UNIVERSIDADE DE SÃO PAULO

Faculdade de Ciências Farmacêuticas

Programa de Tecnologia Bioquímico-Farmacêutica

Área de Tecnologia de Fermentações

Cultivo de bactérias ácido-láticas em meio contendo resíduos de café e obtenção de compostos antimicrobianos de interesse alimentar e farmacêutico

Anna Carolina Meireles Piazentin

São Paulo

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Anna Carolina Meireles Piazentin

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Cultive of lactic acid bacteria in coffee residues elaborated media and obtention of antimicrobial compounds of food and pharmaceutical interest

Commission of Thesis for the degree of Doctor in Science

Prof. Dr. Ricardo Pinheiro de Souza Oliveira
Advisor/President

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3rd Examiner

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DEDICATION

I would like to dedicate this work to my partner Eduardo, my parents, my siblings, and my aunt. Also, I would like to dedicate this work to Titi, you always be in my heart.

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EPÍGRAFE

O que tiver que se ser, será! Mas um café te ajuda a lidar.

Autor desconhecido

ABSTRACT

PIAZENTIN, A. C. M. **Cultive of lactic acid bacteria in coffee residues elaborated media and obtention of antimicrobial compounds of food and pharmaceutical interest.** 2022. 122 p. Thesis (PhD) – Faculty of Pharmaceutical Sciences, University of São Paulo, São Paulo, 2022.

The objective of this study was to evaluate the potential of lactic acid bacteria (LAB) to produce BLIS (Bacteriocin Like Inhibitory Substances) with activity against pathogenic bacteria of food interest and clinical importance, in addition to developing an alternative culture media based on waste from the coffee industry, such as coffee silverskin (CS) and spent coffee grounds (SCG) where BAL could be able to grow and produce BLIS. *Enterococcus faecium* 135, which was isolated from the intestine of a starfish (Order Forcipulatida), stood out as a producer of BLIS with anti-listeric activity. However, its activity was reduced when cultured with the bacteria *Ligilactobacillus salivarius* and *Limosilactobacillus reuteri*. For the elaboration of the alternative media containing the CS and SCG residues, they were subjected to an acid pretreatment with 120 and 100 mg of H_2SO_4/g, for 75 and 45 minutes at 140 ºC in an autoclave. For CS and SCG, the hydrolysates passed through a post-hydrolysis with 4% (v/v) H_2SO_4 at 121ºC for 60 min, after which the hydrolysates were detoxified using a C-18 silica column and the pH was adjusted to 6. The media was prepared based on the commercial MRS medium (by Man, Rogosa and Sharp), and the hydrolyzate concentrations used for the preparation of the media were 0, 25, 50 and 100 % (v/v) in addition to the detoxified hydrolysate. The media was supplemented with sources of nitrogen and salts equal to the commercial MRS, and also sugars for the diluted media, so the concentration of sugars was equal to the 100% hydrolysate. The supplemented detoxified SCG and CS 25% medium stood out from the others, because *E. faecium* 135 obtained a growth of ~ 1.9 log CFU/mL. In addition the antimicrobial activity was superior to the control, being 480 AU/mL for CS 25 % and 428 AU/mL for supplemented detoxified SCG, in addition to lactic acid production, which was 10.51 g/L for 25% CS. The probiotic potential of *E. faecium* 135 was also tested and it showed resistance to low pH (2.5 and 3.0). Apart from resistance to 3% (w/v) bile salts, the strain was able to adhere to Caco-2 cells, and presented negative results for virulence factors. The presence of some genes responsible for the production of enterocins was also observed in the DNA of the bacterium. *E. faecium* 135 was a lactic acid bacterium that could be good candidate as a probiotic, and it can make BLIS, even in an alternative media made with CS and SCG coffee residues. These residues were interesting carbon sources for the growth of the bacterium, and they could be used as an alternative to traditional culture medium.

Keywords: lactic acid bacteria, *Enterococcus faecium*, BLIS, *coffee silverskin*, *spent coffee grounds*, probiotic potential.

RESUMO

PIAZENTIN, A. C. M. **Cultivo de bactérias ácido-láticas em meio contendo resíduos de café e obtenção de compostos antimicrobianos de interesse alimentar e farmacêutico.** 2022. 122 f. Tese (Doutorado) – Faculdade de Ciências Farmacêuticas, Universidade de São Paulo, São Paulo, 2022.

O objetivo desse trabalho foi avaliar o potencial de bactérias ácido-láticas (BAL) produzirem BLIS (*Bacteriocin Like Inhibitory Substances*) com atividade antimicrobiana contra cepas patogênicas de interesse alimentar e importância farmacêutica, além de elaborar um meio de cultivo alternativo baseado nos resíduos da indústria do café, como película prateada (*coffee silverskin* – CS) e a borra do café (*spent coffee grounds* – SCG) onde as BAL pudessem crescer e produzir o BLIS. *Enterococcus faecium* 135, que foi isolada do intestino de uma estrela do mar (Ordem Forcipulatida), se destacou como produtora de BLIS com atividade anti-listérica, entretanto teve sua atividade reduzida quando cultivada com as bactérias *Ligilactobacillus salivarius* e *Limosilactobacillus reuteri*. Para a elaboração do meio alternativo contendo os resíduos CS e SCG, os mesmos foram submetidos um pré-tratamento ácido 120 e 100 mg de H_2SO_4/g, durante 75 e 45 minutos a 140 ºC em autoclave, para CS e SCG respectivamente, os hidrolisados passaram por uma pós-hidrólise com 4% (v/v) H_2SO_4 a 121ºC durante 60 min, após esse período uma parte dos hidrolisados foi detoxificada utilizando uma coluna de sílica C-18 e o pH foi ajustado para 6. Posteriormente, os meios foram elaborados com base no meio comercial MRS (de Man, Rogosa and Sharp), e as concentrações de hidrolisado utilizadas para elaboração do meio foram 0, 25, 50 e 100 % (v/v) além do hidrolisado detoxificado. Os meios tiveram suplementação com fontes de nitrogênio e sais iguais ao meio comercial, e de açúcares para os meios diluídos, a fim de que a concentração de açúcares fosse igual ao meio 100%. Os meios SCG detoxificado suplementado e CS 25% destacaram-se dos demais pois, *E. faecium* 135 obteve um crescimento de ~ 1,9 log UFC/mL, além da atividade antimicrobiana superior ao controle, sendo de 480 AU/mL para CS 25% e 428 AU/mL para SCG detoxificado suplementado, além da produção de ácido lático que foi de 10,51 g/L para CS 25 %. O potencial probiótico de *E. faecium* 135 também foi testado e o mesmo apresentou resistência a pH baixos (2.5 e 3.0), além de resistência a sais de bile 3% (w/v), a cepa foi capaz de se aderir a células Caco-2, e apresentou resultados negativos para fatores de virulência, também foi observada a presença de alguns genes referentes a produção de enterocinas no DNA da cepa. Em conclusão *E. faecium* 135 foi uma bactéria ácido-lática que apresentou potencial probiótico, e produtor de BLIS, mesmo em meio alternativo elaborado com os resíduos do café CS e SCG. Resíduos esses que se mostraram interessantes fontes de carbono para o crescimento da cepa, podendo ser utilizados com alternativa aos meios de cultivo convencionais.

Palavras-chave: bactérias ácido-láticas, *Enterococcus faecium*, BLIS, *coffee silverskin*, *spent coffee grounds*, potencial probiótico

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ABREVIATIONS

AU: arbitrary units

BLIS: bacteriocin like substances

CFU: colony forming unity

CFS: cell-free supernatant

CS: coffee silverskin

GRAS: Generally Recognized As Safe

HPLC: high performance liquid chromatography

LAB: lactic acid bacteria

O.D.: optical density

PAN: primary aminoacids

rpm: rotations per minute

SCG: spent coffee grounds

g: centrifugal force

v/v: volume per volume

w/v: weight per volume

5-HMF: hydroxymethylfurfural

Summary

GENERAL INTRODUCTION

BLIS (bacteriocin like inhibitory substances) are peptides or proteins produced by ribosomes and present antimicrobial activity against several microorganisms (COTTER; HILL; ROSS, 2005). The most common producers of these molecules are the lactic acid bacteria (LAB), mostly because they are usually considered GRAS (Generally Recognized As Safe) strains and a larger number of them are isolated from fermented foods (QIN et al., 2017). The research about BLIS has increased over the years. The applications vary, but in the food industry is where they could have more usage in food preservation against foodborne pathogens (COTTER; ROSS; HILL, 2013; DOBSON et al., 2012; SOLTANI et al., 2021). Recently, BLIS has been studied and applied as an alternative to the use of antibiotics in animal production due to the increasing cases of antibiotic resistance (CROTTA; GEORGIEV; GUITIAN, 2017; POPOVA, 2017). Alternatives have started to be required, so LAB or probiotic strains the are capable of producing BLIS are applied as a supplement to the animal feeding (JAHROMI et al., 2016; MESSAOUDI et al., 2011).

The use of agro-industrial waste is increasing as an alternative carbon source for the growth of probiotic and lactic acid bacteria. Among these residues are the ones from the coffee industry, which increase each year due to the high demand for the beverage (ICO-International Coffee Organization, 2021). Coffee silverskin (CS) and spent coffee grounds (SCG) are two of the principal residues generated in the coffee industry. Both are usually used as fertilizer and for the elaboration of biofuels (MCNUTT; HE, 2019; NARITA; INOUYE, 2014). Although they are sources for cellulose and hemicellulose (BALLESTEROS; TEIXEIRA; MUSSATTO, 2014), CS and SCG show potential as prebiotics (JIMÉNEZ-ZAMORA; PASTORIZA; RUFIN-HENARES, 2015) and alternative growth medium for fungi and yeast (MACHADO et al., 2012). However, using these residues for the production of BLIS is not approached by literature.

Due to the potential of LAB to produce BLIS and the availability of sugar sources from CS and SCG, the aim of this study was to evaluate the capability of LAB to produce BLIS with activity against some pathogens from the food industry and clinical interests, and the

elaboration of an alternative media with CS and SCG that a LAB would grow and produce BLIS.

References

BALLESTEROS, Lina F.; TEIXEIRA, José A.; MUSSATTO, Solange I. Chemical, Functional, and Structural Properties of Spent Coffee Grounds and Coffee Silverskin. **Food and Bioprocess Technology**, *[S. l.]*, v. 7, n. 12, p. 3493–3503, 2014. DOI: 10.1007/s11947-014-1349-z. Disponível em: http://link.springer.com/10.1007/s11947-014-1349-z.

COTTER, Paul D.; HILL, Colin; ROSS, R. Paul. Food microbiology: Bacteriocins: Developing innate immunity for food. **Nature Reviews Microbiology**, *[S. l.]*, v. 3, n. 10, p. 777–788, 2005. DOI: 10.1038/nrmicro1273.

COTTER, Paul D.; ROSS, R. Paul; HILL, Colin. Bacteriocins-a viable alternative to antibiotics? **Nature Reviews Microbiology**, *[S. l.]*, v. 11, n. 2, p. 95–105, 2013. DOI: 10.1038/nrmicro2937.

CROTTA, Matteo; GEORGIEV, Milen; GUITIAN, Javier. Quantitative risk assessment of Campylobacter in broiler chickens – Assessing interventions to reduce the level of contamination at the end of the rearing period. **Food Control**, *[S. l.]*, v. 75, p. 29–39, 2017. DOI: 10.1016/j.foodcont.2016.12.024. Disponível em: http://dx.doi.org/10.1016/j.foodcont.2016.12.024.

DOBSON, Alleson; COTTER, Paul D.; PAUL ROSS, R.; HILL, Colin. Bacteriocin production: A probiotic trait? **Applied and Environmental Microbiology**, *[S. l.]*, v. 78, n. 1, p. 1–6, 2012. DOI: 10.1128/AEM.05576-11.

JAHROMI, Mohammad Faseleh; ALTAHER, Yassir Wesam; SHOKRYAZDAN, Parisa; EBRAHIMI, Roohollah; EBRAHIMI, Mahdi; IDRUS, Zulkifli; TUFARELLI, Vincenzo; LIANG, Juan Boo. Dietary supplementation of a mixture of Lactobacillus strains enhances performance of broiler chickens raised under heat stress conditions. **International Journal of Biometeorology**, *[S. l.]*, v. 60, n. 7, p. 1099–1110, 2016. DOI: 10.1007/s00484-015-1103-x. Disponível em: http://dx.doi.org/10.1007/s00484-015-1103-x.

