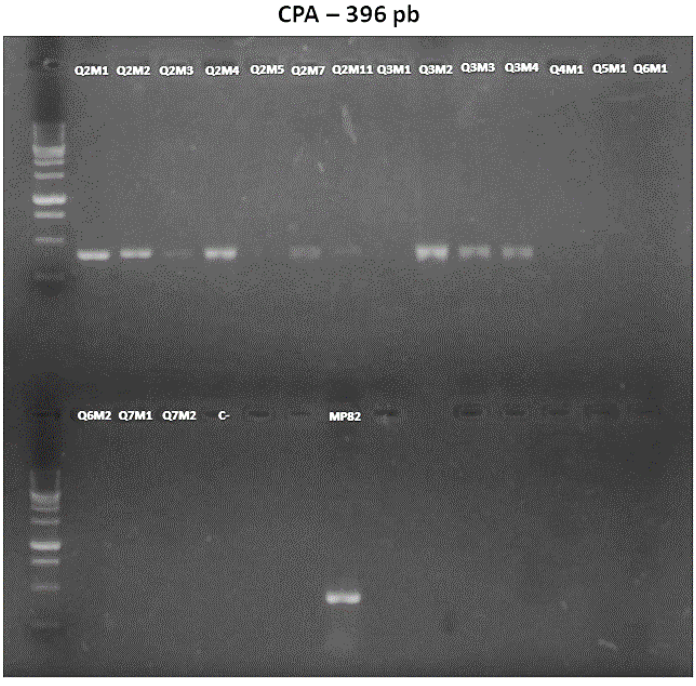

Figure III-7. Agarose gel electrophoresis of PCR products obtained with primer pairs C-dmaTF/C-dmaTR (Cyclopiazonic acid – CPA) and genomic DNA from P. commune reference strain (MP82). First lane with DNA molecular size marker of 1kbp. *P. commune* (lanes Q2M1, Q2M2, Q2M3, Q2M4, Q2M7, Q2M11, Q3M2, Q3M3 and Q3M4); *P. solitum* (lanes Q2M5, Q7M1 and Q7M2), *P. cvjetkovicii* (lane Q3M1); *P. cyclopium* (lane Q4M1); *P. griseofulvum* (lane Q5M1); *P. brevicompactum* (lane Q6M1) and *P. polonicum* (Q6M2); C-, negative control using water.

As for the primer pair FC2/IDH2 (for PAT), it yielded a specific amplicon of 496 bp in almost all *P. commune* (non-patulin producers in our analysis, Table III-5), including the expected isolate *P. griseofulvum* (Q5M1) known to be patulin producer, but it was not detected in *P. commune* (Q2M3), *P. cvjetkovicii* (Q3M1), *P. brevicompactum* (Q6M1) and in the reference strain (MP82) (Figure III-8).

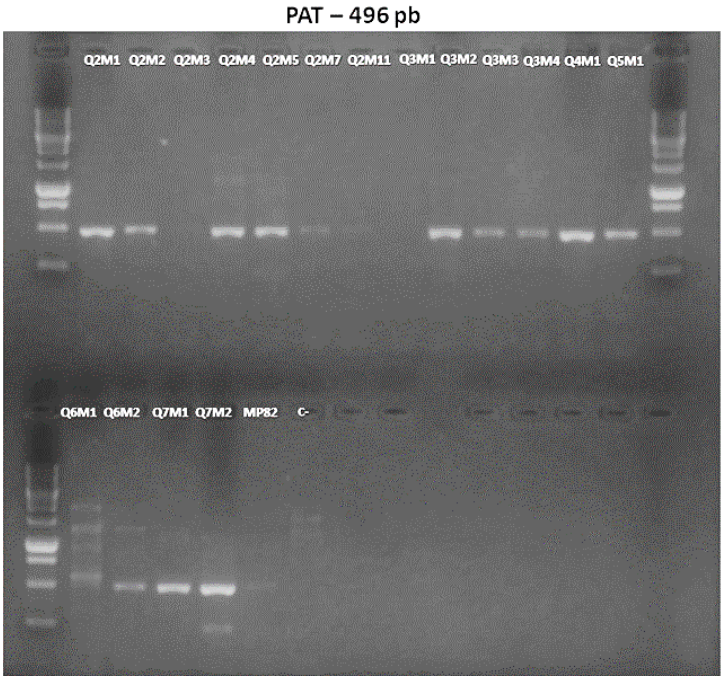

Figure III-8. Agarose gel electrophoresis of PCR products obtained with primer pairs FC2/IDH2 (Patulin – PAT) and genomic DNA from *P. commune*-MP82 reference strain (CBS 468.84). First lane with DNA molecular size marker of 1kbp. *P. commune* (lanes Q2M1, Q2M2, Q2M3, Q2M4, Q2M7, Q2M11, Q3M2, Q3M3 and Q3M4); *P. solitum* (lanes Q2M5, Q7M1 and Q7M2), *P. cvjetkovicii* (lane Q3M1); *P. cyclopium* (lane Q4M1); *P. griseofulvum* (lane Q5M1); *P. brevicompactum* (lane Q6M1) and *P. polonicum* (Q6M2); C-, negative control using water.

4.3. Determination of Mycelium Growth and CPA Production in vitro by P. commune Strains

Mycelium mass weight and CPA production on YES agar (7 d/25 °C) by *P. commune* (*P. biforme*) strains (n=24) are presented on Table III-6. HPLC chromatogram of CPA production by one *P. commune* strain (Q2M2) is shown in Figure III-9.

There were differences in the growth upon the strains of *P. commune* in both degree of growth and CPA production. Looking at the dry mycelium mass, the range is quite large (179-557 mg), which means important differences among the strains tested. The highest mean of mycelium dry weight was presented by isolate M35 (557 mg) while lowest was obtained by the reference strain CBS 468.84 (179 mg) (Table III-6). The dry

mycelium is more accurate than the fresh mycelium in terms of comparison of the growth of the strains (the fresh includes the agar).

As for CPA production, the average was close to 3 mg g^{-1} dry mycelium, with the maximum production being higher than 7 mg g^{-1} dry mycelium. Isolates P5, P1, QP1 and QLM2 were the most producers, close to the value found for the strain collection CBS468.84 (Table III-6). These isolates proceeded from different kind of samples, spoiled and not spoiled cheeses. In contrast, isolate M57 was the one with a lower production (just 106 µg g^{-1} dry mycelium, 1.4% of the maximum production) (Table III-6).

Table III-6. Mycelium mass weight and CPA production on YES agar (7 d/25ºC) by *Penicillium commune* (*P. biforme*) strains (n=24) isolated from Spanish cheeses made in Castilla and León (average ± SD of duplicates) and one collection strain (CBS 468.84).

Isolate	**Dry mycelium mass[a] (g ± SD)**	**Fresh mycelium mass[b] (g ± SD)**	**CPA (µg mL^{-1} ± SD)**	**CPA Dry mycelium[c] (µg g^{-1} ± SD)**	**CPA Fresh mycelium (µg g^{-1} ± SD)**
P5	0.374 ± 0.02	6.11 ± 0.56	2710 ± 126	7477 ± 1715	445 ± 20
P1	0.364 ± 0.04	4.81 ± 0.46	2691 ± 196	7381 ± 277	561 ± 12
CBS 468.84*	0.179 ± 0.01	4.60 ± 0.00	2400 ± 232	7217 ± 3696	253 ± 34
QP1	0.351 ± 0.03	3.43 ± 0.59	2342 ± 275	6668 ± 446	686 ± 39
QLM2	0.302 ± 0.01	4.49 ± 0.58	1865 ± 411	6296 ± 2086	425 ± 146
QP3	0.496 ± 0.10	6.12 ± 0.63	1770 ± 412	5544 ± 624	288 ± 2
M145	0.473 ± 0.03	9.12 ± 0.47	1656 ± 76	4405 ± 782	293 ± 34
M170	0.296 ± 0.03	5.67 ± 0.16	1635 ± 54	3994 ± 199	289 ± 76
QP2	0.380 ± 0.02	5.40 ± 1.12	1517 ± 87	3808 ± 817	231 ± 39
P3	0.296 ± 0.10	4.47 ± 0.91	1294 ± 111	3556 ± 104	288 ± 39
P6	0.313 ± 0.07	5.15 ±0.80	1206 ± 385	3510 ± 210	182 ± 1
P4	0.346 ± 0.12	5.02 ± 0.70	1111 ± 167	3211 ± 434	226 ± 65
QLM1	0.487 ± 0.06	6.79 ± 1.32	532 ± 50	1208 ± 466	75 ± 10
P2	0.352 ± 0.00	5.36 ± 0.49	397 ± 17	1106 ± 237	81 ± 23
M35	0.557 ± 0.16	5.28 ± 0.17	294 ± 9	701 ± 454	42 ± 7
Q2M2**	0.287 ± 0.05	3.15 ± 0.21	181 ± 16	632 ± 93	58 ± 9
Q3M2**	0.309 ± 0.03	3.98 ± 1.20	164 ± 23	598 ± 83	64 ± 7
Q3M3**	0.374 ± 0.02	2.66 ± 0.62	156 ± 8	534 ± 85	56 ± 4
Q2M4**	0.246 ± 0.06	2.28 ± 0.04	145 ± 13	435 ± 54	53 ± 12
Q3M4**	0.333 ± 0.06	2.74 ± 0.21	145 ± 23	431 ± 125	61 ± 17
Q2M3**	0.312 ± 0.03	3.80 ± 0.59	119 ± 34	416 ± 79	32 ± 6
Q2M11**	0.273 ± 0.00	3.49 ± 0.02	113 ± 21	402 ± 192	33 ± 14
Q2M1**	0.298 ± 0.23	3.19 ± 0.15	105 ± 8	351 ± 10	33 ± 4
Q2M7**	0.195 ± 0.09	3.09 ± 0.42	53 ± 4	274 ± 60	17 ± 1
M57	0.475 ± 0.01	4.50 ± 0.33	50 ± 3	106 ± 17	11 ± 1
Average ± SD	**0.347 ± 0.06**	**4.59 ± 0.51**	**986 ± 110**	**2810 ± 533**	**191 ±25**
Maximum	**0.557**	**9.12**	**2710**	**7477**	**686**
Minimum	**0.179**	**2.28**	**50**	**106**	**11**

**P. commune* (CBS 468.84). ***P. commune* strains isolated in this study. [a] One colony; [b] Two extracted colonies; [c] Estimated value.

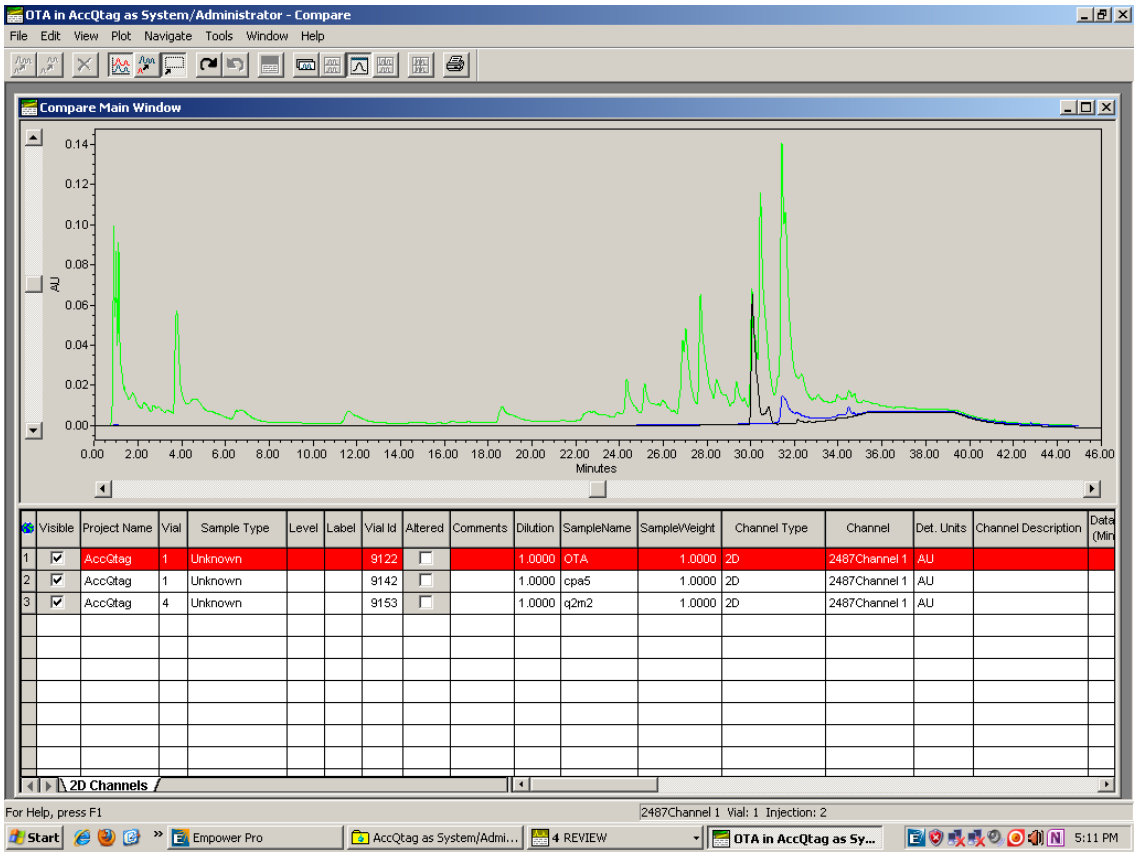

Figure III-9.HPLC chromatogram showing the standard (CPA in blue) and the production of this mycotoxin by one *P. commune* strain (Q2M2).

5. DISCUSSION

5.1. MORPHOLOGICAL AND MOLECULAR IDENTIFICATION OF *PENICILLIUM* GENUS IN SEMI-HARD CHEESES

Based on a recent classification proposed by Houbraken et al. (2020) the *Penicillium* spp. isolated in this study fit into the following taxonomic groups:

- Subgenus *Penicillium*, sections:
    - *Fasciculata* (*P. commune*, *P. cyclopium*, *P. melanoconidium*, *P. solitum*),
    - *Robsamsonia* (*P. griseofulvum*),
    - *Brevicompacta* (*P. brevicompactum*).

- Subgenus *Aspergilloides*, section *Cinnamopurpurea* (*P. cvjetkovicii*).

Macroscopic characters such as colony texture and diameters, obverse and reverse colors, degree of sporulation, the presence and color of soluble pigments and exudates on specific media (CYA, YES, MEA, CREA) are important features for the identification of genus *Penicillium*. All media are incubated at the standard temperature of 25 °C for 7 d,

with additional CYA plates at 30 and 37 ℃ that are useful to distinguish between species (Visagie et al., 2014). Stipe ornamentation and shape, size and ornamentation of conidia are frequently used characters to distinguish the species microscopically.

Within genus *Penicillia* related to food spoilage, the most important subgenus is *Penicillium*. This subgenus is also by far the most difficult taxonomically because of the numerous species and the differences between them are small. New approaches such as extrolite profiles and DNA-based techniques were used to clarify the taxonomy of *Penicillium*. This combination of techniques provides more reliable delimitation at species level (Pitt and Hocking, 2009; Visagie et al., 2014).

In this study, 17 fungus isolates were identified to genus *Penicillium*, 16 of them to subgenus *Penicillium* and 1 to subgenus *Aspergilloides* (Ramos-Pereira et al., 2019; Table III-3). Among them, *P. commune* (53%) was the most frequently found species. This species is quite easy to identify (CPA production is a distinctive feature, among the normal cheese mycobiota). *P. commune* is one of the most common fungi in cheese factories (Kure et al., 2001, 2004). Likewise, several studies (Garnier et al., 2017; Kandasamy et al., 2020; Lund et al. 1995; Panelli et al., 2012; Ramos-Pereira et al., 2019; Tzanetakis et al., 1987) reported the dominance of *P. commune* on cheese. The dominance of this indoor environmental mold could be attributed to its ability to survive under the conditions in the environment of dairy factories (Kandasamy et al., 2020).

Differently from Garnier et al. (2017), the *P. commune* strains identified in this study were all isolated from non-spoiled cheeses which suggests that this species exerts double role in cheeses, both spoilage or favoring maturation. Even being considered a commonly spoilage mold, there are examples that it positively contributes to ripening changes and cheese flavor characteristics (Lund et al., 1995). Some authors consider this species a part of the essential microflora of cheese that possibly contributes to the ripening changes and flavour characteristics of the final product (e.g., Kopanisti cheese and Taleggio cheese) (Hymery et al., 2014; Panelli et al., 2012; Tzanetakis et al., 1987). In the case of the samples of unspoiled cheese analysed in our study, they show a blue coat on the surface that could be due mainly to the presence of *P. commune,* according to our results (the cheeses had not been inoculated artificially and the mold that developed was part of a natural contamination). These cheeses are consumed and enjoyed by consumers, and therefore this fungus seemingly does not influence the organoleptic characteristics (flavour) of these varieties in a negative way. The prevalence of *P. commune* in cheeses

is explained by its ability to grow at low temperatures, low oxygen concentrations, lipolytic activity, and resistance to the action of preservatives (Pitt and Hocking, 2009).

The second most frequently species was *P. solitum*, while *P. cvjetkovicii*, *P. cyclopium*, *P. griseofulvum*, *P. brevicompactum* and *P. melanoconidium* were the less frequently. *P. solitum* was been found the dominant species in both air and cheese crust, and also air-dried meat but has never been reported as mycotoxigenic (Frisvad, 2014; Decontardi et al., 2017, 2018; Hocking and Faedo, 1992; Kure et al., 2001, 2004; Lund et al., 1995). *P. solitum* has been found in spoiled margarines, being responsible for the production of off-flavor components. Its spoiled potential may be a concern for the dairy products (Lund et al., 1995; Delamarre and Batt, 1999).