JIMÉNEZ-ZAMORA, Ana; PASTORIZA, Silvia; RUFIÁN-HENARES, José A. Revalorization of coffee by-products. Prebiotic, antimicrobial and antioxidant properties. **LWT - Food Science and Technology**, *[S. l.]*, v. 61, n. 1, p. 12–18, 2015. DOI: 10.1016/j.lwt.2014.11.031.

INTERNATIONAL COFFEE ORGANIZATION. **Trade Statistics Tables**. Disponível em: https://www.ico.org/trade_statistics.asp. Acessado em: 18/03/2022.

MACHADO, Ercília M. S.; RODRIGUEZ-JASSO, Rosa M.; TEIXEIRA, José A.; MUSSATTO, Solange I. Growth of fungal strains on coffee industry residues with removal of polyphenolic compounds. **Biochemical Engineering Journal**, *[S. l.]*, v. 60, p. 87–90, 2012. DOI: 10.1016/j.bej.2011.10.007. Disponível em: http://dx.doi.org/10.1016/j.bej.2011.10.007.

MCNUTT, Josiah; HE, Quan (Sophia). Spent coffee grounds: A review on current utilization. **Journal of Industrial and Engineering Chemistry**, *[S. l.]*, v. 71, p. 78–88, 2019. DOI: 10.1016/j.jiec.2018.11.054. Disponível em: https://doi.org/10.1016/j.jiec.2018.11.054.

MESSAOUDI, Soumaya; KERGOURLAY, Gilles; ROSSERO, Albert; FERCHICHI, Mounir; PRÉVOST, Hervé; DRIDER, Djamel; MANAI, Mohamed; DOUSSET, Xavier. Identification of lactobacilli residing in chicken ceca with antagonism against Campylobacter. **International Microbiology**, *[S. l.]*, v. 14, n. 2, p. 103–110, 2011. DOI: 10.2436/20.1501.01.140.

NARITA, Yusaku; INOUYE, Kuniyo. Review on utilization and composition of coffee silverskin. **Food Research International**, *[S. l.]*, v. 61, p. 16–22, 2014. DOI: 10.1016/j.foodres.2014.01.023. Disponível em: http://dx.doi.org/10.1016/j.foodres.2014.01.023.

POPOVA, Teodora. Effect of probiotics in poultry for improving meat quality. **Current Opinion in Food Science**, *[S. l.]*, v. 14, p. 72–77, 2017. DOI: 10.1016/j.cofs.2017.01.008. Disponível em: http://dx.doi.org/10.1016/j.cofs.2017.01.008.

QIN, Chubin; ZHANG, Zhen; WANG, Yibing; LI, Shuning; RAN, Chao; HU, Jun; XIE, Yadong; LI, Weifen; ZHOU, Zhigang. EPSP of L. casei BL23 protected against the infection caused by Aeromonas veronii via enhancement of immune response in zebrafish. **Frontiers in Microbiology**, *[S. l.]*, v. 8, n. DEC, p. 1–13, 2017. DOI: 10.3389/fmicb.2017.02406.

SOLTANI, Samira et al. Bacteriocins as a new generation of antimicrobials: toxicity aspects and regulations. **FEMS Microbiology Reviews**, *[S. l.]*, v. 45, n. 1, p. 1–24, 2021. DOI: 10.1093/femsre/fuaa039. Disponível em: http://orcid.org/0000-0002-3685-5639.

OBJECTIVES

GENERAL

Optimization and production of BLIS by *Enterococcus faecium* 135, elaboration of media containing coffee residues and evaluation of the antimicrobial activity against foodborne and clinically important pathogens.

SPECIFICS

- Evaluation of the production of bacteriocin like inhibitory substances (BLIS) by monoculture of *E. faecium* 135 and in co-culture with *Ligilactobacillus salivarius* and *Limosilactobacillus reuteri.*

- Optimization of BLIS production by *E. faecium* 135, and evaluation of the antimicrobial activity against *Listeria monocytogenes*, *Salmonella enterica* serovar Typhimurium, *Salmonella enterica* serovar Choleraesius, *Staphylococcus aureus* and *Streptococcus agalactiae*.

- Elaboration of an alternative growth media using spent coffee grounds and coffee silverskin, and evaluation of the growth and BLIS production by *E. faecium* 135.

- Pre-purification of the BLIS produced by *E. faecium* 135.

- Determination of the probiotic nature and presence of bacteriocin genes in *E. faecium* 135.

PRESENTATION

This thesis is organized in the form of scientific articles (published, and to be submitted for publication, and is divided into the following chapters.

Chapter 1: "*Bacteriocin-like inhibitory substances production by Enterococcus faecium 135 in co-culture with Ligilactobacillus salivarius and Limosilactobacillus reuteri*" - This chapter aimed to evaluate the best conditions for growth and production of antimicrobial compounds produced by the monoculture of *E. faecium* and the ternary culture with *L. salivarius* and *L. reuteri.* The following published scientific article resulted in this chapter: **Piazentin, A.C.M**, Mendonça, C.M.N., Vallejo, M., Mussatto, S.I., Oliveira, R.P.S. (2022) Bacteriocin-like inhibitory substances production by *Enterococcus faecium* 135 in co-culture with *Ligilactobacillus salivarius* and *Limosilactobacillus reuteri*. *Brazilian Journal of Microbiology*, v. 53, p. 131-141. https://doi.org/10.1007/s42770-021-00661-6

Chapter 2: "*Use of coffee residues as alternative media for growth and production of antimicrobial compounds from Enterococcus faecium 135*" - The work presented in this chapter was performed during the internship performed at Denmark Technical University. The aim of the work was to recover sugars from spent coffee grounds and coffee silverskin. Both residues were hydrolysate, and from this hydrolysate it was elaborate an alternative media similar to a commercial medium used in lactic acid growth. It was evaluated the capability of *E. faecium* 135 to grow in this new media and produce antimicrobial compounds. - This content resulted in a manuscript, which will be submitted: **Piazentin, A.C.M**, Yamakawa, C.K., Vallejo, M., Oliveira, R.P.S., Mussatto, S.I. (2022) Use of coffee residues as alternative meda for growth and production of antimicrobial compounds from *Enterococcus faecium* 135. *Bioresource Technology*, **to be submitted.**

Chapter 3: "*Bacteriocinogenic probiotic bacteria isolated from an aquatic environment inhibit the growth of food and fish pathogens*" - This chapter evaluates the probiotic potential of *E. faecium* 135 and other lactic acid bacteria isolated from aquatic environments, besides the presence of bacteriocin production genes and the production of bacteriocin like substances. The first authorship of this article was divided by the first to authors who equally contributed to the work: **Pereira, W.A., Piazentin, A.C.M.**, Oliveira, R.C., Mendonça, C.M.N., Tabata, Y.A.,

Mendes, M.A., Fock, R.A., Makiyama. E.N., Corrêa, B., Vallejo, M., Villalobos, E.F., Oliveira, R.P.S. (2022) Bacteriocinogenic probiotic bacteria isolated from an aquatic environment inhibit the growth of food and fish pathogens. *Scientific Reports*, v. 12:5530. https://doi.org/10.1038/s41598-022-09263-0

CHAPTER
.1.

Bacteriocin-like inhibitory substances production by *Enterococcus faecium* 135 in co-culture with *Ligilactobacillus salivarius* and *Limosilactobacillus reuteri*

Abstract

The use of lactic acid bacteria (LAB) and probiotic cultures in the breeding of animals such as poultry and swine are quite common. It is known that those strains can produce bacteriocins when grown in pure culture. However, the production of bacteriocin using co-culture of microorganisms has not been much studied so far. The present study contributes with innovation in this area by embracing the production of bacteriocin-like inhibitory substances (BLIS) by a newly isolated strain of *Enterococcus faecium* 135. Additionally, the co-cultivation of this strain with *Ligilactobacillus salivarius* and *Limosilactobacillus reuteri* was also investigated. The antimicrobial activity of the produced BLIS was evaluated against *Listeria monocytogenes*, *Listeria innocua*, *Salmonella enterica*, and *Salmonella enterica* serovar Typhimurium using two methods: turbidimetric and agar diffusion. In addition, the presence of enterocin genes was also evaluated. The BLIS produced showed a bacteriostatic effect against the bio-indicator strains, and the highest antimicrobial activities expressed by arbitrary units per mL (AU/mL) were obtained against *L. monocytogenes* in monoculture (12,800 AU/mL), followed by the co-culture of E. faecium with Limosilactobacillus reuteri (400 AU/mL). After concentration with ammonium sulfate, the antimicrobial activity raised to 25,600 AU/mL. Assays to determine the proteinaceous nature of the BLIS showed susceptibility to trypsin and antimicrobial activity until 90 ºC. Finally, analysis of the presence of structural genes of enterocins revealed that four enterocins genes were present in *E. faecium* 135. These results suggest that BLIS produced by *E. faecium* 135 has potential to be a bacteriocin and, after purification, could potentially be used as an antimicrobial agent in animal breeding.

Keywords: *Enterococcus faecium*, antimicrobial activity, co-culture, foodborne pathogens

1. Introduction

The inappropriate and excessive use of antibiotics in the breeding of animals such as poultry and swine, associated with the increased resistance of bacteria to antibiotics, has led to major changes in policies to regulate the use of antibiotics, especially by the European Food Safety Authority (EFSA). These policies mainly imply the prohibition of certain antibiotics used in subtherapeutic doses to assist the growth of animals, in addition to the often inappropriate treatments by breeder (FOOD SAFETY AUTHORITY; CENTRE FOR DISEASE PREVENTION, 2014). With the mass production and processing of these meats, there is a great concern with the potential presence of pathogenic microorganisms (*Salmonella* spp., *Listeria* spp., and *Campylobacter* spp.), which has led to serious cleaning and disinfection measures in animal breeding, slaughtering, and meat handling (GARCÍA-SÁNCHEZ et al., 2017). Therefore, besides the classical methods of prevention such as sanitization and use of antibiotics, other techniques have also been used by breeders including vaccination, use of organic acids, lactic acid bacteria (LAB), probiotics and bacteriocins (CROTTA; GEORGIEV; GUITIAN, 2017; LAKICEVIC; NASTASIJEVIC, 2017). Some of the main reasons for the use of LAB and probiotics in the feeding of these animals are the prevention of growth of enteric pathogens and the improvement of the meat quality (JOHNSON; SHANK; JOHNSON, 2017). Usually, the administration of those probiotics and bacteriocins in animal breeding is made during the feed, and to secure the viability of the strains and action of the bacteriocins, the substances are commonly microencapsulated and administered with ration or water (SOLTANI et al., 2021; STROMPFOVÁ et al., 2019).

Several microorganisms are known to show probiotic properties, including LAB, non-lactic acid bacteria, and some yeasts. From these microorganisms, LAB are the most studied and they generally belong to the genera *Streptococcus* spp., *Lactococcus* spp., *Enterococcus* and the family *Lactobacillaceae* (KHAN; FLINT; YU, 2010; MITSUOKA, 2014; RAMCHANDRAN; SHAH, 2010; ZHENG et al., 2020). In addition to producing organic acids, these LAB can also produce other antimicrobial compounds such as hydrogen peroxide and bacteriocins (GILLOR; ETZION; RILEY, 2008). Bacteriocins or bacteriocin-like inhibitory substances (BLIS) are peptides or proteins produced by the ribosomes and excreted into the extracellular environment, exerting antimicrobial activities against other bacteria (COTTER; HILL; ROSS, 2005; MESSAOUDI et al., 2013). LAB strains that produce bacteriocins or BLIS include strains of the genera *Enterococcus* spp. (KHAN; FLINT; YU, 2010; SUVOROV, 2020). Among these, some strains of *Enterococcus faecium* have been

reported to produce enterocins belonging to different classes, which are circular bacteriocins, members of class I or class II bacteriocins with low molecular weight (<10 kDa) (ACEDO et al., 2018; COTTER; HILL; ROSS, 2005) and excellent activities against *Listeria monocytogenes*, S*almonella enterica* and *Escherichia coli* (CHAKCHOUK-MTIBAA et al., 2018; KHAN; FLINT; YU, 2010; QIAO et al., 2020). From the *Lactobacillaceae* family, *Ligilactobacillus salivarius* is capable of producing salivaricin, a class II bacteriocin with activity against *Salmonella* sp., *Campylobacter jejuni* and *Listeria* sp. [14], while *Limosilactobacillus reuteri* produces reuterin, a class II bacteriocin with an anti-listerical effect (MONTIEL et al., 2014).

The use of co-cultures to produce bacteriocins is a strategy still not much investigated in the literature. Some authors reported that the production of nisin by *Lactococcus lactis* was increased by 85% when the strain was co-cultivated with *Saccharomyces cerevisiae* (LIU et al., 2004). On the other hand, co-cultivation of *Lactococcus lactis* subsp. *cremoris* with a bacteriocin producer strain of *E. faecium* resulted in an interruption of the bacteriocin production (FOULQUIÉ MORENO et al., 2003). In general, when bacteria are used in co-cultivation to produce bacteriocins, the success is strain related (CHANOS; MYGIND, 2016; DOMÍNGUEZ-MANZANO; JIMÉNEZ-DÍAZ, 2013; MALDONADO; RUIZ-BARBA; JIMÉNEZ-DIAAZ, 2004) and the inducing microorganism must be resistant to this particular bacteriocin (GUTIÉRREZ-CORTÉS et al., 2018; KOS et al., 2011; ROJOBEZARES et al., 2007).

Due to the potential of some LAB to produce BLIS or bacteriocins, this study aimed to evaluate the production of BLIS by a newly isolated strain of *E. faecium* 135 and its antimicrobial activity against *Listeria monocytogenes*, *Listeria innocua*, *Salmonella enterica* and *Salmonella enterica* serovar Typhimurium. Also, the presence of enterocin genes in *E. faecium* 135 DNA was investigated. The BLIS production by co-cultivation of *E. faecium* 135 with the two bacteriocin producers *Ligilactobacillus salivarius* and *Limosilactobacillus reuteri,* was also evaluated, with its action tested against the most common pathogens that cause concern to the breeding industry.

2. Materials and methods

2.1. Microorganisms

Enterococcus faecium 135, isolated from the intestines of a starfish (Order Forcipulatida) in Playa Unión, Rawson-Chubut (Patagonia, Argentina) and kindly donated by Prof. Marisol Vallejo, National University of Patagonia San Juan Bosco (Argentina), and was used as BLIS producer. The following strains were used as bio-indicators: *Salmonella enterica* CECT 724 and *Listeria monocytogenes* CECT 934 (acquired from the Spain Collection of Cultures, Spain), *Salmonella enterica* serovar Typhimurium IOC 5551/16 and *Listeria innocua* CLIST 2711 (kindly provided by Fiocruz, Rio de Janeiro, Brazil). For the co-cultivation experiments, the bacteriocin producer strains *Ligilactobacillus salivarius* subsp. *salicinius* ATCC 11742 and *Limosilactobacillus reuteri* ATCC 23272 (acquired from André Tosello Foundation, Campinas, Brazil) were used.

The strains were cryopreserved in tubes containing 1 mL of cell culture with glycerol 20%, at -76 ºC.

2.2. Media, inoculum and cultivation conditions

MRS (de Man, Rogosa, and Sharpe) broth (Difco™, Maryland, USA) was the medium used for the bacteria cultivation. It was prepared following the manufacturer's instructions and had the final pH adjusted to 6 by adding HCl 1N.

The inoculum was prepared by adding 1 mL of stock culture of *E. faecium* 135 in a 250 mL-Erlenmeyer flask containing 50 mL of MRS broth, followed by incubation on an orbital shaker (Tecnal Equipamentos Científicos, Piracicaba, Brazil) at 37 °C, 100 rpm, for 24 h. Then, 10 mL of the inoculum, with optical density (OD) at 600 nm wavelength adjusted to 0.8 (~ 7 log CFU/mL), were transferred to an Erlenmeyer flasks containing 90 mL of MRS broth. In this step, the single cultivation of the strain was performed in a metabolic agitator under different conditions of temperature (30 and 35 ºC) and agitation (100 and 150 rpm).

For the co-cultivation assays, the following strain mixtures were used: (i) *Ligilactobacillus salivarius* ATTC 11742 and *Limosilactobacillus reuteri* ATCC 23272 (5:5 mL); (ii) *Ligilactobacillus salivarius* ATTC 11742 and *E. faecium* 135 (5:5 mL); (iii) *Limosilactobacillus reuteri* ATCC 23272 and *E. faecium* 135 (5:5 mL); (iv) *Ligilactobacillus salivarius* ATTC 11742, *Limosilactobacillus reuteri* ATCC 23272 and *E. faecium* 135 (3.3: 3.3: 3.3 mL). *Ligilactobacillus salivarius* and *Limosilactobacillus reuteri* strains were activated in a similar way to that performed for *E. faecium*. For the experiments, all the strains had the

OD adjusted to 0.8 (~7 log CFU/mL) in order to start with the same amount of cells. The co-cultivation assays were carried out in Erlenmeyer flasks containing 100 mL of MRS broth, at 35 ºC, in an orbital shaker at 100 rpm for 24 h. At the end of the experiments, samples were taken and submitted for antimicrobial activity analysis.

2.3. Antimicrobial activity of potential BLIS

2.3.1. Determination of antimicrobial activity by turbidimetric analysis

Samples were taken at the end of the cultivations, centrifuged at 4470 *g* for 10 min, and the supernatant was filtered through 0.22 µm filter (Analítica, São Paulo, Brazil). Then, the cell-free supernatant (CFS) had the pH adjusted to 6.0-6.5 with NaOH 1M and was thermically treated at 70 ºC for 20 min to be used in the antimicrobial activity assays (CABO et al., 1999).

The antimicrobial activity was verified against the bio-indicator strains *S. enterica*, *S.* Typhimurium, and *L. monocytogenes*. The strains were previously grown in TSB (Tryptic Soy Broth) (Difco™, Le Pont de Claix, France) medium for 16 h. For the analysis, the cultures had the OD adjusted to 0.1 (*S. enterica* and *S.* Typhimurium) and 0.2 (*L. monocytogenes*).

Analyses were performed in 96 well plates using a microplate reader (Synergy HTX, Bio Tek, Winooski, USA). For analysis, the following volumes were added per well: 50 µL of the bioindicator strain, 50 µL of the previously treated CFS, and 100 µL of TSB broth. Assays were also performed with a positive control, i.e., without BLIS. The microplates were maintained at 37 ºC for 24 h. During this period, the OD (600 nm) was measured every 30 min (the plate was agitated at 50 rpm before every reading to homogenize the cell suspension). Analyses were performed in triplicate. After 24 h, a graphic comparing the positive control with the samples containing treated CFS was plotted to observe the action of BLIS on the growth of the bio-indicator strains.

2.3.2. Determination of antimicrobial activity through the agar diffusion method

After obtaining the CFS as described in 2.3.1., BHI (Brain Heart Infusion) (Difco™, Maryland, USA) medium was used to grow *L. monocytogenes*, and TSB (Difco™, Le Pont de Claix, France) medium was used to grow *S. enterica* and *S.* Typhimurium. Initially, 10^8 CFU/mL of each strain were inoculated in 10 mL of BHI or TSB agar and then transferred to a Petri dish. After solidification, 10 µL of CFS were added and the plate was incubated at 37 ºC for 18 h. The antimicrobial activity of the BLIS was expressed in arbitrary units per mL

(AU/mL), the activity was measured by the dilution of the treated CFS in a twofold dilution using phosphate buffer 100 mM (pH 6.5), the highest dilution with a minimum halo of 2 mm of inhibition, was considered to calculate using the equation (1), were a the dilution factor, and b is the highest dilution with an inhibition halo, the value is expressed in mL by multiplying per 100 (TODOROV; DICKS, 2006).

$$\mathrm{AU/mL} = \mathrm{a\text{^}b} \times 100 \quad (1)$$

Treated CFS obtained from co-cultures of *E. faecium*, *L. salivarius* and *L. reuteri* also had its antimicrobial activity tested using the method described above, being the activity also expressed in AU/mL.

2.4. BLIS concentration with ammonium sulfate

To concentrate the produced BLIS, 10 g of ammonium sulfate (Labsynth Produtos para Laboratórios Ltda, Diadema, Brazil) were added in tubes containing 20 mL of treated CFS (50% saturation), then the tubes were agitated vigorously for 1 min, followed by incubation at 10 ºC, 100 rpm, for 1 h. Afterwards, the content of the tubes was centrifuged (4470 *g*, 4 ºC, 30 min), the precipitate was recovered and resuspended with 10% (v/v) of the initial volume (20 mL) of 25 mM (pH 6.5) ammonium acetate (Labsynth Produtos para Laboratórios Ltda, Diadema, Brazil) solution, and filtered through 0.22 µm filter [29]. The antimicrobial activity of the concentrated BLIS was determined through the agar diffusion method.

2.5. Determination of the protein nature of the BLIS

The proteinaceous nature of the antimicrobial compound was determined through the agar diffusion method. Firstly, BLIS was obtained as described in 2.3.1. Then, 10 µL of treated BLIS were poured in a Petri dish containing *L. innocua* as bio-indicator strain. The same volume of trypsin (10 µL) (Sigma-Aldrich, Saint Louis, USA) at 1 mg/mL, was poured in the Petri dish in such a way that it would just partially cover the BLIS (TODOROV; DICKS, 2006). The proteinaceous nature of the BLIS was determined by the absence of antimicrobial activity affecting the formation of the inhibition halo.

2.6. Effect of salts, detergents, and temperature on BLIS stability

The method described by Todorov and Dicks (2006) was used to verify the stability of the produced BLIS. For the assays, the BLIS liquid culture was treated with 1% (w/v) of the

following reagents: NaCl (Cromoline Química Fina LTDA, Diadema, Brazil), EDTA, Triton 100x, SDS, Tween-20 (Inlab, Alamar Tecno Científica Ltda, São Paulo, Brazil), and Tween-80 (Labsynth Produtos para Laboratórios Ltda, Diadema, Brazil)) at 30 ºC for 2 h. Reactions were performed in conical tubes with a working volume of 1 mL. In addition, BLIS was submitted to various heat treatments: 30, 50, 70, and 90 ºC for 1 h, or 120 ºC for 15 min. After treatment, the stability of the BLIS was verified through the agar diffusion method against *L. innocua* as bio-indicator strain.

2.7. Molecular identification of Enterococcus faecium 135 and amplification of enterocin genes

Genotypic identification of *E. faecium* 135 was confirmed through 16S rRNA technique, its DNA was extracted using a Promega Wizard Genomic DNA purification kit (Madison, Wisconsin, USA) following the manufacturer's instructions. The protocols used in amplification were described by Jackson *et al.* (2004) and the primers by Kariyama *et al.* (2000). Each assay had a negative control, and a positive control using a strain of *E. faecium* ATCC 19434. The PCR (Polymerase Chain Reaction) was carried in a thermocycler Mastercycler® (Eppendorf, Hamburg, Germany). Electrophoresis of the products from genetic amplification was performed in agarose gel 1.8% (w/v) (Sigma-Aldrich) at 70 V for 1 h, using TAE (Tris, Acetic acid, EDTA) (Sigma-Aldrich) buffer pH 8. To calculate the molecular size of the products from amplification, a molecular marker of 100-1000 bp (Inbio Highway, Buenos Aires, Argentina) was used. At the end of the run, the gel was transferred to a solution of TAE buffer and Ethidium Bromide (0.5 µg/mL) solution for 20 min. Then, the gel was visualized through UV light using a DNA light transilluminator U1000 (Labnet International Inc.) and photographed.

The presence of enterocin structural genes, was also evaluated by PCR amplification, the primers and protocols used are listed in **Table 1**. PCR products were analyzed by gel electrophoresis as described above.