The other species *P. cyclopium*, *P. griseofulvum*, and *P. brevicompactum* were also previously reported as commonly contaminants in cheese environments and dairy products (Bath et al., 2012; Budak et al., 2015; Garnier et al., 2017; Kandasamy et al., 2020; Kure et al., 2004). As for *P. melanoconidium*, it is usual in barley, wheat, rye, oats, rice (Frisvad & Samson, 2004) and, to our knowledge, is not reported in cheese.

One important feature of fungus isolated from food is the mycotoxigenicity. From the mycotoxigenic species isolated in this study, all *P. commune* strains (9) were CPA producers and *P. griseofulvum* was PAT producer by the extrolite analysis, which is in accordance to the description of these species (Pitt, 2014b; Hocking et al., 2006). Seven species of *Penicillium* are producers of CPA and *P. commune* are the most natural source and also considered an important contaminant of cheese (Pitt, 2014a). It was thought that this species is a wild ancestor of *P. camemberti* (Giraud et al., 2010), which is used in production of white mold cheeses like Brie and Camembert. *P. camemberti* also produces CPA, even though in lower concentrations (Pitt and Hocking, 2009). *P. griseofulvum* is a very efficient producer of high levels of patulin in pure culture, and it may potentially produce patulin in cereals, pasta and similar products. This species is commonly associated to cereals and nuts and may produce also cyclopiazonic acid, roquefortine C and griseofulvin (Pitt and Hocking, 2009).

P. cyclopium is known to be producer of penicillic acid, viomellein, xanthomegnin and vioxanthin mycotoxins. *P. brevicompactum* produces botryodiploidin, and mycophenolic acid. *P. melanoconidium* produces penicillic acid, verrucosidin, santhomegnin and other mycotoxins, while *P. solitum* do not produce mycotoxins (Pitt and Hocking, 2009; Frisvad, 2014).

Regarding identification by DNA barcoding, in mycology, ITS rDNA has been widely used for species recognition and barcoding but is not diverse enough to delimit species of the cheese environment (Boysen et al., 1996; Pedersen et al., 1997; Skouboe et al., 1999). We have researched the three gene markers (*CaM*, B*enA* and *ITS*) to discriminate closely related species of *Penicillium* and have shown that β-tubulin exhibits an appropriate level of divergence between most species in this genus (Ramos-Pereira et al., 2019). The β-tubulin gene has been recommended as a specific barcode for species identification (Samson et al., 2010; Visagie et al., 2014).

BenA phylogenetic analysis (Figure III-5) allowed identification of all isolates but in some occasions the phenotypical analysis was necessary. In the past, *P. biforme* was considered as synonymous of *Penicillium camemberti* and *P. commune* (Frisvad and Samson, 2004), but was defined as a distinct species by Giraud et al., (2010). Giving that information and regarding the phenotypic and ecological characteristics (Ramos-Pereira et al., 2019), these CPA producing strains isolated in this study could not be *P. camemberti* since it usually grows as a white mycelium. Finally, a recent study by Ropars et al. (2020) suggests that wild strains identified as *P. commune* corresponded to either *P. fuscoglaucum* or *P. biforme*, and the differentiation between them are related to the isolation environment, being clustered to P. *fuscoglaucum* those strains isolated from natural environments and clustered to *P. biforme* those isolated from cheese. Following these authors, the strains found in our study would be *P. biforme* (according to the *BenA* analysis and the cheese origin).

Apart from this case, the identification by DNA sequencing using *BenA* as a molecular marker could not distinguish easily *P. solitum* (Q7M1) from *P. discolor* (Table III-4; Figure 5). For instance, strain Q7M1 according to the phylogenetic tree could be *P. discolor*, but it has not roughed conidia and the rest of phenotypic features agree with *P. solitum*, that is very closed (Figure III-5).

We found several strains to belong to series *Viridicata* which are very similar among them. *P. melanoconidium* was found in our study, although cheese is not a substrate for this species. Its identification using just phenotypic features is not easy, being very similar to other close species such as *P. polonicum*. In our study, the *BenA* and the *CaM* sequencing were necessary to identify our strain (Q6M2). As for strain Q4M1, it was identified as *P. cyclopium* (the lack of sporulation on YES agar is characteristic), and the *BenA* and *CaM* analysis confirmed this identification.

Finally, the strain Q3M1 was identified as *P. cvjetkovicii* by molecular analysis and confirmed by phenotypic characteristics (Ramos-Pereira et al. 2019; Table III-3). Morphologically, this fungus is characterized by the *monoverticillata* penicilli and the production of vinaceous to reddish-brown soluble pigments and reverse red on CYA (a feature of our strain, Table II-3). A cheese isolate obtained from Spain (Marín et al., 2014) was identified by Peterson et al. (2015) as *P. cvjetkovicii*, although it had initially been considered to be *P. chermesinum* (Marín et al., 2014; Peterson et al., 2015). The isolate obtained in our work would be the second finding of it in cheese. In our case, the fungus produced a spoilage on the surface of ripened cheeses (Castellano) that was characterized by small dark spots. The spoilage occurred in one factory, and the probable origin was the air (Peterson et al., 2015).

Given the difficulty to identify molds at species level, described in the previous paragraphs, and the time consuming of morphological traditional techniques, a few studies are focused on a rapid and reliable identification of filamentous fungi as a key step for a better management of food safety and quality (Quéro et al., 2019; Quéro et al., 2020). Matrix-assisted laser desorption ionization time of flight mass spectrometry (MALDI-TOF) analysis has been applied as a promising tool for the discrimination of closely related species in various groups of organisms, including food-related fungi (Chalupová et al., 2013; Hettick et al., 2008; Santos et al., 2010).

All of our *P. commune* strains (n=9) were further identified in University of Léon/Department of Food Hygiene and Food Technology with MALDI-TOF (Bruker), (T. M. López, unpublished data) together with another 16 strains previously identified by Ramos-Pereira et al. (2019), giving as a result *P. commune*/*P. camemberti* (or *vice versa*), with a score >2. This shows that this system does not allow the differentiation of these two so closely related species, as has been reported by Queró et al. (2019).

In addition, the MALDI-TOF could not identify several of our strains, due to the limited database that includes only 23 *Penicillium* species. Thus, the *P. solitum* isolates (Q2M5, Q7M1 and Q7M2), and the *P. melanoconidium* (Q6M2) gave a wrong identification (*P. commune*/*P. camemberti*), and the *P. griseofulvum* (Q5M1), *P. cvjetkovicii* (Q3M1) and *P. cyclopium* (Q4M1) gave no identification. As for Q6M1, that had been identified as *P. brevicompactum* by the *BenA* sequence, it gave "no identification", although this species is in the database.

Therefore, in our opinion, we think that the database of this equipment is, by now, too limited to identify food-borne *Penicillium* isolates.

5.1.1. *Penicillium* Mycotoxin-Producing Genes

In our study, we found the *idh* gene in all the wild *P. commune* (*P. biforme*) strains tested, although these were not patulin producers according to our analysis. In contrast, the gene could not be found in the *P. commune* reference strain (CBS 468.84) (Table III-5; Figure III-8).

Luque et al. (2011) found this gene in *P. commune* (2) and *P. camemberti* (3), strains that were all patulin producers according to their analysis. These species are not known to be producers of patulin according to the literature (Frisvad and Samson, 2004; Pitt and Hocking, 2009; Samson et al., 2014). Luque et al. (2011) analyzed the contents of three plates (MEA, 15 d, 25ºC) when usually (as in our case too) only the content of one plate is analyzed. This may explain their findings. To verify if our *P. commune* strains were patulin producers and possibly explain the presence of *idh* gene we tested the same methodology as described by Luque et al. (2011). Our findings confirmed patulin production by Q3M3 strain in HPTLC analysis, although the production was very low. Further analysis in HPLC will be required to confirm that result. In any case, the presence of the *idh* genes in our strains agrees with these authors and should be further investigated (it is interesting in the future studies regarding patulin production).

As for the other genes, the results agree with the mycotoxin production found by us and in the literature. The CPA producers had the CPA genes and none produced OTA and *otanPS* gene was not found, only on the reference strain *P. verrucosum*-MP88 (Table III-5).

5.2. Determination of Mycelium Growth and CPA Production *in vitro* by *P. commune* strains

Table III-6 shows the concentrations of CPA found in our samples (extracts of YES plates/25 ℃/7 d) and the production capacity of the strains (amount per dry/fresh mycelium). As it can be seen, the amounts of CPA that our strains are able to produce range from 50 to 2710 µg mL^{-1}, which means a very high influence of the strain. The average was close to 1000 µg mL^{-1}. As for the toxigenic ability (production per mycelium mass), we can see M57 as the strain with a lower production which could be considered an interesting feature in case of the use of a *P. commune* in cheese production, as we will discussed later. On the other hand, there are some strains that showed maximum toxigenic strength (P5, P1, QP1, QLM2), with a production > 6 mg g^{-1} dry mycelium.

Comparison with other studies is not easy, for the different media/conditions/time of incubation and different units used. Gqaleni et al 1996 found a production in YES 15 days (25 ℃) higher than 3000 μg mL^{-1}. In our study we found amounts close to this (our plates were incubated 7 d). Alapont (2014) found a production of 1168.28 μg g^{-1} culture medium (Czapeck agar, 25 ℃/21 d; average of 24 strains of *P. commune*, the weight of the mycelium analyzed was not reported).

Le Bars (1979) found a production of 272 μg g^{-1} dry mycelium using a medium similar to YES, after 25 d/25 ℃ (average of 20 strains) by *P. camemberti*, the domesticated form of *P. commune*, which is a very low value comparing to most of our *P. commune* strains. This author also found a great influence of incubation time and temperature. Nevertheless, M57 could be of interest in cheese production, with a production of 106 μg g^{-1}, after 7 d, considering that the *P. camemberti* strains used in cheese production are producers of CPA, according to this author results, although the toxigenic strength should be further studied increasing incubation time. In Chapter IV we will discuss the production of CPA on cheese and the possible risks for the consumers.

6. CONCLUSION

The identification of *Penicillium* genus at the species level is still difficult regarding their similarity between species. Other studies about identification and new methods are necessary to increase the fungi database and give more reliable information. Although in our study only a few strains were not possible to distinguish by DNA barcoding, the phenotypical analysis is still one of the best options to use together with molecular analysis. The presence of mycotoxigenic molds on the surface of the investigated cheeses is of concern, in fact, there are studies about CPA, OTA, and PAT in cheese but the levels of mycotoxin that could be harmful to the consumer are unclear.

The investigation of mycotoxin-producing genes was not reliable enough to identify the mold only for the mycotoxin they may produce but it could be useful to rapidly identify the presence or not of mycotoxigenic mold in food, helping in mycological safety and quality of foods.

P. commune (*P. biforme*) is indeed the most common mold isolated in cheese. Besides, these mycotoxigenic strains isolated from unspoiled and spoiled cheeses were CPA producers, with some isolates being good producers. This information is extremely important given the mycotoxin present in food consists of health hazards for consumers.

Our results demonstrate the potential given by those *P. commune* strains to produce such amount on cheeses.

On the other hand, the *Penicillium* found in this study could be of interest to the dairy industry since their mycotoxin production was medium to low, compared to the other *P. commune* (not isolated in this study) and the lipolytic and proteolytic activity from molds are of great interest to the food industry. Further analysis is required to ensure the domestication of these strains to be used as cheese-making fungal cultures and also the evaluation of their technological properties.

7. REFERENCES

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Chapter IV.

LACTOBACILLI EFFECT ON *PENICILLIUM COMMUNE* AND *PENICILLIUM NORDICUM* GROWTH AND MYCOTOXIN PRODUCTION *IN VITRO* AND IN CHEESE

1. INTRODUCTION

Cheese is an excellent substrate for mold growth and its deterioration is a critical concern for dairy industry. Incidence of molds in cheese is mainly attributable to *Penicillium* genera, although it can be contaminated by a wide variety of molds. Among the spoilage mold species isolated from cheeses and even dairy facilities, *P. commune* represents one of the most isolated species (Marín et al., 2015; Garnier et al., 2017; Jahn et al., 2017; Ramos-Pereira et al., 2019).

The major problem about the presence of spoilage molds in dairy products is the resulting defects that include discoloration (visible surface growth of mold) and off-flavors, which represents economic losses and negative impact on sensory properties. The control of fungal contamination is particularly important to also avoid the occurrence of mycotoxins (Stark, 2007; Dalié et al., 2010; Batt and Tortorelo, 2014).

Mycotoxins are not directly essential for fungal growth; they naturally occur as a defense of the producer strain within complex ecosystems (Magan and Aldred 2007; Fox and Howlett 2008). Once mycotoxins are present in food, decontamination may be almost impossible to achieve and some of them are known to be resistant to industrial processing (Kabak, 2010). Most species belonging to *Penicillium* genus associated with spoilage cheeses are producers of mycotoxins. The mycotoxins common isolated from spoilage cheeses are: mycophenolic acid, roquefortine C, PR toxin, isofumigaclavine A, penitrem, patulin, penicillic acid, ochratoxin A and cyclopiazonic acid (Sengun, 2008).

Cyclopiazonic acid (CPA) is a potent mycotoxin that causes necrosis, renal and hepatic disorder, ability to be immunotoxic and mutagenic which were reported in several animal species (Ostry et al., 2018). Although no direct evidence of toxicity has been described in humans but its presence in cheeses has been confirmed (Le Bars, 1979; Schoch et al., 1983; Zambonin et al., 2001; Ansari and Häubl, 2016). CPA can be produced by *Aspergillus* and *Penicillium* species, being *P. commune* and *P. palitans* major producers of CPA within *Penicillium* genus (Moretti and Susca, 2017).

Controlling the contamination of spoilage molds in dairy foods is not an easy task, but it can be achieved through several approaches including: cleaning and sanitation of the processing facilities, control of air quality and use of protective cultures, preservatives and packaging strategies (Martin et al., 2020). Good manufacturing practices is an important strategy that reduces the entry of spoilage molds during the processing of cheeses, but once introduced into the product, it won't work to inhibit mold growth (Buehler et al., 2019). In this case, the use protective cultures (biopreservation),

packaging strategies or even the use of preservatives will be more efficient to control them (Martin et al., 2020).

Therefore, the food industry is concerned about the demand of consumers for natural and preservative-free products. Because of that, they are interested in new strategies that involves the application of natural antimicrobial microorganisms such as lactic acid bacteria (LAB) in food to naturally preserve the product from spoilage microorganisms.

LAB strains are usually used in fermented products as starter cultures and their beneficial and functional importance is associated mainly with their metabolism. Besides the production of organic acids, they also produce other compounds in which some them have antimicrobial properties. Because of that, they are potentially effective as natural substitutes for chemical preservatives (Sadiq et al., 2019).

In Chapter II we have investigated the ability of *Lactobacilli* isolates from Brazilian Artisanal Serrano cheese to inhibit mycotoxigenic and spoilage *Penicillium* sp., which resulted in a selection of two *L. plantarum* strains (L119 and L49). To follow up the investigation, the aim of this work was to evaluate the impact of the application of *Lactiplantibacillus plantarum* on *Penicillium* growth and mycotoxin production *in vitro* and on semi-hard cheese slices.

2. OBJECTIVES

- Quantify the inhibitory activity of two selected *Lactiplantibacillus plantarum* isolates (L119 and L49) on the growth and production of mycotoxins (CPA and OTA) by *Penicillium nordicum*-M32 and *Penicillium commune*-M35 *in vitro* (on agar media);
- Analyze the effect of L119 and L49 on the morphology of *Penicillium nordicum*-M32 and *Penicillium commune*-M35 on agar plates by using Scanning Electron Microscopy (SEM);
- Evaluate the ability of L119 to inhibit the growth and production of mycotoxins (CPA) by *P. commune*-M35 on cheese slices;

3. MATERIALS AND METHODS

As seen in Chapter II, *L. plantarum*-L119 and L49 showed the best results regarding the antagonistic activity against *P. commune*-M35 and *P. nordicum*-M32 on overlay method. In this Chapter, the effect of L119 and L49 on mycelial growth and

mycotoxin production by *P. commune*-M35 and *P. nordicum*-M32, in YES agar and cheese slices was evaluated.

3.1. Effect of *L. plantarum*-L119 and L49 on Growth and Mycotoxin Production by *P. commune*-M35 and *P. nordicum*-M32 on Agar Media

The effect of the L119 and 49 in growth and production of mycotoxins of *P. commune*-M35 and *P. nordicum*-M32 was evaluated using Gqaleni et al. (1996) and Taniwaki et al. (2006) methodology with some modifications (Figure IV-1).

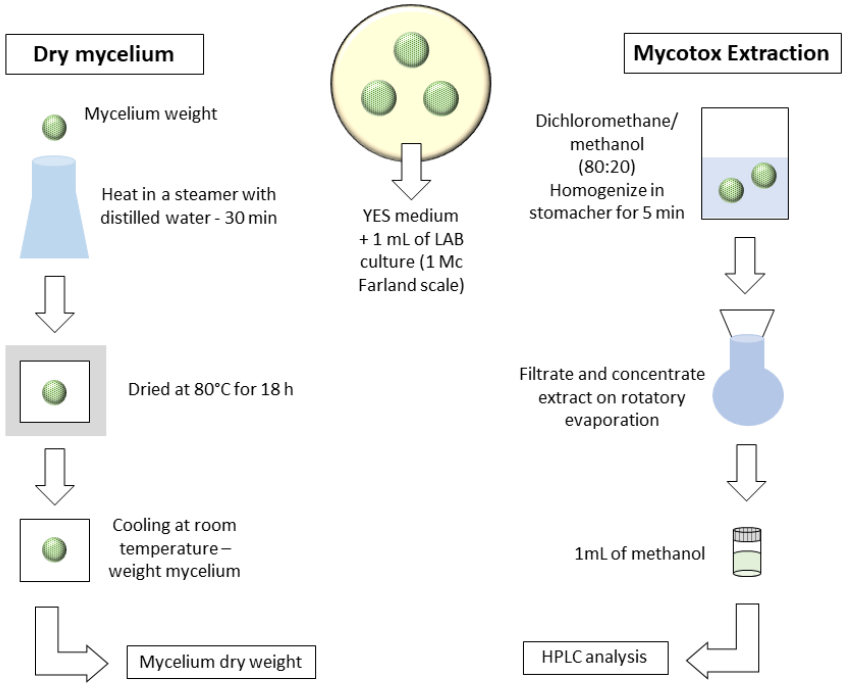

Figure IV-1.Extraction and quantification of mycotoxin and mycelium dry weight protocol in the presence of LAB strains.