Table 1 Primers used for PCR amplification of structural enterocin gen

Enterocin	Target gene and primer sequence (5´- 3´)	PCR conditions			PCR positive control	Size (bp)	Reference
		Temp (ºC)	Duration	No. of cycles			
Enterocin A	*entA* f: GGTACCACTCATAGTGGAAA r: CCCTGGAATTGCTCCACCTAA	95 95 95 72	5 min. 30 s 30 s 5 min.	1 30 1		138	
Enterocin B	*entB* f: CAAAATGTAAAAGAATTAAGTACG r: AGAGTATACATTTGCTAACCC	95 95 95 72	5 min. 30 s 30 s 5 min.	1 30 1	*E. faecium* ETW 20	201	
Enterocin P	*entP* f: GCTACGCGTTCATATGGTAAT r: TCCTGCAATATTCTCTTTAGC	95 95 95 72	5 min. 30 s 30 s 5 min.	1 30 1		87	(DE VUYST, 2003)
Enterocin LB50A	*entL50A* f: ATGGGAGCAATCGCAAAATTA r: TTTGTTAATTGCCCATCCTTC	95 95 95 72	5 min. 30 s 30 s 5 min.	1 30 1	*E. mundti* STw60	274	
Enterocin LB50B	*entL50B* f: ATGGGAGCAATCGCAAAATTA r: TAGCCATTTTTCAATTTGATC	95 95 95 72	5 min. 30 s 30 s 5 min.	1 30 1	*E. faecium* ETW20	274	

Table 1 *Continued*

Enterocin	Target gene and primer sequence (5´- 3´)	PCR conditions			PCR positive control	Size (bp)	Reference
		Temp (ºC)	Duration	No. of cycles			
Enterocin 96	*ent96* f: GTGGAGAGGACGAAAGGAGA r: TTGATTAGTGGAGAGGACGGATTA	95 95 60 72	15 min. 15 s 1 min. 1 min	1 40 1	-	291	(HENNING; GAUTAM; MURIANA, 2015)
Enterocin 31	*Bact31* f: CCTACGTATTACGGAAATGGT r: GCCATGTTGTACCCAACCATT	94 94 58 72	5 min. 30 s 30 s 45s	1 35 1	*E. faecalis* FA 2-2	130	(ÖZDEMIR et al., 2011)
Enterocin 1071	*Ent1071A/B* f: GGGGAGAGTCGGTTTTTAG r: ATCATATGCGGGTTGTAGCC	97 94 55 72 72	2 min. 45 s 30 s 45 s 2 min.	1 35 1	-	273	(MARTÍN et al., 2006)
Enterocin Q	*entqA* f: ATGAATTTTCTTCTTAAAAATGGTATCGCA r: TTAACAAGAAATTTTTTCCCATGGCAA	97 94 55 72	2 min. 1 min. 30 s 2 min.	1 35 1	*E. faecium* L50	105	(BELGACEM et al., 2010)
Mundticin KS	*mun KS* f: TGAGAGAAGGTTTAAGTTTTGAAGAA r: TCCACTGAAATCCATGAATGA	94 94 53 72	3 min. 30 s 30 s 1 min	1 30 1	*E. mundti* STw60	379	
Hiracin JM79	*HirJm79* f: ATGAAAAAGAAAGTATTAAAACATTGTGTTATTCTAGG r:ATAAGTTAAGCTTGTACTACCTTCTAGGTGCCCATGGACC	97 94 61 72 72	2 min. 45 s 30 s 30 s 7 min.	1 35 1	*E. hirae* DCH5	250	(ALMEIDA et al., 2011)

2.8. Statistical analysis

All the experiments were performed in triplicate and the results were evaluated by analysis of variance (ANOVA) using the software Statistica 12.0 (TIBCO, Palo Alto, CA, USA). The main values were compared using the Tukey test for a level of significance $p< 0.05$.

3. Results and discussion

3.1. Antimicrobial activity of potential BLIS produced by E. faecium 135

The results of antimicrobial activity through the turbidimetric method of the potential BLIS produced by *E. faecium* 135 against the bio-indicator strains *Salmonella enterica* (a), *S.* Typhimurium (b), and *L. monocytogenes* (c) are shown in **Fig. 1**. Assays against *S. enterica* revealed that the BLIS was able to reduce 37.7% the OD when compared to the control; however, it was not able to fully inhibit the growth of this pathogen during the 24 h of assay. Similar results were observed against *S.* Typhimurium, with a reduction of 42.4% of OD when compared to the control; but a not complete inhibition of the growth of this pathogen during the 24 h assayed.

Unlike the results obtained against *Salmonella* sp., the activity against *L. monocytogenes* showed a significant delay in the lag phase of this strain **(Fig. 1 c)**, with the OD value remaining at approx. 0.182 during 9 h and 14 h, for cultivations carried out at 30 and 35 ºC, respectively. Only after these long periods of lag phase, the strain was able to reach the exponential phase, achieving an OD of 0.560 after 24 h, which revealed a bacteriostatic effect of the BLIS.

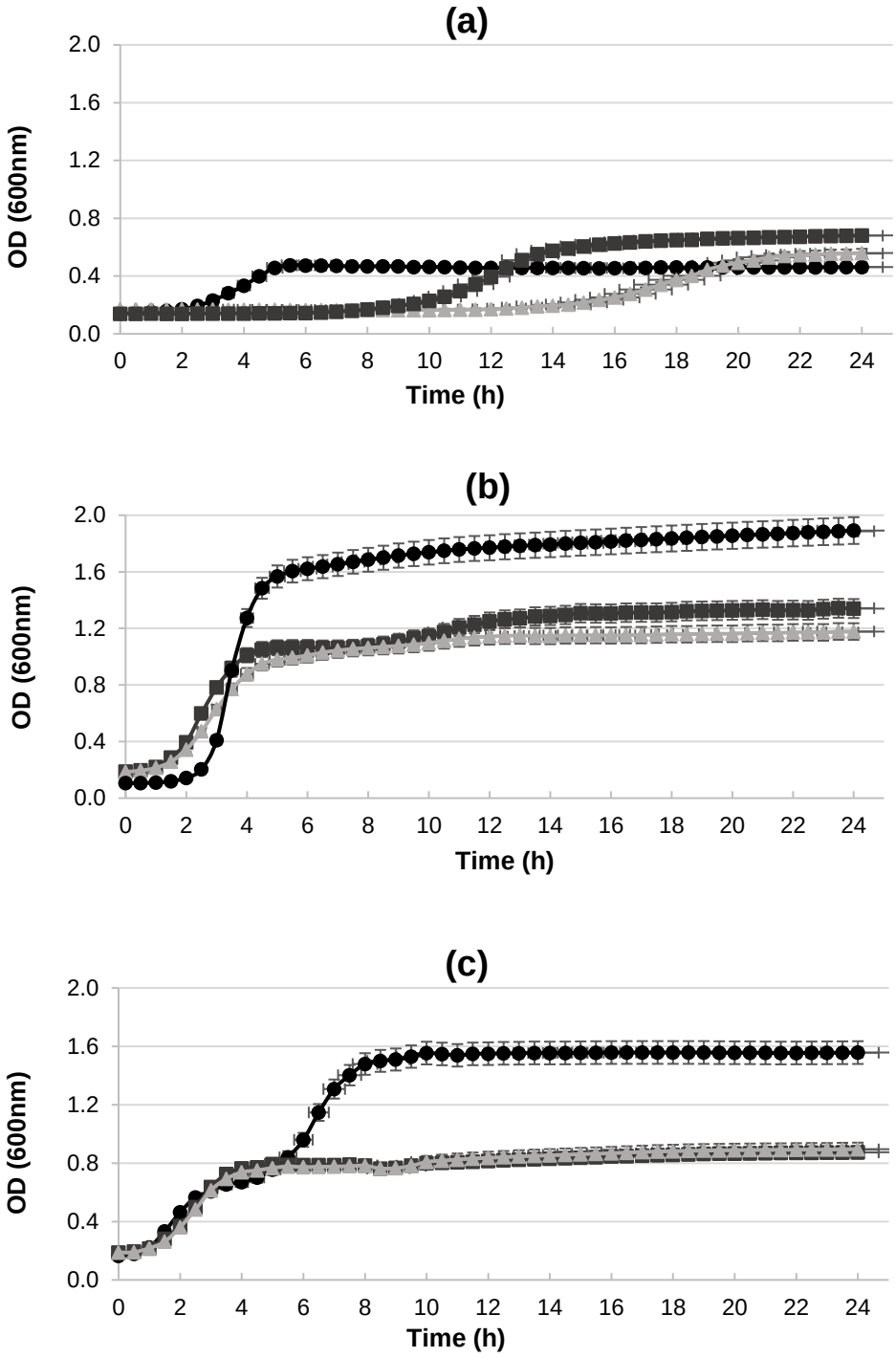

Fig. 1 Antimicrobial activity of BLIS produced by *E. faecium* against the bio-indicator strains *L. monocytogenes* (a), *S. enterica* (b), and *S.* Typhimurium (c). Assays performed with positive controls (●). Samples cultivated at 30 ºC (■) and 35 ºC (▲).

Fig. 2 shows the results of antimicrobial activity obtained by the agar diffusion method. These results confirmed the previous obtained by turbidimetric assay, with the highest inhibitory action of BLIS (activity ranging between 6,400 and 12,800 AU/mL) being observed against *L. monocytogenes* (**Fig. 2 a**). Antimicrobial activity was less pronounced against *S. enterica* (ranging between 200 and 400 AU/mL, **Fig. 2 b**) and for *S.* Typhimurium 400 AU/mL, **Fig. 2 c**) from *E. faecium* 135 cultures carried out at 35 ºC, 100/150 rpm, and without activity at 30 ºC. Overall, these results of antimicrobial activity are in agreement with other studies that have reported the production of BLIS and bacteriocins by other strains *of E. faecium* [9, 18, 38, 39], especially on the action against *L monocytogenes*, once the values of AU/mL were bigger against this strain, conforming the efficacy of the potential BLIS. Similar results were also reported by Baños et al. (2016) for antimicrobial activity of *E. faecalis* UGRA10 enterocin against *L. monocytogenes* in refrigerated raw salmon meat. The antimicrobial activities against the strains of *S. enterica* and *S.* Typhimurium were less pronounced when compared to *L. monocytogenes*, probably because they are Gram-negative bacteria. According to some authors, it is usually difficult to bind the antimicrobial compound to the membrane of Gram-negative bacteria (LAUKOVA¨ et al., 2003; MARTÍNEZ VIEDMA et al., 2008; SUVOROV, 2020).

L. monocytogenes, *S. enterica* and *S. Typhimurium* are considered important pathogens in the meat processing industry, mostly because the contamination can occur during the steps of cutting and evisceration where the pathogens present mostly on the intestinal content could get in contact with the meat (LAKICEVIC; NASTASIJEVIC, 2017), so a molecule that is capable of reduce the effect of these pathogens both in the animal's gastrointestinal tract and at the time it is meat is processed, it is of great interest (BAÑOS et al., 2016). In this sense, the BLIS produced by *E. faecium* 135 presented activity against those pathogens, showing potential for future utilization by this industry. By applying a microencapsulation technique, the structure and activity of the BLIS could remain intact (SOLTANI et al., 2021).

Fig. 2 also shows that the results of antimicrobial activity were not significantly ($p < 0.05$) affected by the variations in temperature (30 or 35 ºC) and agitation (100 or 150 rpm) evaluated for cultivation of *E. faecium*. Therefore, for the subsequent experiments, it was decided to perform the cultivations at 35 ºC and 100 rpm since such conditions gave the best results by the turbidimetric assay (**Fig. 1**) and were also the only ones that showed antimicrobial activity against all the bio-indicator strains tested by the agar

diffusion method (**Fig. 2**). After the positive results of antimicrobial activity, the treated CFS was now denominated as BLIS.

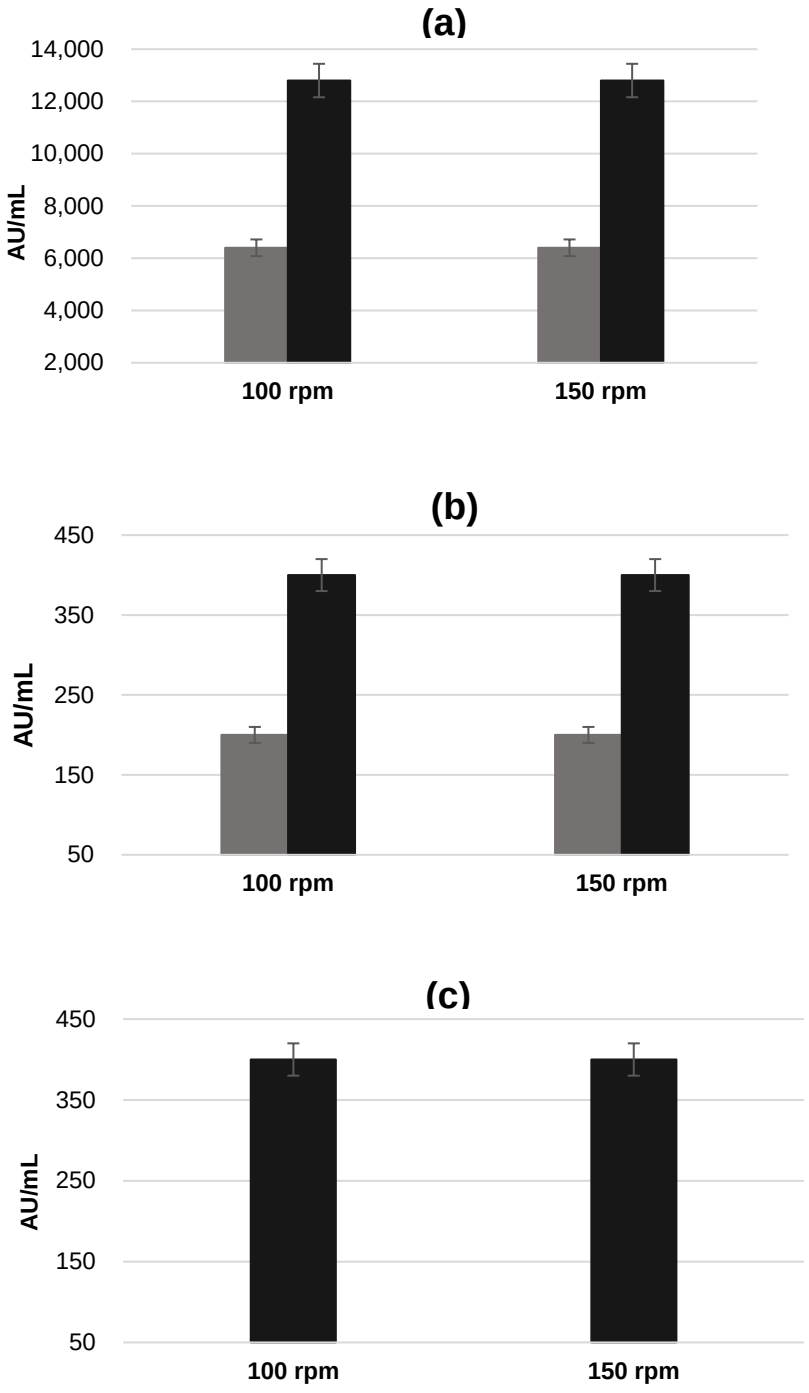

Fig. 2 Antimicrobial activity of BLIS produced by *E. faecium* 135 at 30ºC (■) and 35ºC (■), 100 and 150 rpm, by the agar diffusion method against the strains *L. monocytogenes* (a), *S. enterica* (b), and *S.* Typhimurium (c). Results are expressed in arbitrary units AU/mL.

3.2. BLIS concentration with ammonium sulfate and its proteinaceous nature

In an attempt to increase the activity of the BLIS produced by *E. faecium* 135, the BLIS was concentrated with 50% (w/v) ammonium sulfate solution and then treated with trypsin (1 mg/mL). Results revealed that, after concentrated, the size of inhibition halos from BLIS against *L. monocytogenes* was 25,600 AU/mL, which represented an increase of 50% when compared to non-concentrated BLIS (12,800 AU/mL).

Concentration with ammonium sulfate is usually used to precipitate proteins (TODOROV; DICKS, 2006) or as a pre-purification step for certain bacteriocins (MESSAOUDI et al., 2012; QIAO et al., 2020). In the present study, the isolation of proteins present in BLIS by treatment with ammonium sulfate resulted in a more active BLIS fraction against the bio-indicator strain since the interference of other compounds was reduced. It is worth mentioning that when the BLIS of *E. faecium* 135 was treated with trypsin (1 mg/mL), its antimicrobial activity ceased. This result indicates the proteinaceous nature of the antimicrobial compound present in the sample. Since trypsin is an enzyme with proteolytic activity, then the antimicrobial activity from *E. faecium* 135 probably came from the BLIS produced by this strain (remembering that BLIS and bacteriocins are small peptides). However, more tests using other proteases, as well as the application of more purification steps, would be useful to confirm the protein origin.

3.3. Stability of the BLIS produced by E. faecium 135

The stability of the BLIS is an important aspect to be evaluated since the use of salts and detergents during the purification of bacteriocins may interfere in the stability of the molecule (TODOROV; DICKS, 2006). Usually, solutions of 1% EDTA (w/v), SDS (w/v), Triton 100x (v/v), Tween-20 (v/v) or Tween-80 (v/v) present antimicrobial effect against the bio-indicator strain *L. innocua* CLIST 2711 (TODOROV; DICKS, 2006; TODOROV et al., 2011). In fact, when the reagents SDS, Tween 20, Tween 80, and Triton 100x were incubated with BLIS from *E. faecium* 135, a synergistic effect in the antimicrobial activity was observed (**Table 2**), with the halos increasing about two/three millimeters after BLIS interaction with those chemicals, when compared to the control. On the other hand, the presence of 1% NaCl did not affect the bio-indicator strain nor resulted in an additional effect when combined with BLIS from *E. faecium* 135. The same behavior was observed when EDTA was used, which was an unexpected result since EDTA usually has a certain antimicrobial activity because it is a chelating agent and has

ability to destabilize cell membranes (KHAN et al., 2015; MASTROMATTEO et al., 2010).

Regarding the thermic treatment, the use of temperature up to 90 ºC for 1 h did not affect the stability of the BLIS, revealing some heat stability, which is an important information for future purification of this biomolecule. A negative effect was only observed for higher temperature (120 ºC), in this case, even for a shorter period (15 min only) (**Table 2**). Overall, the results presented in **Table 2** were similar to those obtained by Todorov et al. (2006; 2011) when evaluating the stability of a bacteriocin produced by *Lactobacillus spp.*

Table 2 Effect of detergents, salts, and temperature on the stability of the BLIS produced by *Enterococcus faecium* 135.

Treatment	**Inhibition zone***
	Listeria innocua CLIST 2711
Control	++
Triton 100x	+++
SDS	+++
Tween-80	+++
Tween-20	+++
EDTA	++
NaCl	++
Thermic treatment:	
30, 50, 70 or 90 ºC for 1 hour	++
120 ºC for 15 minutes	-

* (+++) > 12 millimeters, (++) 11.0 – 11.99 millimeters, (+) 10.0 – 10.99 millimeters, and (-) did not show inhibition zone. Control: BLIS from *E. faecium* 135 without addition of any salt or detergent. The concentration of salts and detergents used for the experiments was of 1% (w/v) or (v/v).

3.4. Production of BLIS by microbial co-cultivation

E. faecium, Ligilactobacillus salivarius, and *Limosilactobacillus reuteri,* the strains used in co-cultivation in the present study, are commonly found as part of the microbiota of several animals and humans (KHAN; FLINT; YU, 2010; SUVOROV, 2020; ZHENG et al., 2020), which supports the idea that they could work in co-culture. The results of antimicrobial activity obtained from their co-cultivation are summarized in **Table 3.** As can be seen, when *Ligilactobacillus salivarius* and *Limosilactobacillus reuteri* were used in co-culture, no inhibitory activity was observed against any of the bio-indicator strains. However, the results were improved when *E. faecium* was used in co-cultivation with the other strains. The best results were obtained by co-cultivation of *E. faecium* with *Limosilactobacillus reuteri,* especially against *L. monocytogenes* (400 AU/mL) and *S. enterica* (200 AU/mL). Binary culture of *E. faecium* with *Ligilactobacillus salivarius* resulted in half of the antimicrobial activity against *L. monocytogenes* than the co-culture with *Limosilactobacillus reuteri*. Ternary culture of *E. faecium*, *Limosilactobacillus reuteri* and *Ligilactobacillus salivarius* also gave lower (200 AU/mL) antimicrobial activity when compared to the binary culture of *E. faecium* and *Limosilactobacillus reuteri* (400 AU/mL), and when comparing the ternary culture with the binary culture of *E. faecium* and *Ligilactobacillus salivarius*, both presented a similar antimicrobial activity (200 AU/mL). None of the tested co-cultures showed antimicrobial activity against *S.* Typhimurium.

Overall, the results obtained by co-cultures were less efficient than the antimicrobial activities obtained by monoculture. This would suggest that the strains compete by the carbon source. As it is known that the biosynthesis of many bacteriocins is regulated by quorum sensing trigger (DIEP; H»VARSTEIN; NES, 1995; NES et al., 1996), rather than presence of other species, the carbon competition may have prevented bacterial cells from reaching a critical number that would trigger bacteriocin production. In addition, the strain itself may produce compounds that interfere with the growth of the other strain such as organic acids and even BLIS (MALDONADO; RUIZ-BARBA; JIMÉNEZ-DIAAZ, 2004). *L. reuteri* INIA P579 and *L. salivarius* SMXD5, for example, are producers of bacteriocins (MESSAOUDI et al., 2012; MONTIEL et al., 2014). Similar to the results obtained in the present study, Giraffa et al. (1995) also observed a decrease in the production of enterocin when *E. faecium* 7C5 (an enterocin producer strain) was used in co-culture with *S. thermophilus* and *L. bulgaricus* (generally used in the preparation of cheeses and yogurts). According to the authors, when these three strains

are used directly for food production, they can produce organic acids, which are able to inhibit the growth of pathogenic strains such as *L. monocytogenes* and *S. enterica*. Taking this into account, it is possible to conclude that if live strains were used in the present study instead of their BLIS, probably different results would have been obtained, since these strains are also able to produce organic acids (GILLOR; ETZION; RILEY, 2008) that have antimicrobial activity.

Table 3 Inhibitory activity of BLIS produced by the binary and ternary cultures of *E. faecium*, *L. salivarius,* and *L. reuteri* against the bio-indicator strains *L. monocytogenes*, *S. enterica*, and *S.* Typhimurium. Results are expressed in arbitrary units per mL (AU/mL).

BLIS producing strains	**Bio-indicator strains**		
	L. monocytogenes	*S. enterica*	*S.* Typhimurium
L. salivarius + L. reuteri	-	-	-
E. faecium + L. reuteri	400 [a]	200 [a]	-
E. faecium + L. salivarius	200 [b]	-	-
E. faecium + L. reuteri + L. salivarius	200 [b]	-	-

Different letters in the same column mean statistically different values according to the Tukey's test ($p < 0.05$).

3.5 Molecular identification of enterocin genes in Enterococcus faecium 135

Analysis for molecular identification revealed that *E. faecium* 135 presents structural genes for the enterocins A (136 bp), B (198 bp), P (86 bp) and Mundticin KS (379 bp), which are classified as class II bacteriocins (FRANZ et al., 2007; NES; DIEP; HOLO, 2007). Enterocin P is the most prevalent in *Enterococcus* species, followed by enterocin A, whose production is usually associated to enterocin B. Those enterocins are known for the high activity against *L. monocytogenes* (ALMEIDA et al., 2011; FRANZ et al., 2007; HENNING; GAUTAM; MURIANA, 2015). Mundticin KS is produced by *E. mundti* NFRI 7393, for example, and it is known to have action against *L. monocytogenes* and *Clostridium botulinum* (FRANZ et al., 2007).