3.1.1. LAB and Mold Growth Conditions

One milliliter of L119 and 49 cultures in Mc Farland 1 scale was added to 19 mL of melted YES agar. One microliter of spore suspension was taken and seeded in three different spots on the YES plates with *L. plantarum* strains. For the control group, LAB was not added in YES medium. Plates were incubated at 25℃ for 7 days.

3.1.2. Mycelium Dry Weight

The dry mycelial weight was determined according to Taniwaki et al. (2006). A colony and surrounding agar were cut from the Petri dish, transferred to a beaker with

distilled water (50 ml), then heated for 30 minutes to melt the agar. The mycelium, which remained intact, was transferred to a heavy filter paper and dried in an oven at 80 ℃ for 18h. After cooling to room temperature in a desiccator, the filter papers and mycelium were weighed and the dry weight was calculated by difference.

3.1.3. Extraction and Quantification of Mycotoxins

The extraction and quantification of CPA and OTA were carried out according to Gqaleni et al. (1996). Two colonies were mixed with 50 mL of the solvent (dichloromethane: methanol, 80:20 v:v) using the Stomacher, then it was filtered on filter paper with 1 g of anhydrous sodium sulfate and then it was concentrated by evaporation in vacuo. The final extract was resuspended in 1 mL of methanol, filtered (nylon filters, 0.22 µm pore size, Pall Life Sciences Corporation) and analyzed by HPLC. All samples were made in duplicate.

3.1.3.1. *HPLC analysis*

The extracts were analyzed by HPLC at the Institute of Food Science and Technology (ICTAL) of the University of León.

Equipment: Waters 2690 (Waters 2487 Dual Absorbance Detector, Waters 474 Scanning Fluorescence Detector, Waters, Milford, USA). Supelco Ultrabase C18 column with a pore size of 5 µm (150 x 4.6 mm) (Sigma Merck). A linear gradient was applied (Frisvad, 1987): solvent A (water) and solvent B (0.05% trifluoroacetic acid in acetonitrile).

HPLC conditions: the initial percentage of B was 10%, which rose to 50% in 3 min, then 90% in 10 min, remained at 90% for 3 min, decreased again to 10% in 6 min and it was maintained at 10% in 1 min at a flow rate of 2.0 ml/min. CPA detection was performed at 254 nm (retention times were 32.6 min, respectively). The detection limits for OTA and CPA were, respectively: 1.30 and 0.83 µg mL^{-1}; repeatability, 88.21 and 90.41; linearity (R2), 0.9926 and 0.9995.

3.2. ANTIFUNGAL ACTIVITY: SCANNING ELECTRON MICROSCOPY (SEM) ANALYSIS

SEM was employed to visualize the morphology of *P. commune*-M35 and *P. nordicum*-M32 and the possible antifungal mechanism of L119 and L49 between them.

From a fresh culture (48 hours), LAB was inoculated as two lines on MRS agar and incubated at 30ºC for 48h. The plates were overlaid with 9 mL of PDA containing 10^4 spores mL^{-1} of each of the two species of *Penicillium* tested (*P. commune*-M35 and *P. nordicum*-M32), and were incubated at 25 ºC for 7 days, taking photographs at the end of the incubation period (Magnusson and Schnürer, 2001).

Agar plugs (after 7 days) were cut out from the antifungal activity assay. The samples were fixed by immersion in 2.5% (v/v) glutaraldehyde in 0.1M sodium cacodylate buffer pH 7.2 overnight at 4 ℃. Them, washed three times in 0.1M sodium cacodylate buffer pH 7.2 for 15 min per change, post-fixed with 1% (m/v) osmium tetroxide for 30 min and washed three times again in 0.1 M sodium cacodylate buffer pH 7.2. The samples were then dehydrated in a graded ethanol series (30%, 50%, 70%, 90% for 15 min and 100% three times for 15 min) and transferred to a critical point dryer process with carbon dioxide (BALTEC CPD 030 Critical Point Dryer). Finally, the agar plugs were mounted on aluminum stubs, with a double-stick carbon tape and coated with gold (BALTEC SDC 050 Sputter Coater) and observed under a FEI Quanta 200 scanning electron microscope at Laboratory of Electron Microscopy and Microanalysis (LMEM) at Londrina State University (UEL).

3.3. Effect of *L. plantarum*-L119 on Growth and Mycotoxin Production by *P. commune*-M35 in Cheese Slices

This analysis consisted in evaluating the ability of the selected *L. plantarum*-L119 to inhibit *P. commune*-M35 in a challenge test using slices of semi-hard cheese. The experiments were based on Cheong et al. 2014 (modified). The L119 were cultivated in the MRS broth for 48 h at 30 ℃. After incubation, 2 mL were centrifuged for one minute to obtain bacterial cell pellets. The supernatant was discarded and the bacterial cells were resuspended in peptone water (0.1%), to obtain a standard number of cells using Mc Farland standard as reference. Each LAB suspension was estimated to contain $1.5x10^9$ CFU mL^{-1} (4 McFarland standard).

The spore suspension was prepared from 5-7 days culture at 25 ℃ in MEA medium (Malt Extract Agar, OXOID) in a slant agar bottle by adding 5 ml of a sterile 0.05% Tween 80 solution. The spore counts were made in a Neubauer chamber and the dilutions in peptone water until containing approximately 10^7, 10^5 and 10^3 spores mL^{-1}. The final concentration on the cheese slices was approximately 10^5, 10^3 and 10 spores g^{-1}, respectively.

Commercially cheese slices of semi-hard cheese weighting 12 grams were inoculated with 1 mL of L119 suspension on the surface and mixed with a sterile *drigalski* spatula for one minute to ensure uniform distribution of bacterial cells. Three controls were prepared, the first was cheese without inoculation of L119 and mold, the second was cheese inoculated only with L119 and the third was cheese inoculated with *P. commune*-M35 in different concentrations. The petri dishes containing the cheese slices were incubated at 10 ℃ for two days and after that 0.1 mL of *P. commune* suspension was inoculated into each slice of cheese. The controls 1 and 2 were prepared by inoculating one mL of peptone water (0.1%). The plates were incubated at 10 ℃ with 85-90% humidity and photographs were taken every 7 days until mold growth had covered the slice of cheese.

3.4. Quantification of Cyclopiazonic Acid on Cheese Slices

The *P. commune* strains used were M35 and M57 (originally isolated from spoiled cheese) and P5 (from an unspoiled cheese). To obtain a parameter of the amount of CPA production by *P. commune*-M35, two cheese isolates that were previously classified as high (*P. commune*-P5) and low (*P. commune*-M57) CPA producers on Chapter III were included in the study. One gram of cheese with 28 days of incubation containing 10^3 spores mL^{-1} of *P. commune*-M35 were selected for the extraction and quantification of CPA. The method used was described by Ansari and Haubl (2016) with some modifications.

The cheese sample was mixed with acetonitrile + 1% formic acid (1:10) using the Stomacher, then the sample was shaken on an orbital shaker for two hours at 30 rpm, centrifuged for 5 minutes at 10,000 rpm, and concentrated in vacuum rotary evaporation. The final extract was resuspended in 1 mL of methanol, filtered (nylon filters, 0.22 µm pore size, Pall Life Sciences Corporation) and analyzed by HPLC (see 3.1.3.1.). All samples were made in duplicate. The cheese slices and inoculation of *P. commune*-P5 and *P. commune*-M57 were prepared as the description of 3.3 item. The spore suspensions were also evaluated in three different concentrations (10^7, 10^5 and 10^3 spores mL^{-1}).

4. RESULTS

4.1. EFFECT OF *L. PLANTARUM* STRAINS ON GROWTH AND MYCOTOXIN PRODUCTION BY *P. COMMUNE*-M35 AND *P. NORDICUM*-M32 ON AGAR MEDIA

As it is shown on Figures IV-2 and IV-3, both *L. plantarum*-L119 and L49 produced a visible reduction effect on mycelium growth of *P. commune*-M35 and *P. nordicum*-M32; besides, the sporulation did not occur (white colonies).

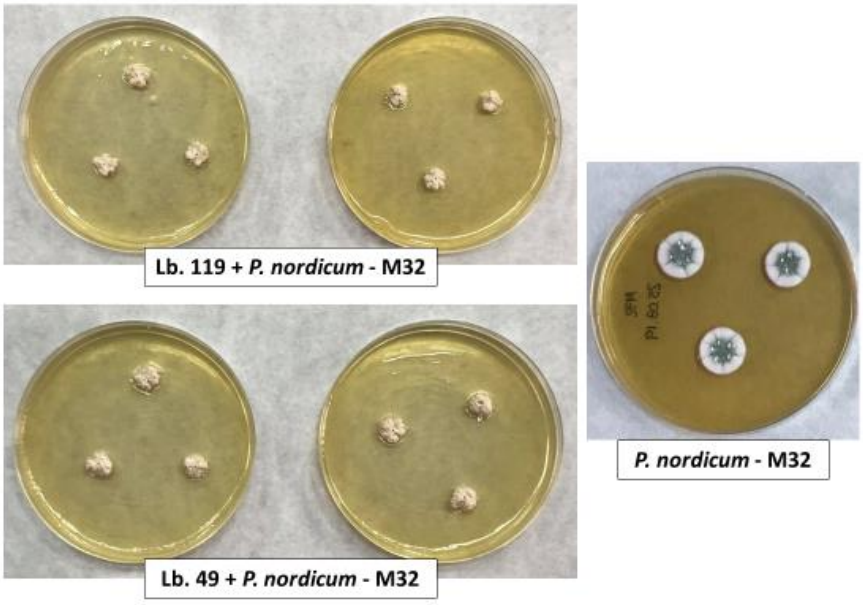

Figure IV-2.Growth inhibition of *P. nordicum*-M32 on YES agar (25 ℃/7 d) in the presence of *L. plantarum*-L119 and L49 strains.

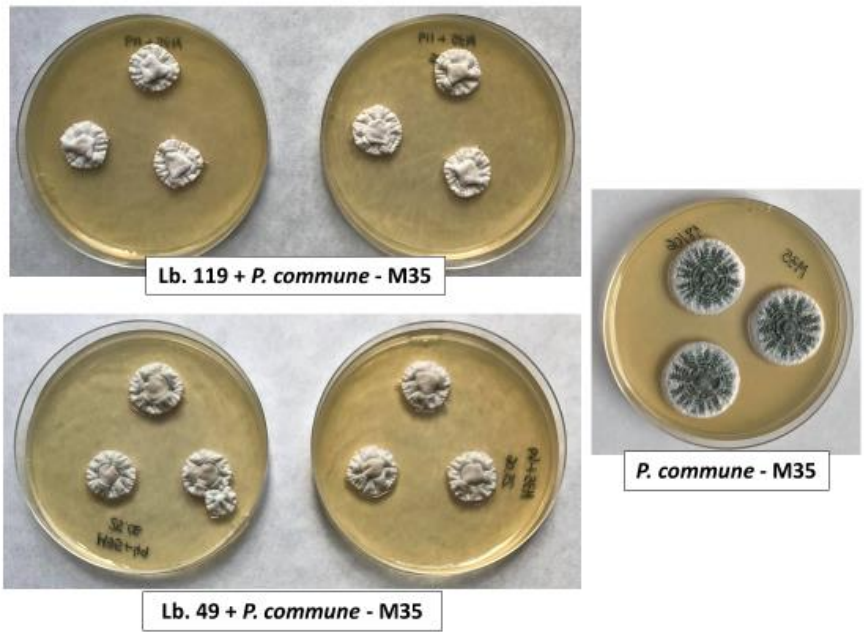

Figure IV-3.Growth inhibition of *P. commune*-M35 on YES agar (25 ℃/7 d) in the presence of *L. plantarum*-L119 and L49 strains.

The presence of L119 in YES medium reduced in 41.87% the dry mycelium weight of *P. commune*-M35 while L49 reduced in 53.68% (Table IV-1). Meanwhile, *P. nordicum*-M32 had its mycelium weight reduced in 82.70% by L119 and 75.20% by L49, compared to the control.

Table IV-1. Effect of *L. plantarum*-L119 and L49 on the growth and mycotoxin production by *P. commune*-M35 (CPA) and *P. nordicum*-M32 (OTA) on YES agar.

Sample	**Mycellium dry weight (g)**	**Growth Reduction (%)**	**Mycotoxin production (µg/mL ± SD)***	**Mycotoxin Reduction (%)**
			CPA	
Penicillium commune - M35	0.29	-	957.82 ± 76.16	-
L119 + M35	0.17	41.87	162.94 ± 17.68	82.99
L49 + M35	0.14	53.68	282.51 ± 3.80	70.51
			OTA	
Penicillium nordicum - M32	0.15	-	3508,40 ± 197.49	-
L119 + M32	0.03	82.70	2336,71 ± 40.87	33.40
L49 + M32	0.04	75.20	3086,67 ± 310.05	12.02

*Average and standard deviation of duplicates.

As for mycotoxin production, consequently, the amount of CPA and OTA found in the samples was reduced, although in a greater manner in the case of CPA. Thus, L119 reduced in 82.99% of the total production of CPA by *P. commune*-M35 while L49 reduced 70.51%. This indicates that apart from a reduction on the mycelium mass there is a reduction on CPA production. As for *P. nordicum*-M32, both strains reduced the production, although in a lesser extent (33.40 and 12.02%, respectively) than the CPA production. In addition, in this case, the effect on the growth of *P. nordicum* was much higher than on the OTA production (Table IV-1).

4.2. Antifungal Activity: Scanning Electron Microscopy (SEM) Analysis

SEM analysis revealed the effect of L119 and L49 on the morphology of hyphae and conidia. The control group had regular hyphal growth displaying tubular, smooth surfaced, elongated structures and conidia showed a typical ovoid shape (Figures IV-5a and IV-6a, IV-6c). The presence of L119 and L49 resulted in deformation of the hyphal structures for both *Penicillia*. The conidial surface was disrupted (Figure IV-6b). SEM also provided a decreasing germination of *P. commune*-M35 conidia in the presence of L119 and L49 (Figures IV-4b, IV-6b and IV-6d); in contrast any germination of *P.*

nordicum-M32 was observed (Figures IV-4d, IV-5b, IV-5d). The extended damage was observed to be very high in *P. nordicum*-M32.

Therefore, the antifungal activity showed by L119 and L49 was not only effective in restricting mycelial growth of both molds, but also morphological changes in hyphae and inhibition of sporulation, mainly in *P. nordicum*-M32 (Figures IV-5b and IV-5d). The two LAB caused prominent changes such as wrinkled and collapsed hyphae and the damage on the hyphae of *P. nordicum*-M32 was more severe than in *P. commune*-M35.

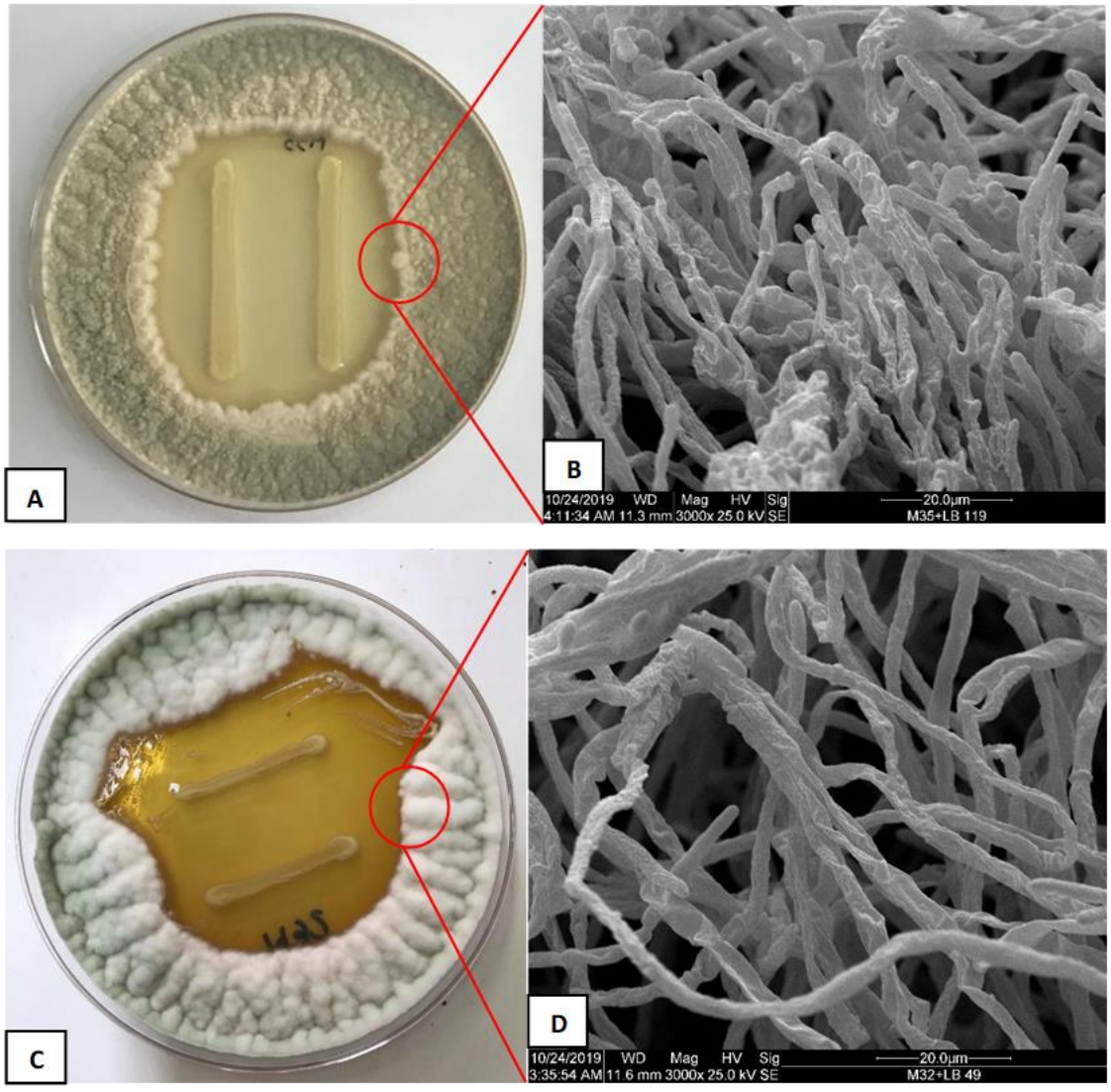

Figure IV-4. Antifungal activity of *L. plantarum* isolates against *Penicillium* sp. in overlay method analyzed by SEM. 1a. Antifungal activity of *L. plantarum*-L119 against *P. commune*-M35 on YES plates (7 d/25 °C). 1b. Scanning Electron Microscopy (SEM) of the growth inhibition of the same plate. 1c. Antifungal activity of L49 against *P. nordicum*-M32 on YES plates (7 d/25 °C). 1d. SEM of the growth inhibition of the same plate.