Other studies have also reported that some species of *Enterococcus* have more than one enterocin gene in their structure (ALMEIDA et al., 2011; HENNING; GAUTAM; MURIANA, 2015). However, the presence of different genes does not imply the

production of different bacteriocins or even the production of bacteriocins at all (FRANZ et al., 2007; LIU et al., 2011b). Liu *et al.* (2011a), for example, have purified two different enterocins produced by *E. faecium* LM-2 with proved action against pathogens such as *L. monocytogenes* and *Staphylococcus aureus.* In another study, Liu *et al.* (2011b) characterized two bacteriocins (Ent7A and Ent7B) produced by *E. faecalis* 710C, which had action against *Clostridium sporogenes* and *L. monocytogenes.* The results obtained in the present study suggest that probably some of these four enterocin genes could have been produced by *E. faecium* 135, due to the excellent activity observed against *L. monocytogenes.* However, purification of the BLIS is necessary to verify if some of these enterocins are being produced.

4. Conclusion

This study demonstrated that a newly isolated strain of *Enterococcus faecium* 135 is able to produce BLIS with antimicrobial activity against *L. monocytogenes* and also against *S. enterica* and *S.* Typhimurium. In addition, the produced BLIS was stable under high temperatures (up to 90 ^{o}C) as well as in the presence of several salts and detergents used for purification purposes, and showed susceptibility to trypsin, which suggest a proteinaceous nature of the BLIS produced by *E. faecium* 135. The presence of four structural enterocin class II genes was confirmed, suggesting the production of some type of enterocin by *E. faecium* 135. Production of BLIS using monoculture of *E. faecium* 135 resulted in better antimicrobial activity compared to the production by some co-cultures tested. However, further studies are needed to elucidate the behavior of this new strain when used in co-culture. Studies on the characterization of this BLIS, as well as on the purification of this molecule, would also give better indications on the properties of this BLIS or potential bacteriocin, which could be a promising molecule with great applicability's in animal breeding, and process of the meat, preventing contamination for the most common food-borne pathogens.

References

ACEDO, Jeella Z.; CHIOREAN, Sorina; VEDERAS, John C.; VAN BELKUM, Marco J. The expanding structural variety among bacteriocins from Gram-positive bacteria. **FEMS Microbiology Reviews**, *[S. l.]*, v. 42, n. 6, p. 805–828, 2018. DOI:

10.1093/femsre/fuy033. Disponível em: https://academic.oup.com/femsre/article/42/6/805/5063573.

ALMEIDA, Tereza et al. Identification of bacteriocin genes in enterococci isolated from game animals and saltwater fish. **Journal of Food Protection**, *[S. l.]*, v. 74, n. 8, p. 1252–1260, 2011. DOI: 10.4315/0362-028X.JFP-11-016.

BAÑOS, Alberto; GARCÍA-LÓPEZ, José David; NÚÑEZ, Cristina; MARTÍNEZ-BUENO, Manuel; MAQUEDA, Mercedes; VALDIVIA, Eva. Biocontrol of Listeria monocytogenes in fish by enterocin AS-48 and Listeria lytic bacteriophage P100. **LWT - Food Science and Technology**, *[S. l.]*, v. 66, p. 672–677, 2016. DOI: 10.1016/j.lwt.2015.11.025.

BELGACEM, Zouhaier Ben; ABRIOUEL, Hikmate; OMAR, Nabil Ben; LUCAS, Rosario; MARTÍNEZ-CANAMERO, Magdalena; GÁLVEZ, Antonio; MANAI, Mohamed. Antimicrobial activity, safety aspects, and some technological properties of bacteriocinogenic Enterococcus faecium from artisanal Tunisian fermented meat**. Food Control**, [S. l.], v. 21, n. 4, p. 462–470, 2010. DOI: 10.1016/j.foodcont.2009.07.007.

CABO, M. L.; MURADO, M. A.; GONZALEZ, M. P.; PASTORIZA, L. A method for bacteriocin quantification. **Journal of Applied Microbiology**, *[S. l.]*, v. 87, n. 6, p. 907–914, 1999. DOI: 10.1046/j.1365-2672.1999.00942.x. Disponível em: http://doi.wiley.com/10.1046/j.1365-2672.1999.00942.x.

CHAKCHOUK-MTIBAA, Ahlem; SELLEM, Imen; KAMOUN, Yosra; SMAOUI, Slim; KARRAY-REBAI, Ines; MELLOULI, Lotfi. Safety Aspect of Enterococcus faecium FL31 Strain and Antibacterial Mechanism of Its Hydroxylated Bacteriocin BacFL31 against Listeria monocytogenes. **BioMed Research International**, *[S. l.]*, v. 2018, p. 1–10, 2018. DOI: 10.1155/2018/5308464. Disponível em: https://www.hindawi.com/journals/bmri/2018/5308464/.

CHANOS, Panagiotis; MYGIND, Tina. Co-culture-inducible bacteriocin production in lactic acid bacteria. **Applied Microbiology and Biotechnology**, *[S. l.]*, v. 100, n. 10, p. 4297–4308, 2016. DOI: 10.1007/s00253-016-7486-8. Disponível em: http://dx.doi.org/10.1007/s00253-016-7486-8.

COTTER, Paul D.; HILL, Colin; ROSS, R. Paul. Food microbiology: Bacteriocins: Developing innate immunity for food. **Nature Reviews Microbiology**, *[S. l.]*, v. 3, n. 10, p. 777–788, 2005. DOI: 10.1038/nrmicro1273.

CROTTA, Matteo; GEORGIEV, Milen; GUITIAN, Javier. Quantitative risk assessment of Campylobacter in broiler chickens – Assessing interventions to reduce the level of contamination at the end of the rearing period. **Food Control**, *[S. l.]*, v. 75, p. 29–39, 2017. DOI: 10.1016/j.foodcont.2016.12.024. Disponível em:

http://dx.doi.org/10.1016/j.foodcont.2016.12.024.

DE VUYST, L. Screening for enterocins and detection of hemolysin and vancomycin resistance in enterococci of different origins. **International Journal of Food Microbiology**, [S. l.], v. 84, n. 3, p. 299–318, 2003. DOI: 10.1016/S0168-1605(02)00425-7.

DIEP, Dzung Bao; H»VARSTEIN, Leiv Sigve; NES, Ingolf F. A bacteriocin-like peptide induces bacteriocin synthesis in Lactobacillus plantarum C11. **Molecular Microbiology**, *[S. l.]*, v. 18, n. 4, p. 631–639, 1995. DOI: 10.1111/j.1365-2958.1995.mmi_18040631.x. Disponível em: http://doi.wiley.com/10.1111/j.1365-2958.1995.mmi_18040631.x. Acesso em: 14 maio. 2021.

DOMÍNGUEZ-MANZANO, Jesús; JIMÉNEZ-DÍAZ, Rufino. Suppression of bacteriocin production in mixed-species cultures of lactic acid bacteria. **Food Control**, *[S. l.]*, v. 30, n. 2, p. 474–479, 2013. DOI: 10.1016/j.foodcont.2012.09.014. Disponível em: https://linkinghub.elsevier.com/retrieve/pii/S0956713512005014.

FOOD SAFETY AUTHORITY, European; CENTRE FOR DISEASE PREVENTION, European. The European Union summary report on antimicrobial resistance in zoonotic and indicator bacteria from humans, animals and food in 2014 European Food Safety Authority European Centre for Disease Prevention and Control. *[S. l.]*, 2014. DOI: 10.2903/j.efsa.2016.4380. Disponível em: www.efsa.europa.eu/efsajournal. Acesso em: 25 nov. 2020.

FOULQUIÉ MORENO, M. R.; REA, M. C.; COGAN, T. M.; DE VUYST, L. Applicability of a bacteriocin-producing Enterococcus faecium as a co-culture in Cheddar cheese manufacture. **International Journal of Food Microbiology**, *[S. l.]*, v. 81, n. 1, p. 73–84, 2003. DOI: 10.1016/S0168-1605(02)00167-8.

FRANZ, Charles M. A. P.; VAN BELKUM, Marco J.; HOLZAPFEL, Wilhelm H.; ABRIOUEL, Hikmate; GÁLVEZ, Antonio. Diversity of enterococcal bacteriocins and their grouping in a new classification scheme. **FEMS Microbiology Reviews**, *[S. l.]*, v. 31, n. 3, p. 293–310, 2007. DOI: 10.1111/j.1574-6976.2007.00064.x.

GARCÍA-SÁNCHEZ, Lourdes; MELERO, Beatriz; JAIME, Isabel; HÄNNINEN, Marja Liisa; ROSSI, Mirko; ROVIRA, Jordi. Campylobacter jejuni survival in a poultry processing plant environment. **Food Microbiology**, *[S. l.]*, v. 65, p. 185–192, 2017. DOI: 10.1016/j.fm.2017.02.009.

GILLOR, O.; ETZION, A.; RILEY, M. A. The dual role of bacteriocins as anti- and probiotics. **Applied Microbiology and Biotechnology**, *[S. l.]*, v. 81, n. 4, p. 591–606, 2008. DOI: 10.1007/s00253-008-1726-5.

GIRAFFA, Giorgio; CARMINATI, Domenico; TARELLI, Giovanna Torri. Inhibition of listeria innocua in milk by bacteriocin-producing enterococcus faecium 7C5. **Journal of Food Protection**, *[S. l.]*, v. 58, n. 6, p. 621–623, 1995. DOI: 10.4315/0362-028X-58.6.621.

GUTIÉRREZ-CORTÉS, Carolina; SUAREZ, Héctor; BUITRAGO, Gustavo; NERO, Luis Augusto; TODOROV, Svetoslav Dimitrov. Enhanced Bacteriocin Production by Pediococcus pentosaceus 147 in Co-culture With Lactobacillus plantarum LE27 on Cheese Whey Broth. **Frontiers in Microbiology**, *[S. l.]*, v. 9, n. December, p. 1–12, 2018. DOI: 10.3389/fmicb.2018.02952.

HENNING, Chris; GAUTAM, Dhiraj; MURIANA, Peter. Identification of Multiple Bacteriocins in Enterococcus spp. Using an Enterococcus-Specific Bacteriocin PCR Array. **Microorganisms**, *[S. l.]*, v. 3, n. 1, p. 1–16, 2015. DOI: 10.3390/microorganisms3010001.

JACKSON, Charlene R.; FEDORKA-CRAY, Paula J.; BARRETT, John B. Use of a genus- and species-specific multiplex PCR for identification of enterococci. **Journal of Clinical Microbiology**, *[S. l.]*, v. 42, n. 8, p. 3558–3565, 2004. DOI: 10.1128/JCM.42.8.3558-3565.2004.

JOHNSON, Tylor J.; SHANK, Janette M.; JOHNSON, Jeremiah G. Current and potential treatments for reducing Campylobacter colonization in animal hosts and disease in humans. **Frontiers in Microbiology**, *[S. l.]*, v. 8, n. MAR, p. 1–14, 2017. DOI: 10.3389/fmicb.2017.00487.

KARIYAMA, R.; MITSUHATA, R.; CHOW, J. W.; CLEWELL, D. B.; KUMON, H. Simple and reliable multiplex PCR assay for surveillance isolates of vancomycin-resistant enterococci. **Journal of Clinical Microbiology**, *[S. l.]*, v. 38, n. 8, p. 3092–3095, 2000. DOI: 10.1128/jcm.38.8.3092-3095.2000.

KHAN, Avik; VU, Khanh Dang; RIEDL, Bernard; LACROIX, Monique. Optimization of the antimicrobial activity of nisin, Na-EDTA and pH against gram-negative and gram-positive bacteria. **LWT - Food Science and Technology**, *[S. l.]*, v. 61, n. 1, p. 124–129, 2015. DOI: 10.1016/j.lwt.2014.11.035.

KHAN, Haider; FLINT, Steve; YU, Pak-Lam. Enterocins in food preservation. **International Journal of Food Microbiology**, *[S. l.]*, v. 141, n. 1–2, p. 1–10, 2010. DOI: 10.1016/j.ijfoodmicro.2010.03.005.

KOS, Blaženka; BEGANOVIĆ, Jasna; JURAŠIĆ, Lina; ŠVADUMOVIĆ, Martina; LEBOŠ PAVUNC, Andreja; UROIĆ, Ksenija; ŠUŠKOVIĆ, Jagoda. Coculture-inducible bacteriocin biosynthesis of different probiotic strains by dairy starter culture Lactococcus lactis. **Mljekarstvo**, *[S. l.]*, v. 61, n. 4, p. 273–282, 2011.