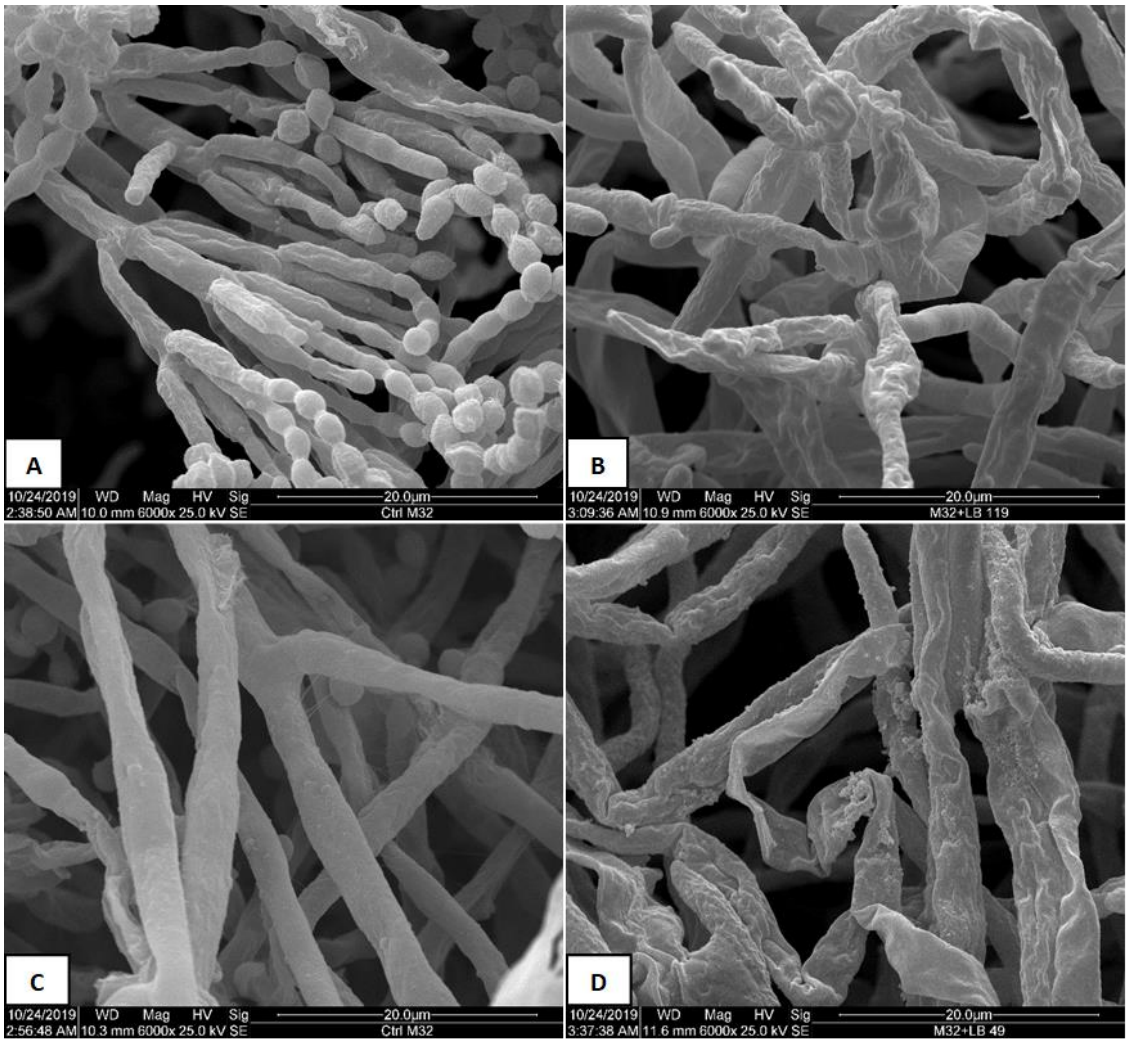

Figure IV-5.Effect of *L. plantarum*-L119 and L49 against *P. nordicum*-M32 observed by Scanning Electron Microscopy (SEM) after 7 days at 25 °C. 2a, c M32 control. 2b M32 + L119 and 2d M32 + L.49.

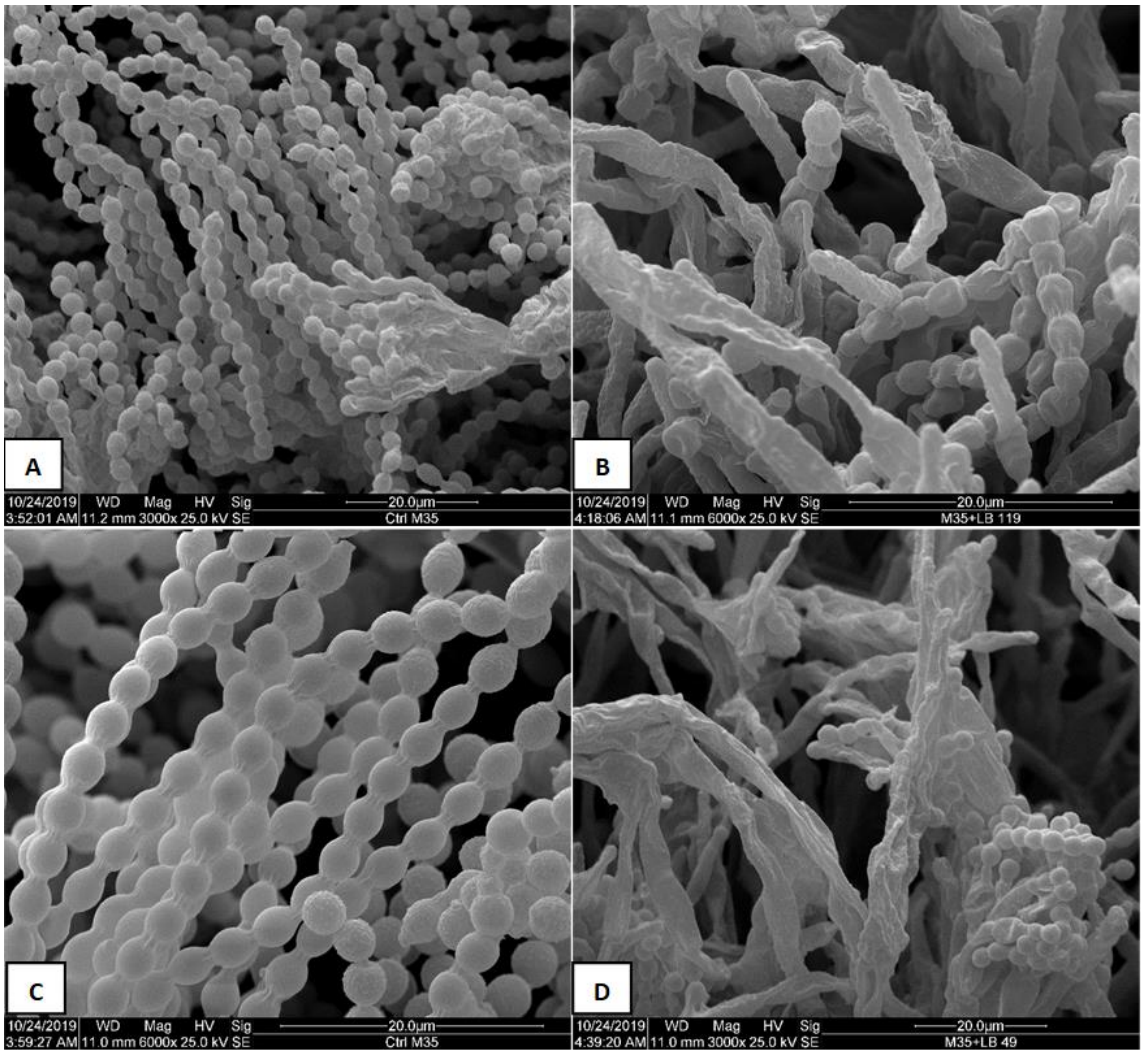

Figure IV-6.Effect of *L. plantarum*-L119 and L49 against *P. commune*-M35 observed by Scanning Electron Microscopy (SEM) after 7 days at 25 ℃. 3a, c M35 control. 3b M35 + L119. 3d M35 + L.49.

4.3. Effect of *L. plantarum*-L119 on Growth and Mycotoxin Production by *P. commune*-M35 in Cheese Slices

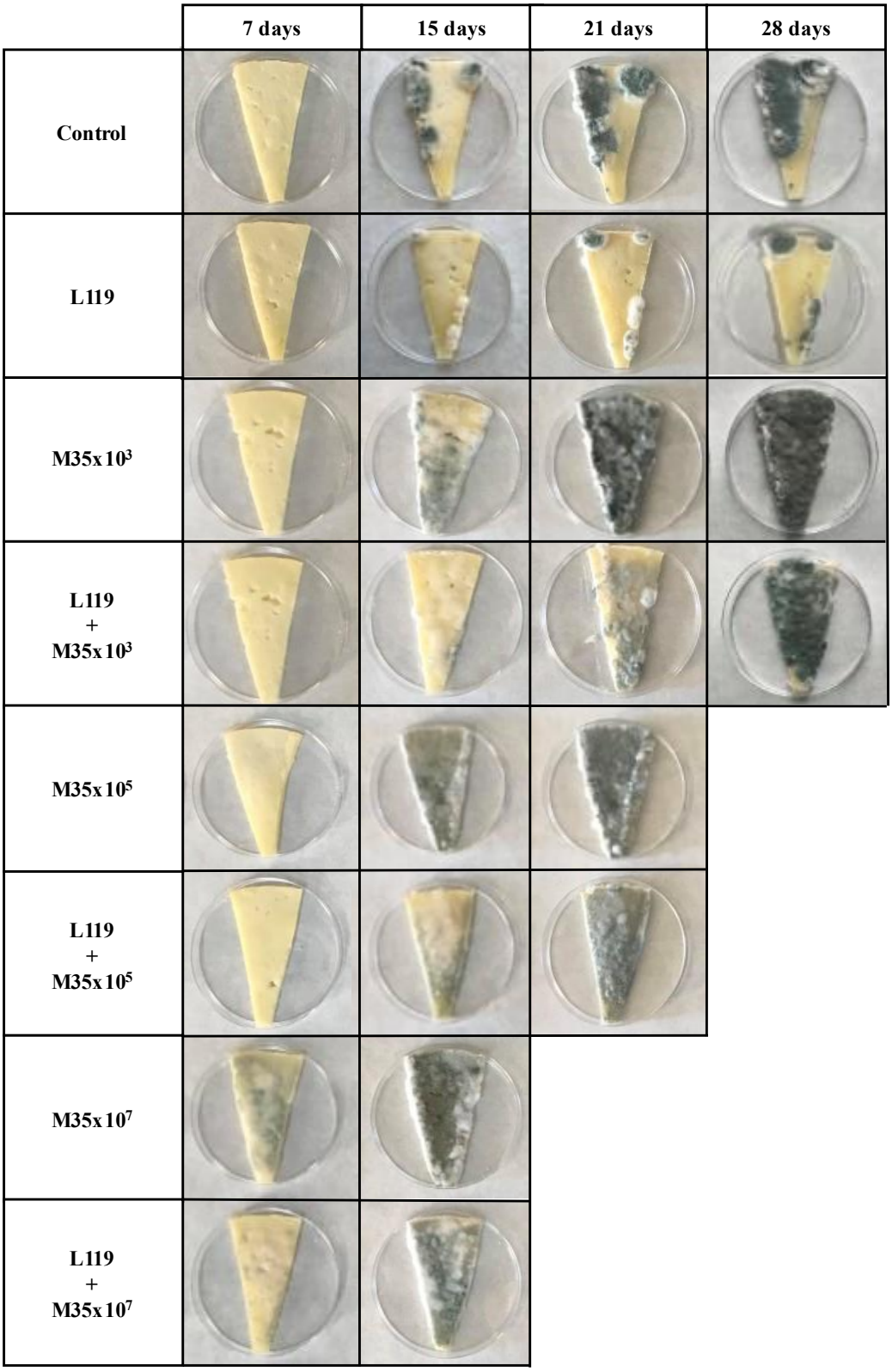

Figure IV-7. Photographs showing the antifungal effect of *L. plantarum*-L119 on cheese slices inoculated with different concentrations of *Penicillium commune*-M35 and not inoculated (control) at 10 °C at 4 stages.

The results of the challenge of *L. plantarum*-L119 against *P. commune*-M35 inoculated at different concentrations (10^3, 10^5 and 10^7 spores mL^{-1}) on slices of semi-hard cheese are shown in Figure IV-7 and Appendix I. At day 7, only *P. commune*-M35 at concentration of 10^7 spores mL^{-1} presented visible mold growth and in the presence of L119 it was less visible. At day 15 all samples presented visible mold growth, including the control samples (L119 inoculated).

The slices of semi-hard cheese without any inoculation (control) presented a visible mold growth starting at day 15 and *L. plantarum*-L119 inhibited this contaminant until the last day of the experiment (28 d) (Figure IV-7 and Figure 13 Appendix I). The presence of this contaminant mold (not inoculated) requires attention to the ease that cheeses can be contaminated by spoilage molds. This unidentified mold was probably industry contamination and, not surprisingly, L119 (row 2) showed signs of antifungal activity controlling mold growth.

Table IV-2. CPA Quantification by *P. commune* (M35; M57; P5) in semi-hard cheese slices and the effect of *L. plantarum*-L119 on CPA production by *P. commune*-M35 (28 d incubation at 10 ℃).

Strain	**CPA (mg kg^{-1})***
M35 × 10^{3**}	20.96 ± 2.24
M57 × 10^3	6.44 ± 0.00
M57 × 10^5	8.27 ± 0.25
M57 × 10^7	9.15 ± 1.17
P5 × 10^3	15.67 ± 0.89
P5 × 10^5	26.81 ± 0.72
P5 × 10^7	28.63 ± 1.89
Control (not inoculated mold)	nd
L119 (control LAB)	nd
L119 + M35 × 10^3	nd

nd, not detected.
*Average and standard deviation of duplicates.**Spores per mL.

As it is shown on Table IV-2, at a concentration of 10^3 spores mL^{-1} an amount of 20.96 mg kg^{-1} of CPA was produced by *P. commune*-M35, while at the same concentration *P. commune*-M57 and *P. commune*-P5 the production was much lower (6.44 mg kg^{-1} and 15.67 mg kg^{-1}, respectively). These results demonstrate that *P.*

commune-M35 is a high producer of CPA compared to the other strains of *P. commune* tested. In addition, we found the maximum production by *P. commune* P5 (at 10^7 spores mL^{-1}), and also the ability of L119 to inhibit CPA production on cheese could be confirmed.

5. DISCUSSION

5.1. Effect of *L. plantarum* Strains on the Growth and Mycotoxin Production by *Penicillium commune* on Agar Media

The current knowledge of the benefits of lactic acid bacteria allowed their use to improve the shelf life and safety of foods. Studies have already pointed out the great potential of lactic acid bacteria with antimicrobial effect. In the genus *Lactobacilli*, *L. plantarum* is one of the most studied strain that exhibits stronger inhibition against different targeted fungi (Cheong et al., 2014; Prema et al., 2010; Rather et al., 2013; Wang et al., 2011; Luz et al., 2017; Guimarães et al., 2018; Bukhari et al., 2020).

The screening results of antifungal activity of *Lactobacilli* strains obtained in Chapter II enable us to select the two strains with broad antimicrobial activity identified as *L. plantarum*-L119 and L49. These two strains, previously isolated from Brazilian Artisanal Serrano cheese, were evaluated against two spoilage molds found in cheese, *P. commune*-M35 and *P. nordicum*-M32, using a not conventional media for the growth of lactic acid bacteria (LAB), the Yeast Extract Sucrose (YES) Agar. As noted in Figure IV-2 and 3, the two *L. plantarum* strains remained active against the molds tested, causing inhibition on the germination and mycelial growth. Laref and Guessas (2013) obtained the same results testing strains of *Lactobacilli* against *Penicillium* spp. and some other fungi (*Aspergillus* spp., *Fusarium roseum*, *Trichoderma* spp., and S*temphylium* spp.).

A similar experiment was done by Kim (2005), where the two strains tested (*L. sakei* and *L. casei*) inhibited the mycelium growth of *Aspergillus fumigatus* in Potato Dextrose Agar (PDA) and in MRS broth. Muhialdin and Hassan (2011) also achieved the antifungal activity of the supernatant produced by *Limosilactobacillus fermentum*, *Levilactobacillus brevis* and *Pediococcus pentosaceus* on conidia and mycelium growth of *Aspergillus oryzae*, using the well-diffusion method. Besides the different methods used, our results, in accordance with other similar studies, demonstrated that L119 and L49 steal have inhibition activity on different media.

Regarding mycotoxin production, it is important to analyze it in experiments that evaluate interaction of LAB and mycotoxigenic molds, because mycotoxins are

secondary metabolites that can be produced as a response of a stress factor. Therefore, for a LAB to be considered a bio-control agent, it is expected to be able to control not only mold growth but also mycotoxin production. Analyzing the two *L. plantarum*-L119 and L49 which caused a reduction of more than 70% in mycelium weight of *P. nordicum*-M32, the presence of both LAB did not show such influence on OTA production. Our results are in agreement with those of Hassan and Bullerman (2008), who also reported a significant reduction on mycelium growth and mycotoxin production by *F. proliferatum*. El-Gazzar, Rusul and Marth (1987) testing different concentrations of lactic acid in growth media, showed that mycelium weight and mycotoxin production by *Aspergillus parasiticus* can be reduced depending on the concentration of the lactic acid.

Dogi et al. (2013) analyzing the interaction of *L. plantarum* and *F. graminearum* in different conditions (aw; pH; temperature and oxygen availability) found that the presence of LAB resulted in reduction of Zearalenone production in all the interacting conditions assayed, compared with the control. These findings are in agreement with our results where the two strains of *L. plantarum*-L119 and L49 were effective inhibiting the growth and mycotoxin production of *P. commune*-M35 and *P. nordicum*-32. Notably, L119 and L49 showed that both were capable to bio-control fungi *in vitro*.

5.2. Antifungal Activity: Scanning Electron Microscopy (SEM) Analysis

LAB compounds are known to have great antimicrobial effect and some of them are very elucidated while others are still no identified yet. Depending on the media LAB grow, it is hard to select one compound that has a great effect as antifungal compound. The results regarding mycelium growth and mycotoxin production showed the importance of evaluating the interaction between LAB and mold. Therefore, in our study, we used SEM analysis to show the interaction and the effect of L119 and L49 in both *Penicillium* strains.

The SEM observation showed a shriveled, crinkled cell wall, and flattened hyphae in both molds after being exposed to *L. plantarum* strains. This morphological alteration may explain not occurrence of sporulation and also mold growth surrounding LAB. Sangamanee and Hongpattarakere (2014) showed irreversible damage and morphological alteration on hyphal structures of *Aspergillus flavus* and *Aspergillus parasiticus* on exposure to 5.87 mg mL^{-1} of *L. plantarum* supernatant. Adebayo and Aderiye (2011) testing the effect of bateriocion brevicin against *Penicillium citrinum* resulted in inhibition growth, hyphal formation and mycelia damage. As suggested by Crowley et al.

(2013b), the antifungal substances produced by LAB induces a general cell metabolic shutdown culminating on mold growth arrest and Siedler, Balti and Neves (2019) say that all compounds together result in synergistic or additive effect.

5.3. Effect of *L. plantarum*-L119 on Growth and Mycotoxin Production by *P. commune*-M35 in Cheese Slices

In order to assess the real potential of *L. plantarum* for food preservation, L119 was selected based on the previously results obtained. Although our results did not focus on shelf-life, the presence of L119 resulted in a delay of 7 days in mold growth. Similarly occurred with Lynch et al. (2013) using *L. amylovorus* as adjunct culture in cheese production delayed *P. expansum* growth on Cheddar cheese in 4 days and when exposed to natural airborne fungi delayed in 6 days. While, Fernandez et al. (2017) obtained with *L. rhamnosus* a shelf-life of 12 days longer in cottage cheese contaminated with *P. chrysogenum*. Sedaghat et al. (2016) obtained a delay of 19 and 22 days on mycelia growth of *A. flavus* and *A. parasiticus*, respectively, with the application of *L. plantarum* as fresh cheese starter culture. Cheong et al. (2014) prevented the growth of *P. commune* on cottage cheese for up to 20 days with *L. plantarum* inoculated.

In a different experiment, Guimarães et al. (2020) tested *L. buchneri* as an antifungal coating to control *P. nordicum* on cheese and no mold growth and OTA was detected. Interestingly, our study also demonstrated that *P. commune*-M35 was not able to produce CPA at the end of the experiment (28 days) but L119 did not completely inhibit mold growth. Probably, the different techniques used regarding the antifungal potential of LAB may obtain different results focused on growth inhibition. Another point, few studies evaluate mold growth and mycotoxin production which difficult comparison with other studies.

As for CPA production on cheese, it increased with the spore concentration inoculated, and comparing the three strains tested (M35, M57 and P5), at 10^3 spores' concentration, it ranged from 6.44 (M57) to 20.96 mg kg^{-1} (M35) (Table IV-2). These amounts are much higher than those found by different authors in white cheeses (reviewed by Burdock and Flamm, 2000), where Le Bars (1979) found, for *P. camemberti*, only 0.05-1.5 mg kg^{-1}; while Schoch et al. (1983, cited by Weidenbörner 2008) found 80-370 µg kg^{-1} in mold ripened cheeses; and Zambonin et al. (2001) found 20-80 µg kg^{-1} in white surface Italian cheeses. Ansari and Haübl (2016) found as much as 3.7 mg kg^{-1} in Camembert cheese, a value closer to our results. On the other hand, Finoli et al. (1999),

analyzed Taleggio cheese, an Italian variety with different *Penicillium* spp. growing on the surface (among them, *P. commune*), and found a maximum CPA concentration of 0.25 mg kg^{-1} (in this case, the crust is not eaten).

As for the risk assessment, according to Burdock and Flamm (2000), the Acceptable Daily intake (ADI) for humans is approximately 10 µg kg day^{-1} or 700 µg day^{-1}. In the context of human exposure, for a maximum of 28.6 µg g^{-1} found in our study, considering a consumption of 50 g of cheese daily, this allows an intake of 1430 µg that is double the ADI (Burdock and Flamm, 2000). In the Spanish market, particularly rural or local, there are artisanal or semi-industrial cheeses covered with a blue coat. We can assume, according to our results, that *P. commune* may be the responsible for this blue coat. For this reason, consumers should be advised to avoid the consumption of the crust/rind of these cheeses or remove at least 2.5 cm below (Dobson, 2017). We also encourage the use of cultures of *P. commune* with a low toxigenicity strength, such as M57 (in this case, with a yield of 6.44 µg g^{-1}, the ADI is not overcome). We will continue the study of this particular strain and its possible use as a fungal culture.

6. CONCLUSION

This study demonstrate that *Penicillium* spp. and mycotoxin production was reduced in the presence of the two *L. plantarum* strains (L119 and L49), confirming their ability to inhibit mycotoxigenic mold. In addition, L119 was capable to inhibit mycotoxin production and reduce *Penicillium* sp. growth on cheese slices. L119 is a good candidate to be used as biopreservative in dairy products, for that, further analyses should be investigated such as the impact of the strain on product organoleptic properties.

Finally, the presence of CPA in cheeses slices was found, and a risk assessment was performed, showing the need of preventing the consumption of cheese with uncontrolled *P. commune* growth and either recommending the use of selected strains.

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CONCLUDING REMARKS

This thesis presents the antifungal properties of *Lactobacilli* against *Penicillium* sp. and also confirms the ability to inhibit common pathogenic bacteria found in milk. The overall conclusion from this study is that the two strains of *L. plantarum* seem to be able to inhibit *Penicillium* spp. growth by a set of factors and other compounds than lactic acid such as phenyllactic acid could be involved in. However, it must be considered that the substances produced by LAB probably act synergistically and that other metabolic products could also contribute to the overall inhibition. The inhibition mode of action has not yet been established and further work on this topic is desirable.

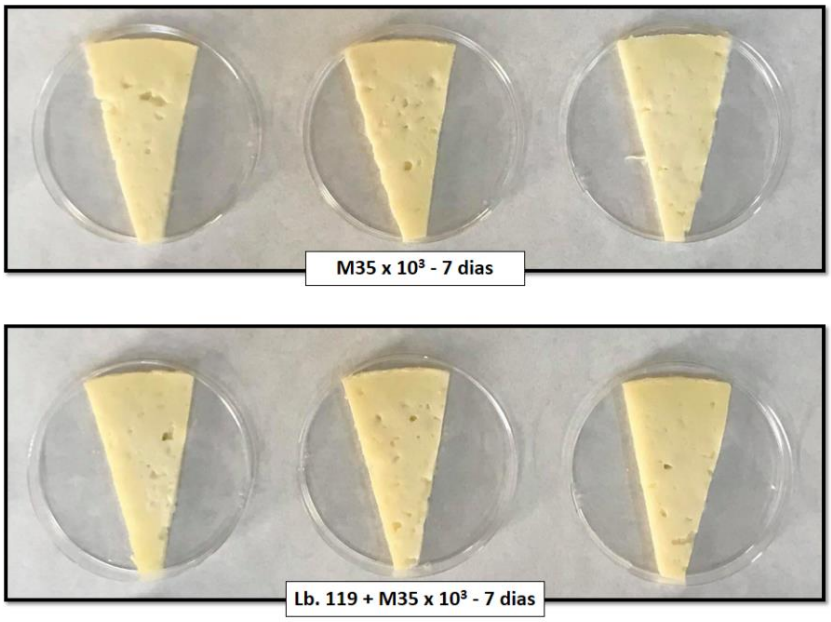
Owing to the importance of *Penicillium* for the dairy industry both for their applicability in cheese-making and also to avoid spoilage, we isolated and identified strains from this genus present on semi-hard Spanish cheeses made in Castilla and León (Spain). Studies regarding this topic are essential for the food industry because through this study we could identify the dominant species (*P. commune*/*P. biforme*) and also their ability to produce mycotoxins *in vitro* and in cheese. We consider that some strains present in cheese can cause harm to the consumer. On the other hand, some wild *Penicillium commune* produce low concentrations of mycotoxin which could be of interest for dairy industry as cheese-making fungal cultures. It is notably that some molds are indispensable for their applicability in dairy industry so, with these findings we can advise consumers to remove the cheese rind before ingestion.

To verify the ability of the *L. plantarum* strains to inhibit *Penicillium* growth and mycotoxin production, we set up a methodology where LAB and *Penicillium* sp. were co-cultivated *in vitro* and in cheese. This part of the experiment shows two important conclusions: first, our *L. plantarum* strains are capable of reducing *Penicillium* growth and mycotoxin production *in vitro* and completely inhibit mycotoxin production in cheese; and second, the ability of *Penicillium commune* producing mycotoxin in cheese which confirms our concern about wild mold growth in cheeses. These findings indicate that *L. plantarum* could be useful as a biopreservative agent in the cheese industry. It would be interesting to evaluate the application of *Lactobacilli* in other food to biocontrol undesirable microorganisms.

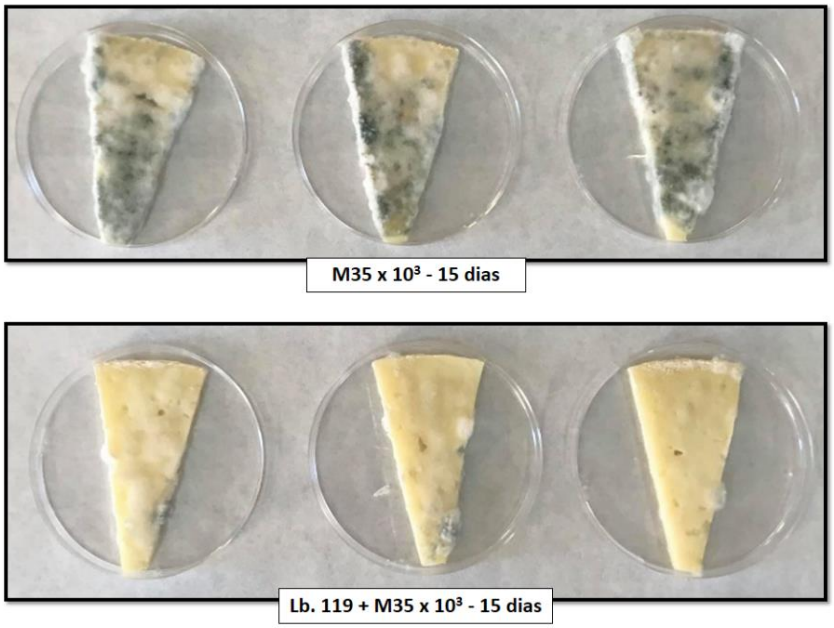
APPENDIX

Appendix I.

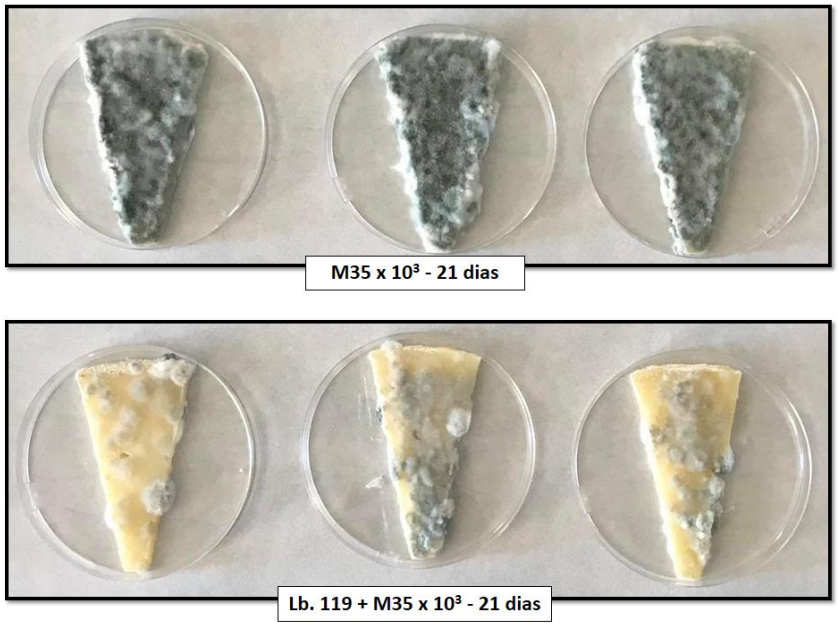
Photographs of the effect of *L. plantarum*-L119 on *P. commune*-M35 growth in cheese slices.



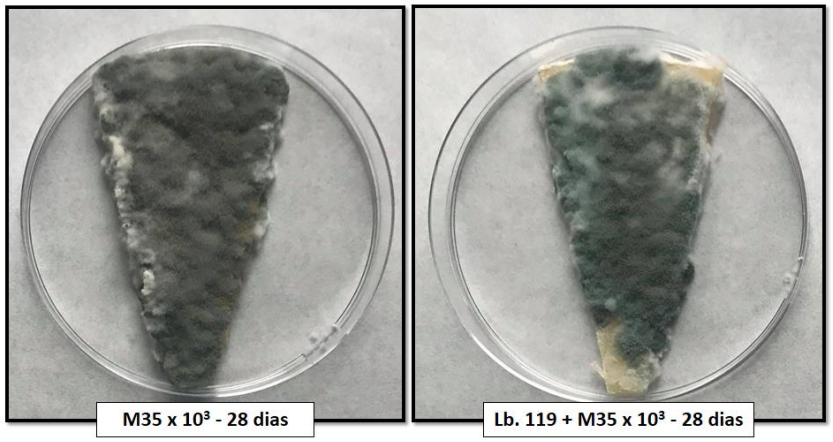
1. Antifungal effect of *L. plantarum*-L119 on cheese slices inoculated with *Penicillium commune*-M35 at 10^3 concentration of spores mL^{-1} compared to the mold control (no LAB) at 10 ℃ for 7 days.



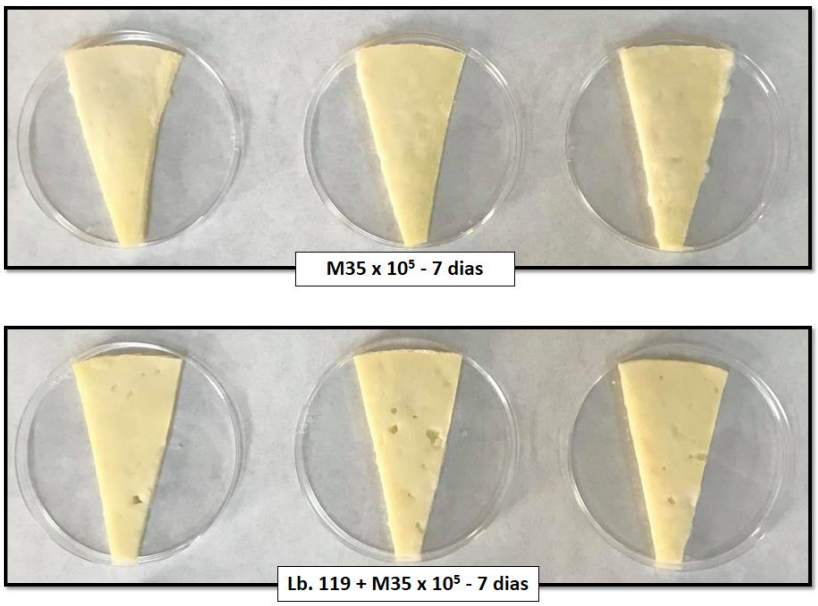
2. Antifungal effect of *L. plantarum*-L119 on cheese slices inoculated with *Penicillium commune*-M35 at 10^3 concentration of spores mL^{-1} compared to the mold control (no LAB) at 10 ℃ for 15 days.