LAKICEVIC, Brankica; NASTASIJEVIC, Ivan. Listeria monocytogenes in retail establishments: Contamination routes and control strategies. **Food Reviews International**, *[S. l.]*, v. 33, n. 3, p. 247–269, 2017. DOI: 10.1080/87559129.2016.1175017.

LAUKOVA¨, Laukova¨; GUBA, A.; NEMCOVA¨, P.; NEMCOVA¨, R.; VASILKOVÄ, Z. Reduction of Salmonella in gnotobiotic Japanese quails caused by the enterocin A-producing EK13 strain of *Enterococcus faecium*. **Veterinary Research Communications**. [s.l: s.n.]. DOI: https://doi.org/10.1023/A:1024027923824.

LIU, Chuanbin; LIU, Yan; LIAO, Wei; WEN, Zhiyou; CHEN, Shulin. Simultaneous production of nisin and lactic acid from cheese whey: Optimization of fermentation conditions through statistically based experimental designs. **Applied Biochemistry and Biotechnology - Part A Enzyme Engineering and Biotechnology**, *[S. l.]*, v. 114, n. 1–3, p. 627–638, 2004. DOI: 10.1385/ABAB:114:1-3:627.

LIU, Guorong; GRIFFITHS, Mansel W.; WU, Pengpeng; WANG, Haifeng; ZHANG, Xiangmei; LI, Pinglan. Enterococcus faecium LM-2, a multi-bacteriocinogenic strain naturally occurring in “Byaslag”, a traditional cheese of Inner Mongolia in China. **Food Control**, *[S. l.]*, v. 22, n. 2, p. 283–289, 2011. a. DOI: 10.1016/j.foodcont.2010.07.023.

LIU, Xiaoji; VEDERAS, John C.; WHITTAL, Randy M.; ZHENG, Jing; STILES, Michael E.; CARLSON, Denise; FRANZ, Charles M. A. P.; MCMULLEN, Lynn M.; VAN BELKUM, Marco J. Identification of an N-Terminal Formylated, Two-Peptide Bacteriocin from Enterococcus faecalis 710C. **Journal of Agricultural and Food Chemistry**, *[S. l.]*, v. 59, n. 10, p. 5602–5608, 2011. b. DOI: 10.1021/jf104751v.

MALDONADO, Antonio; RUIZ-BARBA, José Luis; JIMÉNEZ-DIAAZ, Rufino. Production of plantaricin NC8 by Lactobacillus plantarum NC8 is induced in the presence of different types of gram-positive bacteria. **Archives of Microbiology**, *[S. l.]*, v. 181, n. 1, p. 8–16, 2004. DOI: 10.1007/s00203-003-0606-8.

MARTÍN, María; GUTIÉRREZ, Jorge; CRIADO, Raquel; HERRANZ, Carmen; CINTAS, Luis M.; HERNÁNDEZ, Pablo E. Genes encoding bacteriocins and their expression and potential virulence factors of enterococci isolated from wood pigeons (*Columba palumbus*). **Journal of Food Protection**, [S. l.], v. 69, n. 3, p. 520–531, 2006. DOI: 10.4315/0362-028X-69.3.520.

MARTÍNEZ-CANAMERO, Magdalena; GÁLVEZ, Antonio; MANAI, Mohamed. Antimicrobial activity, safety aspects, and some technological properties of bacteriocinogenic Enterococcus faecium from artisanal Tunisian fermented meat. **Food Control**, [S. l.], v. 21, n. 4, p. 462–470, 2010. DOI: 10.1016/j.foodcont.2009.07.007.

MARTÍNEZ VIEDMA, Pilar; SOBRINO LÓPEZ, Angel; BEN OMAR, Nabil; ABRIOUEL, Hikmate; LUCAS LÓPEZ, Rosario; VALDIVIA, Eva; MARTÍN BELLOSO, Olga; GÁLVEZ, Antonio. Enhanced bactericidal effect of enterocin AS-48 in combination with high-intensity pulsed-electric field treatment against Salmonella enterica in apple juice. **International Journal of Food Microbiology**, *[S. l.]*, v. 128, n. 2, p. 244–249, 2008. DOI: 10.1016/j.ijfoodmicro.2008.08.014.

MASTROMATTEO, Marianna; LUCERA, Annalisa; SINIGAGLIA, Milena; CORBO, Maria Rosaria. Synergic antimicrobial activity of lysozyme, nisin, and EDTA against listeria monocytogenes in ostrich meat patties. **Journal of Food Science**, *[S. l.]*, v. 75, n. 7, 2010. DOI: 10.1111/j.1750-3841.2010.01732.x.

MESSAOUDI, S.; MANAI, M.; KERGOURLAY, G.; PRÉVOST, H.; CONNIL, N.; CHOBERT, J. M.; DOUSSET, X. Lactobacillus salivarius: Bacteriocin and probiotic activity. **Food Microbiology**, *[S. l.]*, v. 36, n. 2, p. 296–304, 2013. DOI: 10.1016/j.fm.2013.05.010. Disponível em: http://dx.doi.org/10.1016/j.fm.2013.05.010.

MESSAOUDI, Soumaya et al. Purification and characterization of a new bacteriocin active against Campylobacter produced by Lactobacillus salivarius SMXD51. **Food Microbiology**, *[S. l.]*, v. 32, n. 1, p. 129–134, 2012. DOI: 10.1016/j.fm.2012.05.002.

MITSUOKA, Tomotari. Development of functional foods. **Bioscience of Microbiota, Food and Health**, *[S. l.]*, v. 33, n. 3, p. 117–128, 2014. DOI: 10.12938/bmfh.33.117.

MONTIEL, R.; MARTÍN-CABREJAS, I.; LANGA, S.; EL AOUAD, N.; ARQUÉS, J. L.; REYES, F.; MEDINA, M. Antimicrobial activity of reuterin produced by Lactobacillus reuteri on Listeria monocytogenes in cold-smoked salmon. **Food Microbiology**, *[S. l.]*, v. 44, p. 1–5, 2014. DOI: 10.1016/j.fm.2014.05.006.

NES, Ingolf F.; DIEP, Dzung B.; HOLO, Helge. Bacteriocin Diversity in Streptococcus and Enterococcus. **Journal of Bacteriology**, *[S. l.]*, v. 189, n. 4, p. 1189–1198, 2007. DOI: 10.1128/JB.01254-06.

NES, Ingolf F.; DIEP, Dzung Bao; HAVARSTEIN, Leiv Sigve; BRURBERG, May Bente; EIJSINK, Vincent; HOLO, Helge. Biosynthesis of bacteriocins in lactic acid bacteria. **Antonie van Leeuwenhoek**, *[S. l.]*, v. 70, n. 2–4, p. 113–128, 1996. DOI: 10.1007/BF00395929.

ÖZDEMIR, Gamze Başbülbül; ORYAŞIN, Erman; BIYIK, H. Halil; ÖZTEBER, Melihcan; BOZDOĞAN, Bülent. Phenotypic and Genotypic Characterization of Bacteriocins in Enterococcal Isolates of Different Sources. Indian **Journal of Microbiology**, [S. l.], v. 51, n. 2, p. 182–187, 2011. DOI: 10.1007/s12088-011-0143-0.

QIAO, Xiaoxiao; DU, Renpeng; WANG, Yu; HAN, Ye; ZHOU, Zhijiang. Purification,

characterization and mode of action of enterocin, a novel bacteriocin produced by Enterococcus faecium TJUQ1. **International Journal of Biological Macromolecules**, *[S. l.]*, v. 144, p. 151–159, 2020. DOI: 10.1016/j.ijbiomac.2019.12.090.

RAMCHANDRAN, Lata; SHAH, Nagendra P. Characterization of functional, biochemical and textural properties of synbiotic low-fat yogurts during refrigerated storage. **LWT - Food Science and Technology**, *[S. l.]*, v. 43, n. 5, p. 819–827, 2010. DOI: 10.1016/j.lwt.2010.01.012.

ROJOBEZARES, B.; SAENZ, Y.; NAVARRO, L.; ZARAZAGA, M.; RUIZLARREA, F.; TORRES, C. Coculture-inducible bacteriocin activity of Lactobacillus plantarum strain J23 isolated from grape must. **Food Microbiology**, *[S. l.]*, v. 24, n. 5, p. 482–491, 2007. DOI: 10.1016/j.fm.2006.09.003.

SOLTANI, Samira et al. Bacteriocins as a new generation of antimicrobials: toxicity aspects and regulations. **FEMS Microbiology Reviews**, *[S. l.]*, v. 45, n. 1, p. 1–24, 2021. DOI: 10.1093/femsre/fuaa039.

STROMPFOVÁ, Viola; KUBAŠOVÁ, Ivana; ŠČERBOVÁ, Jana; MAĎARI, Aladár; GANCARČÍKOVÁ, Soňa; MUDROŇOVÁ, Dagmar; MILTKO, Renata; BELZECKI, Grzegorz; LAUKOVÁ, Andrea. Oral administration of bacteriocin-producing and non-producing strains of Enterococcus faecium in dogs. **Applied Microbiology and Biotechnology**, *[S. l.]*, v. 103, n. 12, p. 4953–4965, 2019. DOI: 10.1007/s00253-019-09847-3.

SUVOROV, Alexander. What Is Wrong with Enterococcal Probiotics? **Probiotics and Antimicrobial Proteins**, *[S. l.]*, v. 12, n. 1, p. 1–4, 2020. DOI: 10.1007/s12602-020-09633-y.

TODOROV, S. D.; DICKS, L. M. T. Screening for bacteriocin-producing lactic acid bacteria from boza, a traditional cereal beverage from Bulgaria. **Process Biochemistry**, *[S. l.]*, v. 41, n. 1, p. 11–19, 2006. DOI: 10.1016/j.procbio.2005.01.026.

TODOROV, Svetoslav Dimitrov; FURTADO, Danielle Nader; SAAD, Susana Marta Isay; GOMBOSSY DE MELO FRANCO, Bernadette Dora. Bacteriocin production and resistance to drugs are advantageous features for Lactobacillus acidophilus La-14, a potential probiotic strain. **The new microbiologica**, *[S. l.]*, v. 34, n. 4, p. 357–70, 2011.

ZHENG, Jinshui et al. A taxonomic note on the genus Lactobacillus: Description of 23 novel genera, emended description of the genus Lactobacillus Beijerinck 1901, and union of Lactobacillaceae and Leuconostocaceae. **International Journal of Systematic and Evolutionary Microbiology**, *[S. l.]*, v. 70, n. 4, p. 2782–2858, 2020. DOI: 10.1099/ijsem.0.004107.

CHAPTER
.2.

Use of coffee residues as alternative media for growth and production of antimicrobial compounds from *Enterococcus faecium* 135

Abstract

Coffee is among the most consumed beverages worldwide, with consumption and production increasing annually. The generation of residues is an important concern in this field, with spent coffee grounds (SCG) and coffee silverskin (CS) the most common coffee residues produced. Both residues are known for being sources of cellulose and hemicellulose, presenting the potential to be used as alternative carbon sources. SCG was pre-treated with 100 mg H_2SO_4/g for 45 min and CS with 120 mg H_2SO_4/g for 75 min, both treatments performed in autoclave at 140º C. Then, the hydrolysates had their pH changed to 2.5, were autoclaved at 121º C for 60 min, had their pH adjusted to 6.0 using NaOH, and finally were filtered (0.22 µm). To reduce the number of phenolic compounds, part of the hydrolysates was also detoxified with a C-18 silica cartridge. The elaborated growth media has the same amount of nitrogen sources and salts present on MRS (de Man, Rogosa and Sharp), and used 0, 25, 50, and 100% (v/v) of SCG and CS hydrolysates as carbon source, as well as the detoxified version. The total sugar recovered was 43.15 g/L for SCG, and 27.61 g/L for CS 5 times concentrated. 5-HMF (hydroxymethylfurfural) and furfural concentrations were reduced from 0.53 and 0.01 g/L on SCG to 0.001 and 0.0001 g/L, respectively. For CS the values were from 0.48 and 0.03 g/L to 0.28 and 0.02 g/L. *Enterococcus faecium* 135, was used due to its BLIS production and anti-listeric activity. The strain was cultivated on the elaborated media and MRS (control) at orbital agitator 100 rpm, at 35 ºC for 24 hours. After this period *E. faecium* was able to grow above 1 log CFU/mL on the elaborated media and showed antimicrobial activity of 480 and 428 AU/mL for the cultivations carried out in CS 25% and SCG detox supplemented, respectively. Furthermore, the acid lactic production was 10.51 and 8.26 g/L for CS 25% and SCG detox supplemented. Tangential filtration (30 and 2 kDa) was performed to concentrate the BLIS, resulting in an increase in antimicrobial activity as well as the presence of a band on the Tris-Tricine gel. In conclusion, the use of coffee residues as a carbon source can be an alternative to conventional growth media. The preliminary results show that it can be used for the growth of important bacteria, such as acid lactic bacteria, and that the production of BLIS and organic acids was not altered.