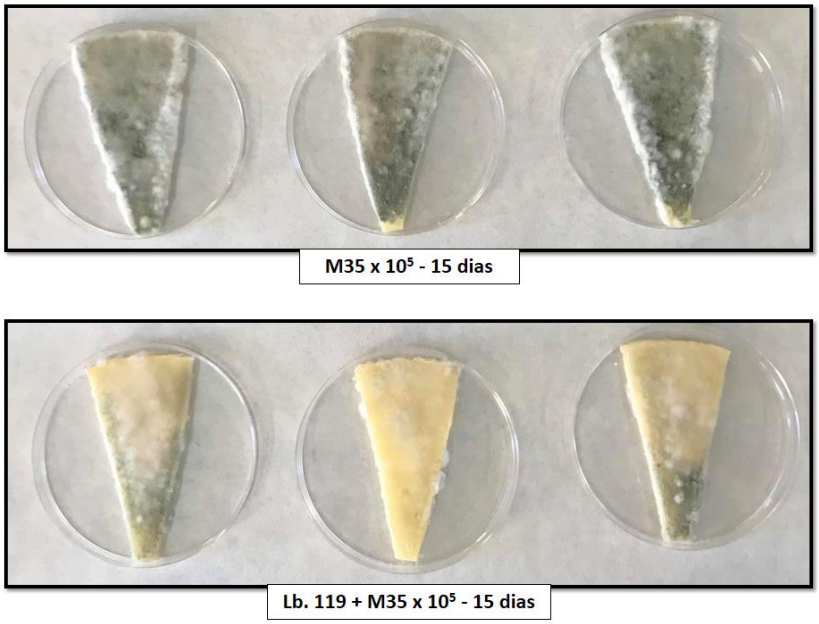
3. Antifungal effect of *L. plantarum*-L119 on cheese slices inoculated with *Penicillium commune*-M35 at 10^3 concentration of spores mL^{-1} compared to the mold control (no LAB) at 10 ℃ for 21 days.



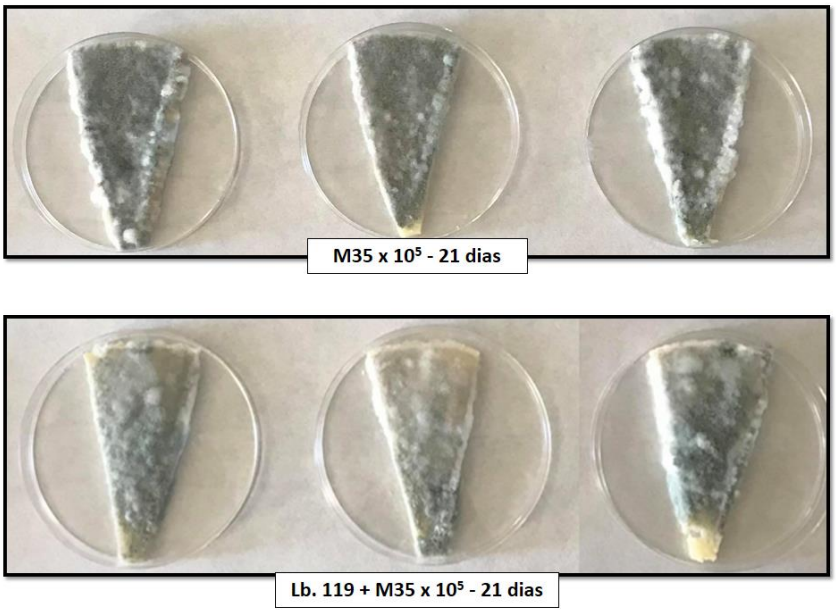
4. Antifungal effect of *L. plantarum*-L119 on cheese slices inoculated with *Penicillium commune*-M35 at 10^3 concentration of spores mL^{-1} compared to the mold control (no LAB) at 10 ℃ for 28 days.



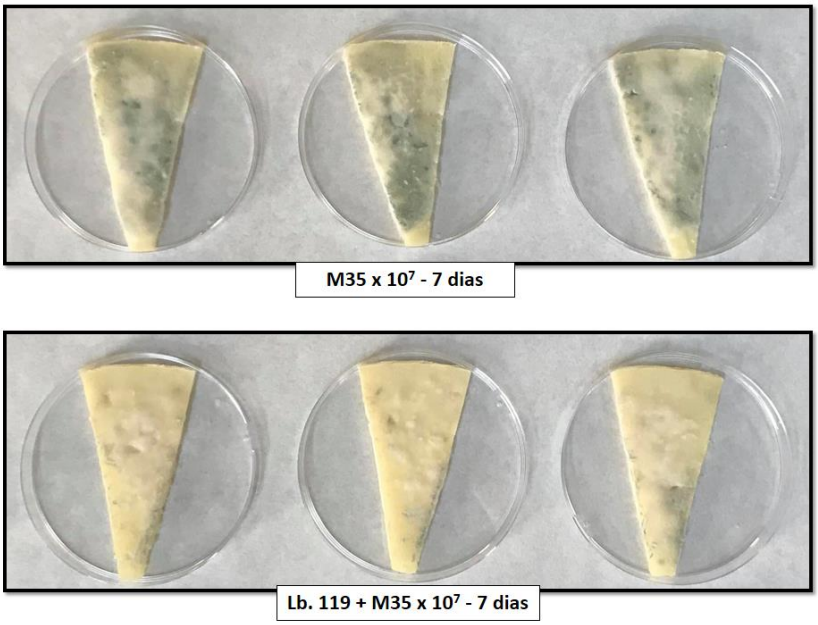
5. Antifungal effect of *L. plantarum*-L119 on cheese slices inoculated with *Penicillium commune*-M35 at 10^5 concentration of spores mL^{-1} compared to the mold control (no LAB) at 10 ℃ for 7 days.



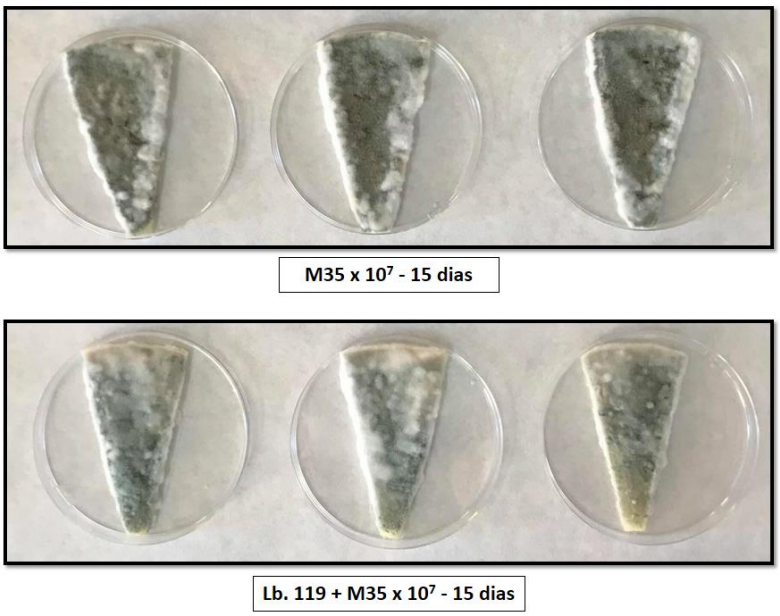
6. Antifungal effect of *L. plantarum*-L119 on cheese slices inoculated with *Penicillium commune*-M35 at 10^5 concentration of spores mL^{-1} compared to the mold control (no LAB) at 10 ℃ for 15 days.



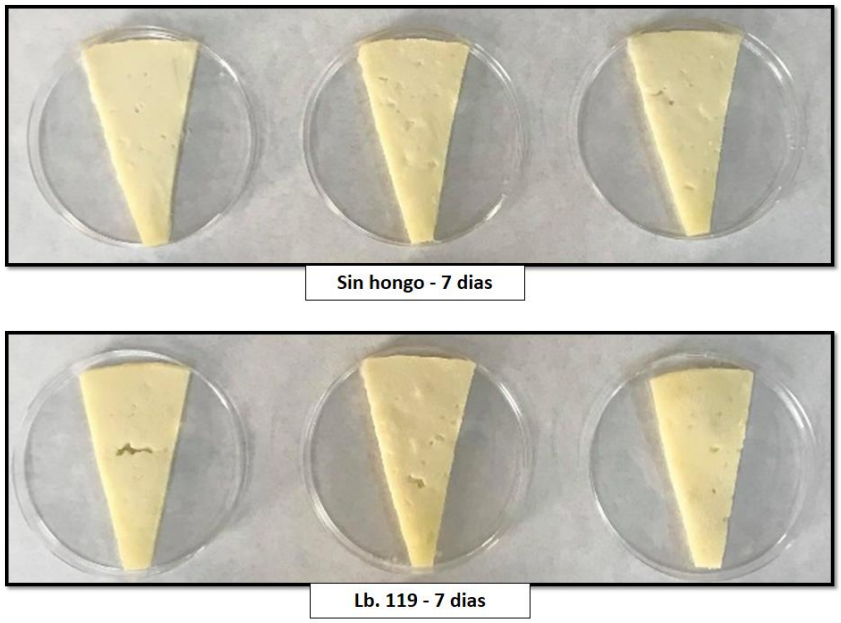
7. Antifungal effect of *L. plantarum*-L119 on cheese slices inoculated with *Penicillium commune*-M35 at 10^5 concentration of spores mL^{-1} compared to the mold control (no LAB) at 10 ℃ for 21 days.



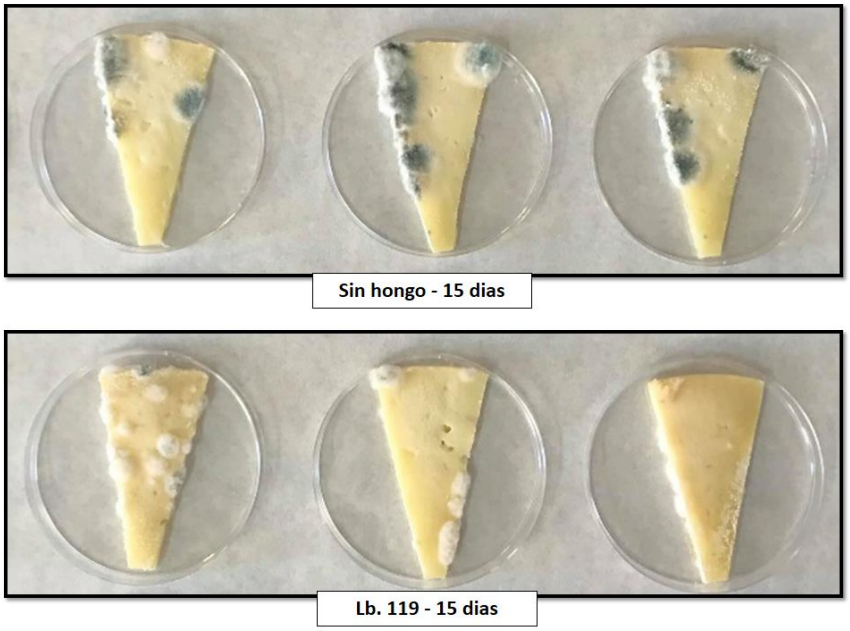
8. Antifungal effect of *L. plantarum*-L119 on cheese slices inoculated with *Penicillium commune*-M35 at 10^7 concentration of spores mL^{-1} compared to the mold control (no LAB) at 10 ℃ for 7 days.



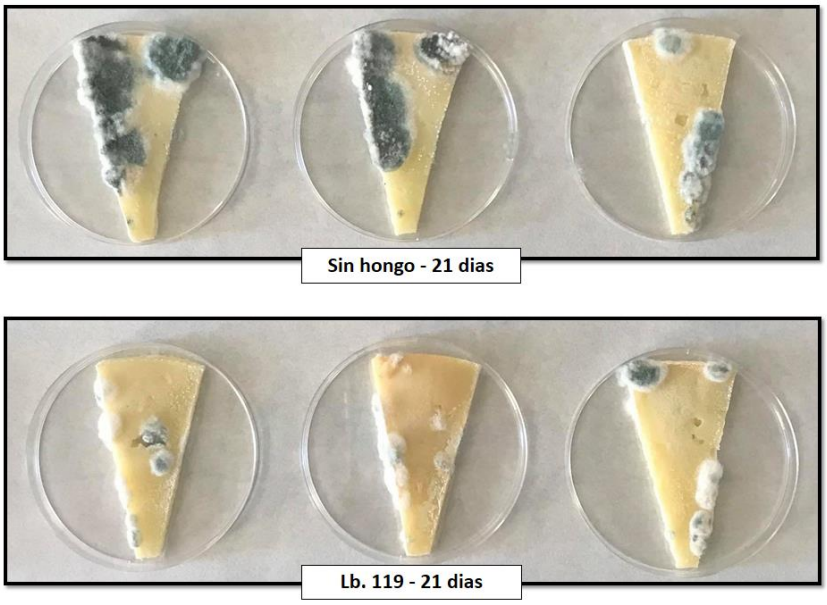
9. Antifungal effect of *L. plantarum*-L119 on cheese slices inoculated with *Penicillium commune*-M35 at 10^7 concentration of spores mL^{-1} compared to the mold control (no LAB) at 10 ℃ for 15 days.



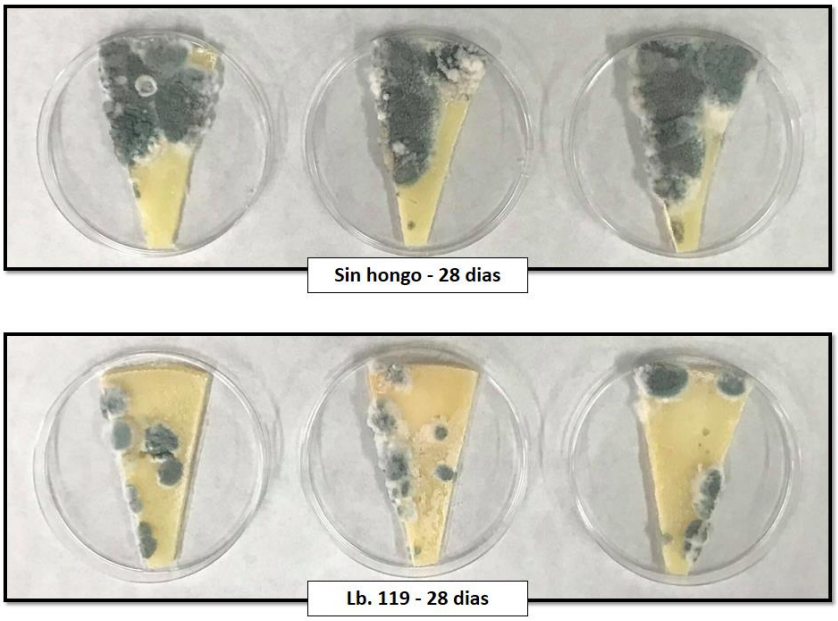
10. Antifungal effect of *L. plantarum*-L119 on cheese slices against contaminant mold (not inoculated mold) at 10 ℃ for 7 days.



11. Antifungal effect of *L. plantarum*-L119 on cheese slices against contaminant mold (not inoculated mold) at 10 ℃ for 15 days.



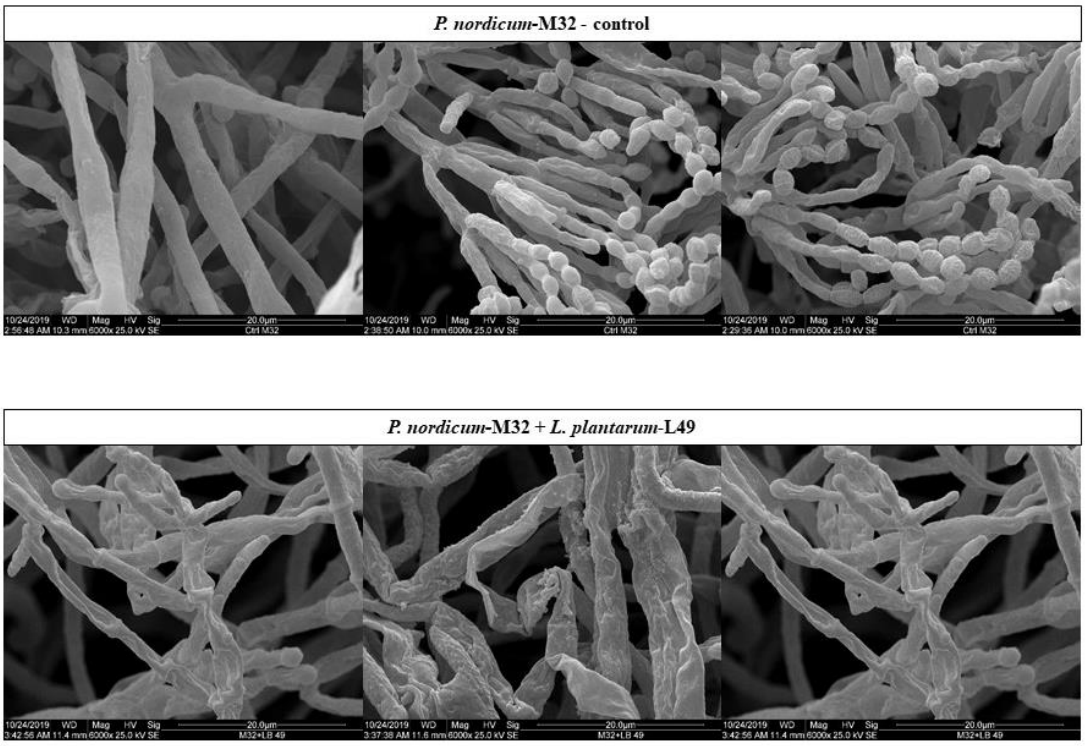
12. Antifungal effect of *L. plantarum*-L119 on cheese slices against contaminant mold (not inoculated mold) at 10 ℃ for 21 days.



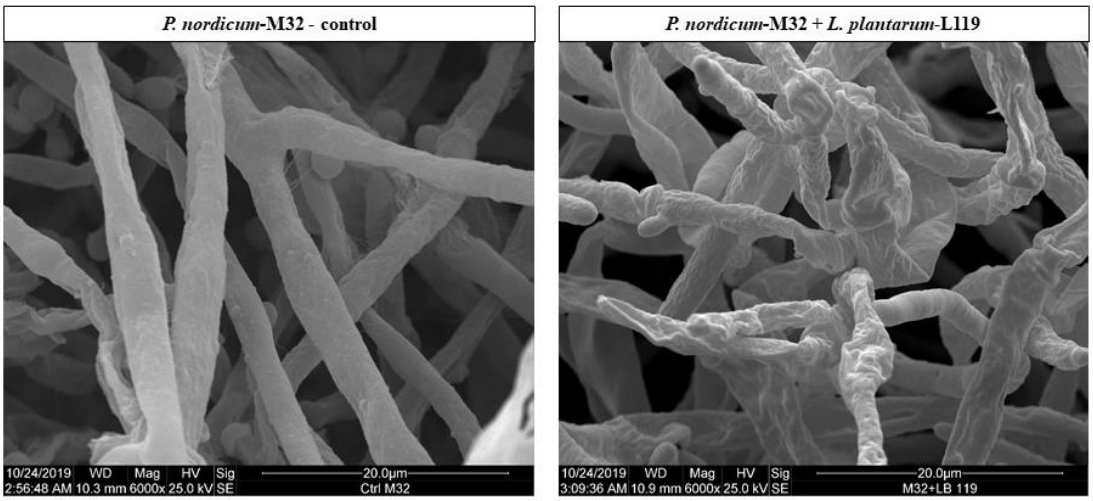
13. Antifungal effect of *L. plantarum*-L119 on cheese slices against contaminant mold (not inoculated mold) at 10 ℃ for 28 days.

Appendix II.

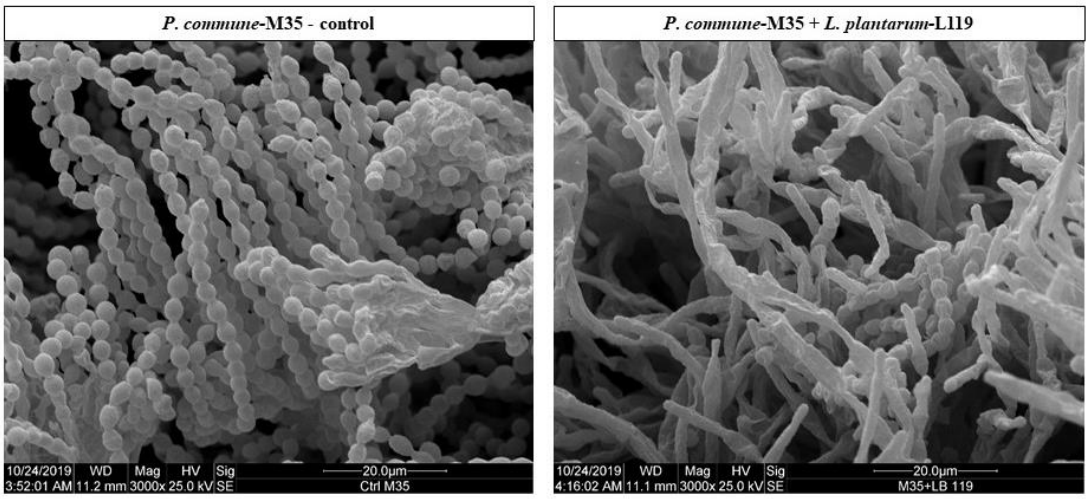
Photographs of Scanning Electron Microscopy (SEM) showing the effect of *L. plantarum* strains (L49 and L119) against *P. commune*-M35 and *P. nordicum*-M32 after 7 days at 25 ℃.



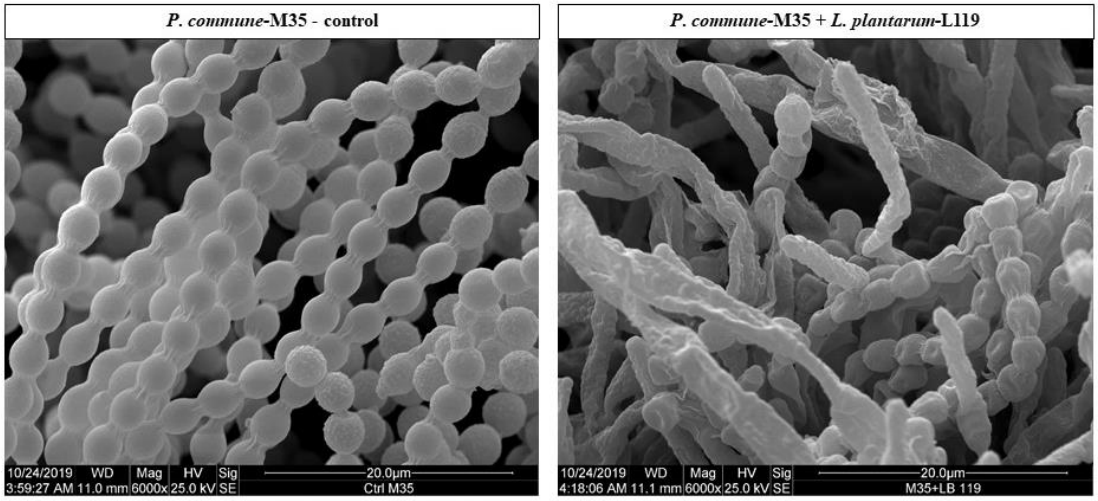
14. Effect of *L. plantarum*-L49 against *P. nordicum*-M32 observed by Scanning Electron Microscopy (SEM) after 7 days at 25 at 6000X magnification.



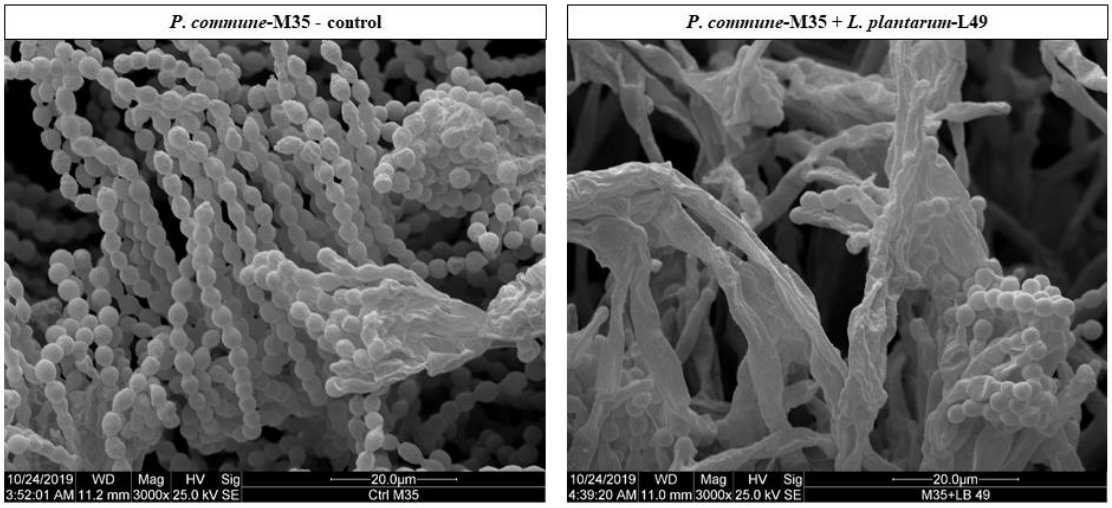
15. Effect of *L. plantarum*-L119 against *P. nordicum*-M32 observed by Scanning Electron Microscopy (SEM) after 7 days at 25 ℃ at 6000X magnification.



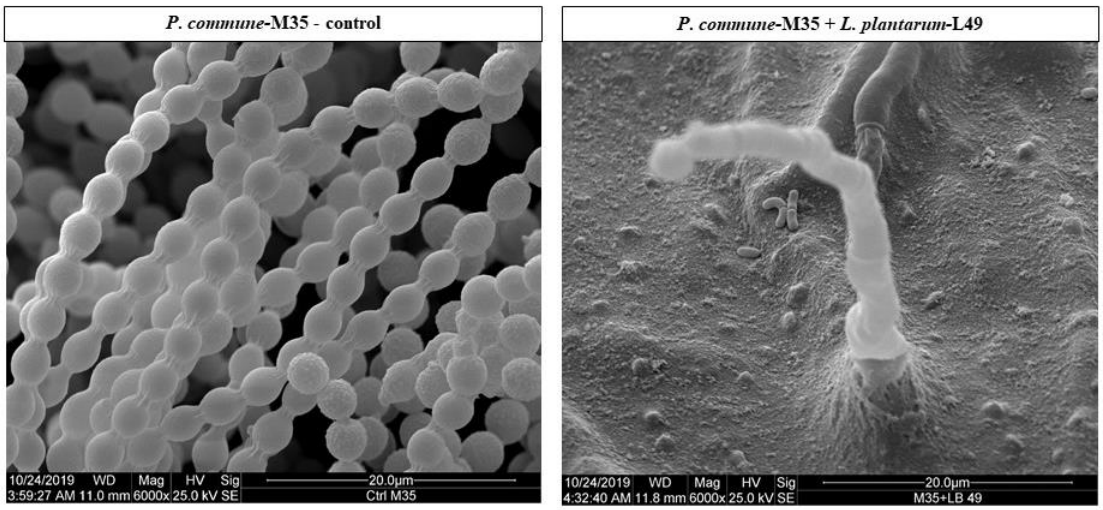
16.Effect of *L. plantarum*-L119 against *P. nordicum*-M35 observed by Scanning Electron Microscopy (SEM) after 7 days at 25 ℃ at 3000X magnification.



17. Effect of *L. plantarum*-L119 against *P. nordicum*-M35 observed by Scanning Electron Microscopy (SEM) after 7 days at 25 ℃ at 6000X magnification.



18. Effect of *L. plantarum*-L49 against *P. nordicum*-M35 observed by Scanning Electron Microscopy (SEM) after 7 days at 25 ℃ at 3000X magnification.



19. Effect of *L. plantarum*-L49 against *P. nordicum*-M35 observed by Scanning Electron Microscopy (SEM) after 7 days at 25 ℃ at 6000X magnification.

Appendix III.

Data sheet for identification of taxa of subgenus *Penicillium*, according to Frisvad and Samson (2004).

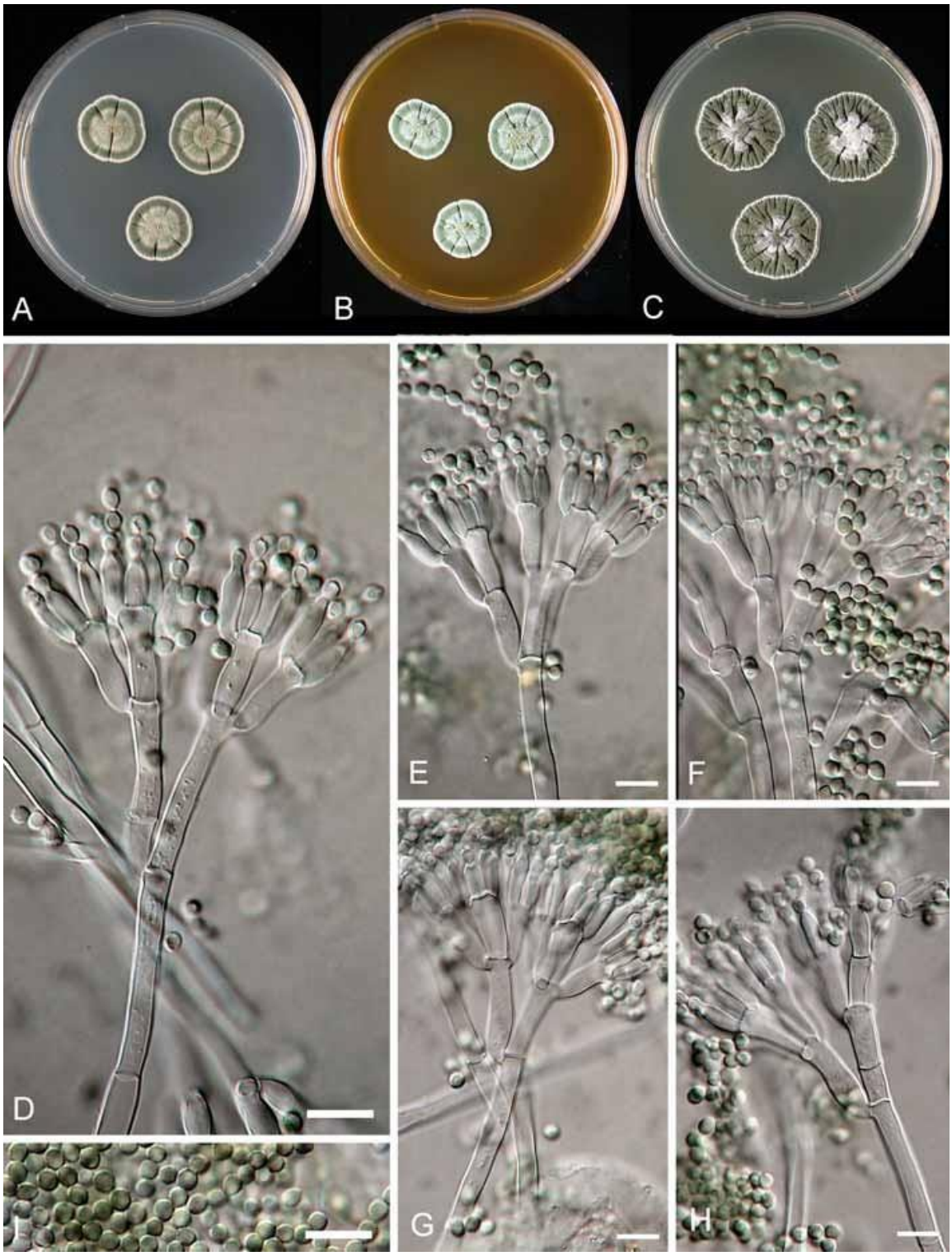
CYA – Incubation 7 days at 25°C	Colony diameter in mm: ……
Colour obverse*	blue green / dark green / dull green / grey green / olive green / yellow; pure green / white
Colour reverse*	pale / yellow / orange / red / beige-light brown / brown / dark brown;blackish green
Ehrlich reaction	no reaction / violet / red-brown / yellow
CYA – Incubation 7 days at 30°C	Colony diameter in mm: ……
MEA – Incubation 7 days at 25°C	Colony diameter in mm: ……
Colony texture	velvety / granulate / weak fasciculate / fasciculate / weakly floccose /floccose
Colour colony obverse*	blue green / dark green / dull green / grey green / olive green / yellow; pure green / white
Prepare microscopic slide	One slide at the colony edge (for conidiophore) and one in the colony (forconidia)
CREA – Incubation 7–10 (14) days at 25°C	Colony diameter in mm: ……
Degree of growth	No or very weak / Weak to moderate / Moderate / Moderate to good / goodNone / Weak / Moderate / Good / High
Acid production	Absent / PresentAbsent / Present
Base production: after 7 days after 10-14 days	
YES – Incubation 7 days at 25°C	Colony diameter in mm: ……
Degree of sporulation	None / Weak / Moderate / Strong
Colour colony reverse*	pale / yellow / orange / red / beige-light brown / brown / dark brown;blackish green
Microscopic characters Conidia	
Length/width in µm	…….µm
Ornamentation	Smooth / fine roughened / rough-walled / echinulate
Phialide length in µm	…….µm
Metulae length in µm	…….µm
Stipe width in µm Ornamentation	…….µm
Conidiophore adpressednes	Smooth / finely roughened / rough-walled / warted
Conidiophore branching pattern*	Strongly adpressed / adpressed / neither adpressed nor divergent / diver-gent / strongly divergent Monoverticillate / biverticillate / terverticillate / quaterverticillate

*More than one character is possible to enter into the database

Reference:

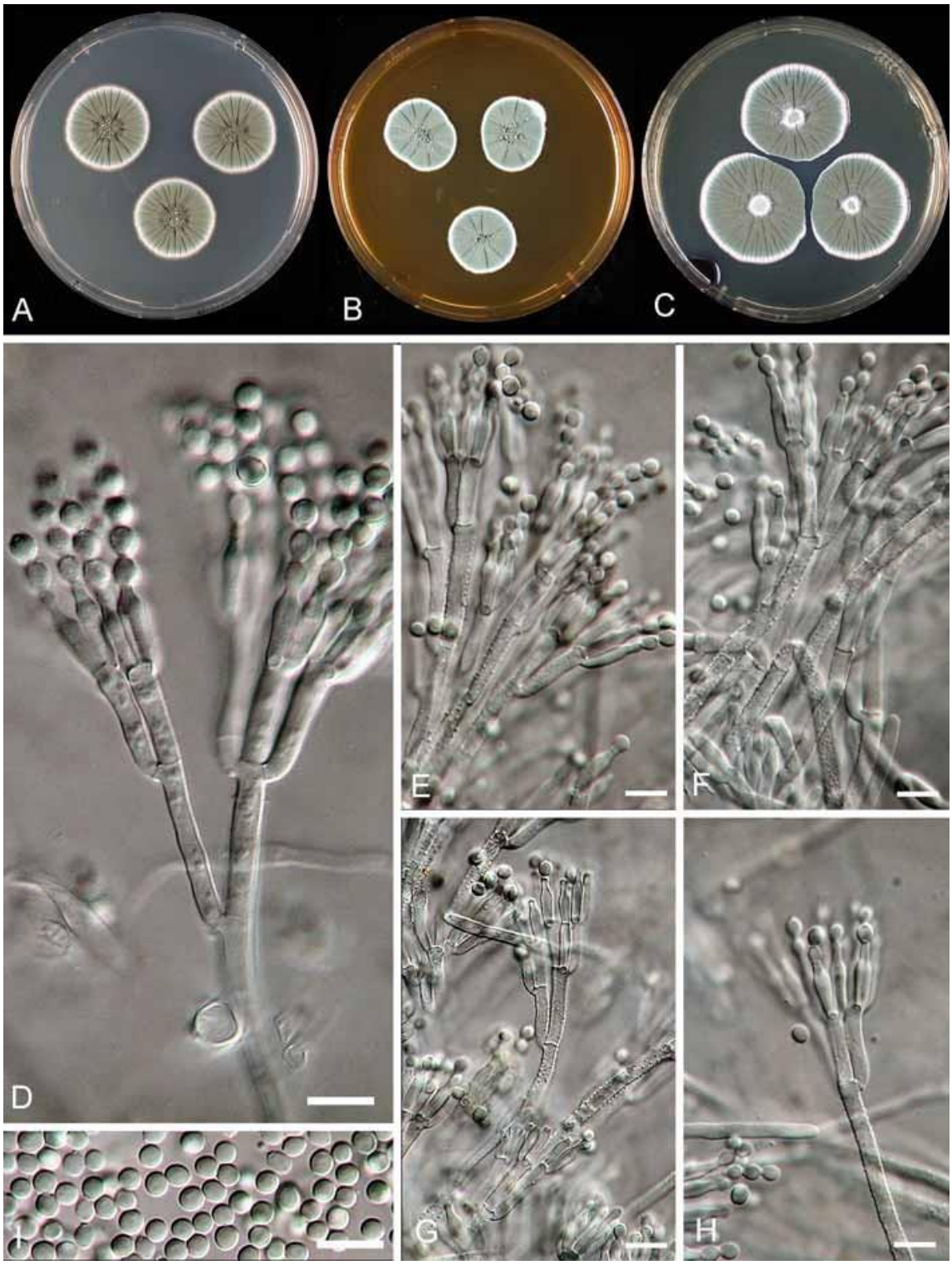
Frisvad, J. C. and Samson, R. A. (2004). Polyphasic taxonomy of *Penicillium* subgenus *Penicillium*. A guide to identification of food and air-borne terverticillate Penicillia and their mycotoxins. Studies in mycology, 49(1), 1-174.

Appendix IV. Photographs of *Penicillium* spp.



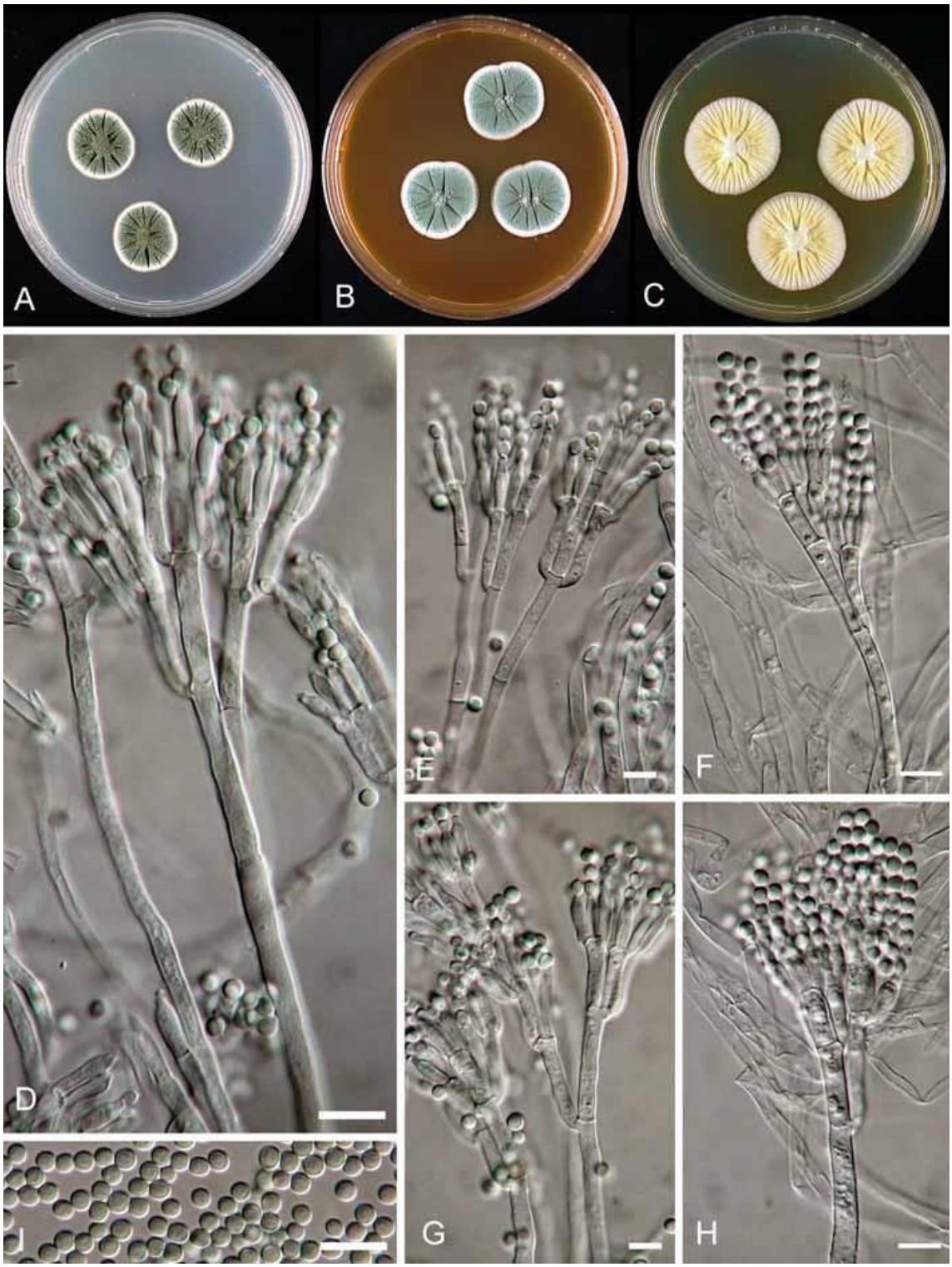
Penicillium brevicompactum. 7-day old colonies at (A) CYA, (B), MEA, (C) YES, D-H. Conidiophores. I. Conidia. White bar = 10 µm.

Source: Frisvad and Samson (2004).



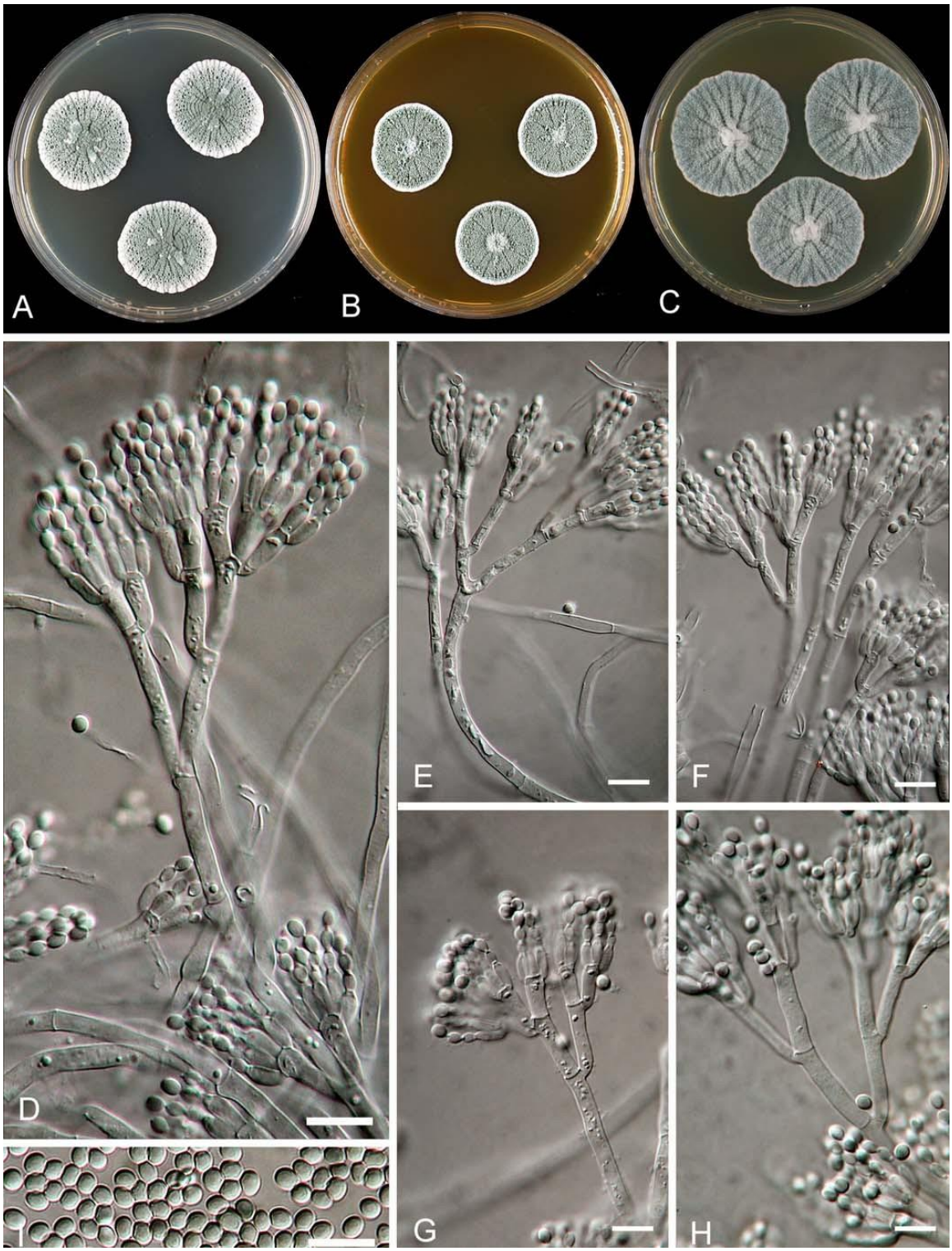
Penicillium commune. 7-day old colonies on A. CYA, B. MEA, C. YES, D-H. Conidiophores. I. Conidia. White bar =10 µm.

Source: Frisvad and Samson (2004).



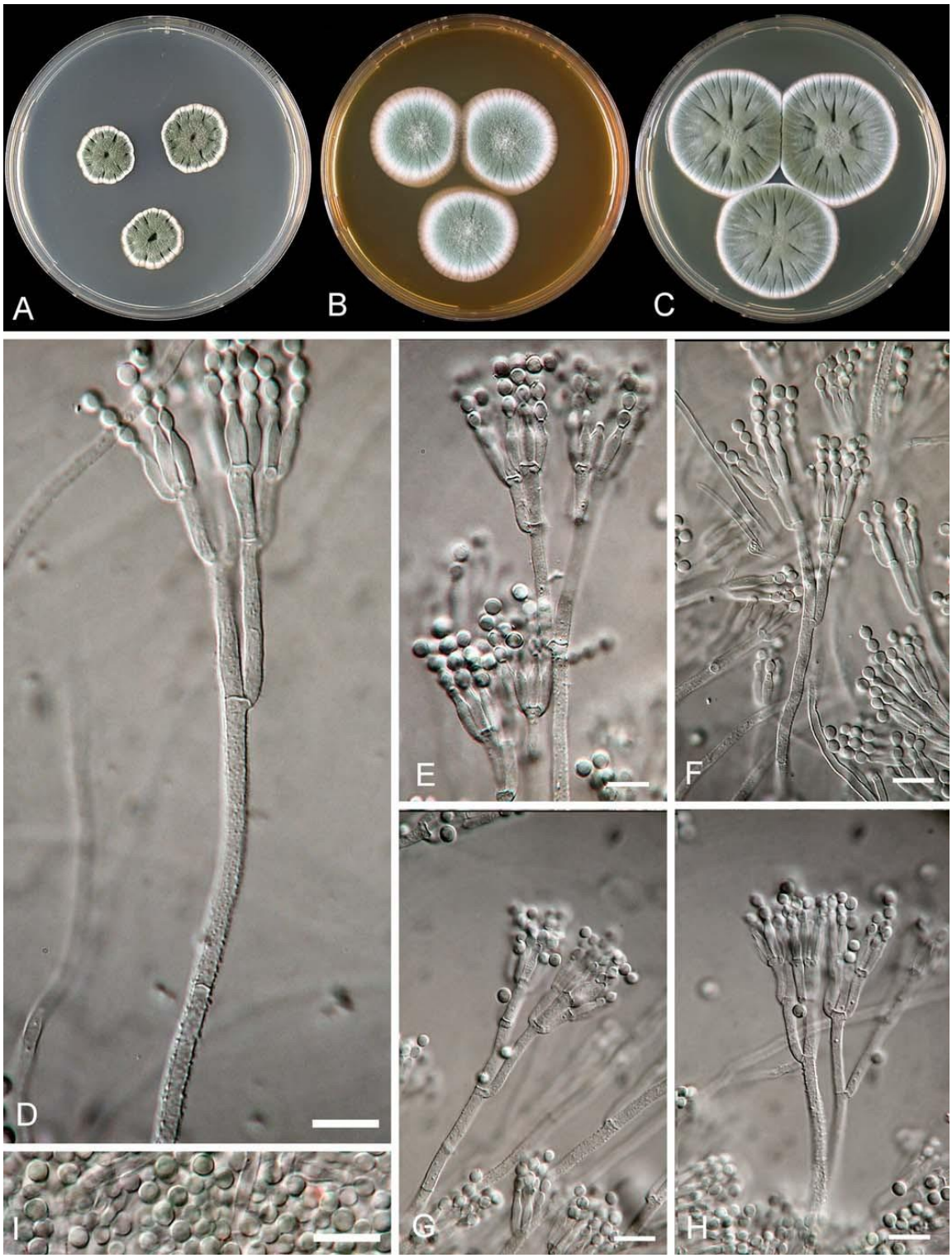
Penicillium cyclopium. 7-day old colonies on A. CYA, B. MEA, C. YES, D-H. Conidiophores. I. Conidia. White bar =10 µm.

Source: Frisvad and Samson (2004).



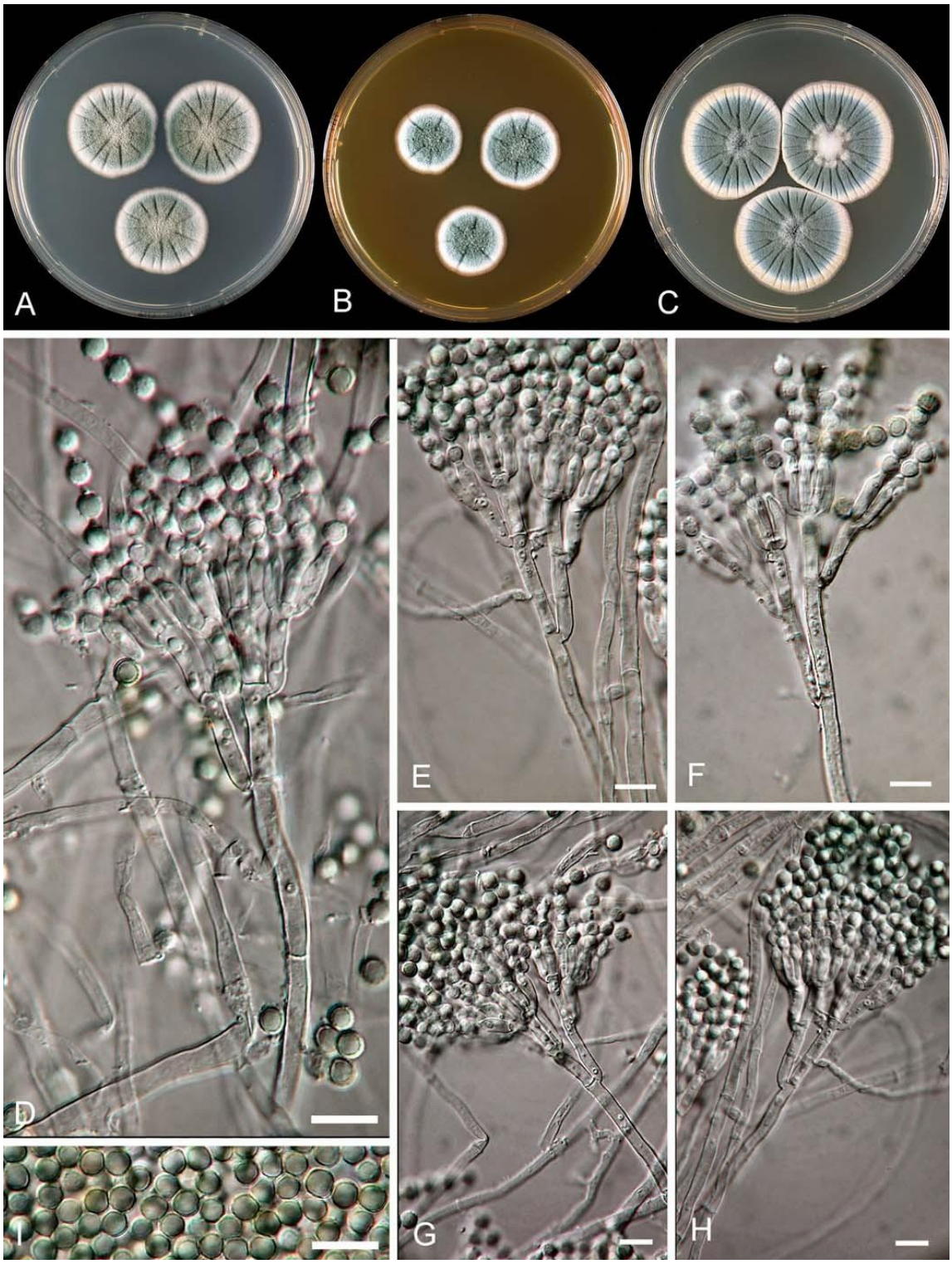
Penicillium griseofulvum. 7-day old colonies on A. CYA, B. MEA, C. YES, D-H. Conidiophores. I. Conidia. White bar = 10 µm.

Source: Frisvad and Samson (2004).



Penicillium melanoconidium. 7-day old colonies on A. CYA, B. MEA, C. YES, D-H. Conidiophores. I. Conidia. White bar = 10 µm.

Source: Frisvad and Samson (2004).



Penicillium solitum. 7-day old colonies on A. CYA, B. MEA, C. YES, D-H. Conidiophores. I. Conidia. White bar = 10 µm.

Source: Frisvad and Samson (2004).