Keywords: coffee residues, hydrolysate, carbon source, bacteria growth, antimicrobial activity.

Introduction

Coffee production and consumption increase every year. According to ICO (International Coffee Organization), only in 2020 the total production was about 175,347 in thousand bags (60 kg), being Brazil the largest producer with 69 million bags in 2020 (ICO - International Coffee Organization, 2021). Between the generated residues from coffee production are the spent coffee grounds (SCG), a solid residue generated after the process of coffee processing, and the coffee silverskin (CS), a residue obtained after the roast of the coffee beans (KLEINWÄCHTER; BYTOF; SELMAR, 2015). The CS has been used as soil fertilizer (SAENGER et al., 2001), as antioxidant sources for cosmetical applications (NARITA; INOUYE, 2014), and as prebiotic for microorganisms (BORRELLI et al., 2004). The SCG has been used on the production of biofuels, bio-active compounds (phenolic compounds, carotenoids and fertilizers), and materials (plastics and adsorptive filtering). Due to its potential of recover sugars, SCG can be used in the growth of microorganisms such as fungi and yeasts (JIMÉNEZ-ZAMORA; PASTORIZA; RUFIÁN-HENARES, 2015; MACHADO et al., 2012; MATA; MARTINS; CAETANO, 2018; MCNUTT; HE, 2019). As described in the literature both CS and SCG could have many applications, however its use as a potential media for the growth of microorganisms is still underrated.

Lactic acid bacteria (LAB) are known for their ability to produce BLIS (bactericion like inhibitory substances) and bacteriocins (WORAPRAYOTE et al., 2016). This group of bacteria is extensively used in the formulation of food products because they are considered safe (GRAS, Generally Reported as Safe) (QIN et al., 2017). The bacteriocins or BLIS are peptides or proteins produced by the ribosomes and excreted into the extracellular environment. They exert antimicrobial activity against other bacterial species (COTTER; HILL; ROSS, 2005). Many strains of LAB produce BLIS/bacteriocins and one of the most studied is the bacteria of the genus *Lactobacillus* sp. Due to its use in the fermented food industry (COTTER; HILL; ROSS, 2005). *Lactococcus* sp. (PEREIRA et al., 2022; SABO et al., 2018), *Pediococcus* sp. (KUNIYOSHI et al., 2021), and *Enterococcus* sp. (PIAZENTIN et al., 2022; SINGHAL et al., 2019) are other important LAB that have been studied for their ability to produce BLIS/bacteriocins.

Enterococcus faecium has been cited in the literature as an important producer of enterocins (DE VUYST, 2003; HENNING; GAUTAM; MURIANA, 2015), mainly with anti-listeria activity (LI et al., 2017; MARTÍNEZ VIEDMA et al., 2008). Although *E. faecium* has sometimes been described as a pathogen (Oliveira & Bettcher, 2010), some studies have shown its probiotic potential (KHAN; FLINT; YU, 2010; PEREIRA et al., 2022). *E. faecium* has the ability to consume different carbohydrates for its growth, being mannose one of the most consumed, even more than glucose (BLAJMAN et al., 2020; PETRUT et al., 2019).

Thus, the aim of this study was to evaluate the possible use of CS and SCG residues as carbon sources for the elaboration of an alternative growth media for *E. faecium* 135. This strain is already characterized as a BLIS producer with anti-listeria activity (PIAZENTIN et al., 2022). The effect of phenolic compounds presents in CS and SCG on the growth of *E. faecium* was also evaluated.

2. Material and methods

2.1 Spent Coffee Grounds (SCG) and Coffee Silverskin (CS)

The SCG was obtained from the coffee machines at the de Novo Nordisk Center for Biosustainability, at the Technical University of Denmark (DTU), Kongens Lyngby campus. The coffee powder was obtained from Jacobs Douwe Egberts company (JDE), Amsterdam, The Netherlands. SCG was dried overnight at 60 oC until the humidity was less than 10% (w/w).The CS was donated by a local Danish company that roasted green coffee beans.

2.2 Pre-treatment and detoxification of SCG and CS

Based on the results presented by Mussatto et al. (2011b), SCG was pre-treated with 100 mg H2SO4/g for 45 minutes and CS with 120 mg H_2SO_4/g for 75 minutes, both treatments performed in an autoclave at 140 ^{o}C. The hydrolysates were filtered to remove solid residues, and the recovered liquid was adjusted to 4% (w/w) H_2SO_4 concentration

before being autoclaved at 121 ºC for 60 minutes. Due to the low concentration of total sugars of CS hydrolyses (9.67 g/L), the CS was concentrated five times using a rote evaporator (IKA, Germany) to eliminate the water. For detoxification (BONFIGLIO et al., 2021), the hydrolysates were adjusted to 2.47 with NaOH pellets, then passed through a silica column C-18 Cartridges (Waters, Ireland), and finally, the detoxified fraction had the pH adjusted to 6.0 with NaOH pellets and was filtered and sterilized (0.22 µm) (**Figure 1**).

2.3 Analytical methodology

The concentrations of glucose, arabinose, mannose, galactose, xylose, cellobiose, acetic acid, lactic acid, furfural and hydroxymethylfurfural, presented on the hydrolysates and after fermentation, were determined by the methodology described by (LIU et al., 2021). Phenolic compounds were determined using the Folin-Ciocalteu reagent, using the methodology described by Mussatto, et al. (2011a) using gallic acid as standard. The nitrogen content in hydrolysates was measured using a primary nitrogen assay and a urea and ammonia assay kit (Megazyme International, Wicklow, Ireland).

2.4 Elaboration of alternative media

The alternative media were based on the salts and nitrogen sources (**Table 1**) from commercial media MRS (de Man, Rogosa and Sharp), using the hydrolysates detoxified or not as a sugar source. In this case, the media were elaborated with 0, 25, 50 and 100 % (v/v) of non-detoxicated hydrolysate. In this case, the amounts of sugar were supplemented, to be equivalent to the amount present in 100% of the hydrolysate. In the detoxicated version, it was used only 100% (v/v). Commercial MRS (Difco, USA), was used as a control.

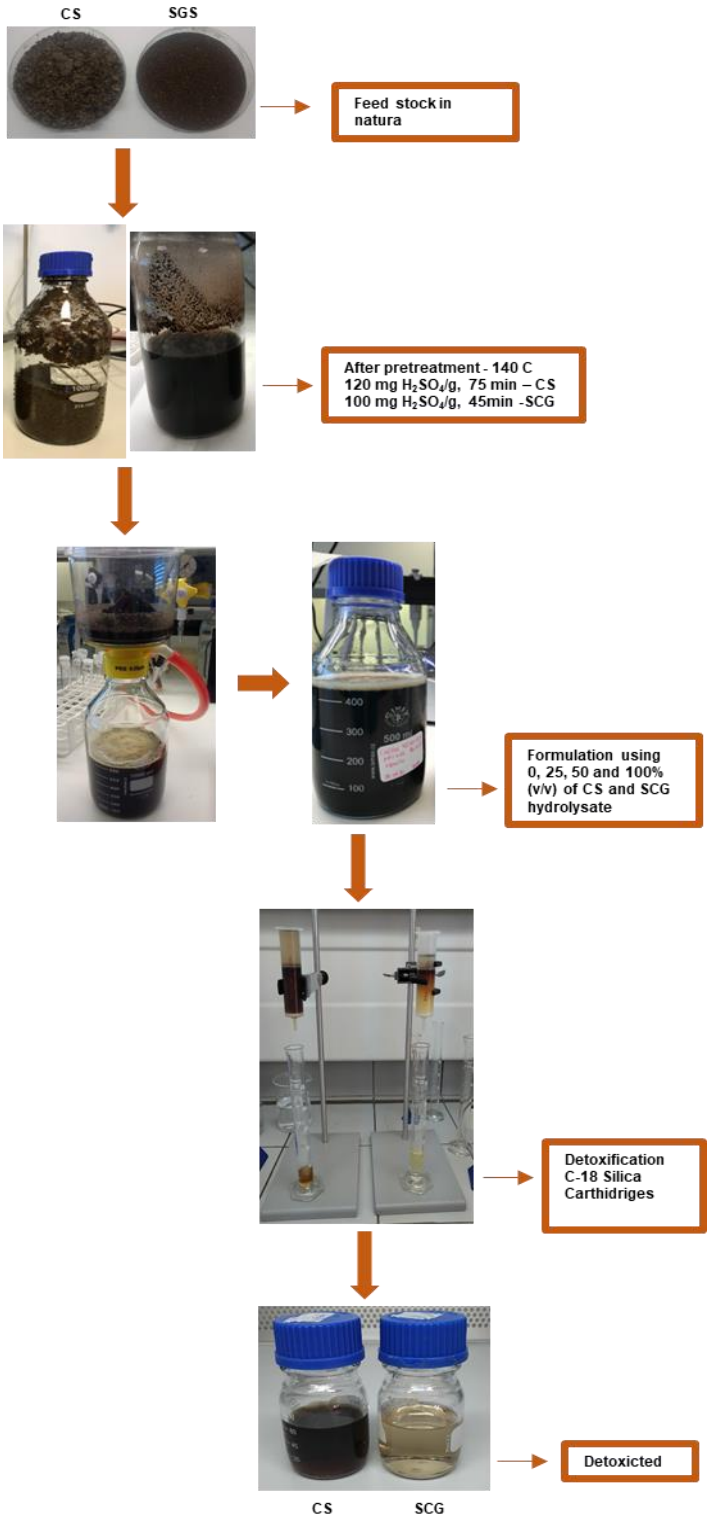

Figure 1. Schematic representation of the pretreatment and detoxification of CS and SCG

Table 1. MRS **(**de Man, Rogosa and Sharp) medium composition (g/L).

Compound	**Concentration g/L**
Peptone	10.00
Beef extract	10.00
Yeast extract	5.00
Polysorbate 80	1.00
Ammonium citrate	2.00
Sodium acetate	5.00
Magnesium sulfate	0.10
Manganese sulfate	0.05
Dipotassium phosphate	2.00
Sugar (dextrose)	20.00

2.5 Cultivation of E. faecium

The *E. faecium* 135 was isolated from a starfish (Order Forcipulatida) in Playa Unión, Rawson-Chubut (Patagonia, Argentina) and was kindly donated by Professor Marisol Vallejo from the National University of Patagonia San Juan Bosco (Argentina). *E. faecium* was chosen due to its capability to produce BLIS (Bacteriocin like inhibitory substances) (PIAZENTIN et al., 2022). The frozen strain was reactivated in commercial MRS (Difco, MD, USA), where 0.5 mL of the strain was added to 10 mL of MRS broth. The tube was maintained at 37 C overnight. The strain was then washed with 0.9% w/v saline solution, settled to 800 nm OD (~ 7 log CFU/mL), and resuspended on the elaborated media. Cultures were performed on Erlenmeyer flasks containing 20 mL of medium and placed in an orbital shaker at 100 rpm for 24 h. After the culture was centrifuged (Thermo Scientific, Germany) at 4,470 g, at 4 C for 15 min, the supernatant had the pH changed to 6.5 and filtered in a 0.45 µm, and submitted to a thermal bath of 80º C for 10 min.

2.6 Viability of cells

Cell counting was performed at the beginning of cultivation and after 24 hours. Aliquots of 1.0 mL were taken and homogenized with 9.0 mL of peptone water, 0.1% (w/v), and serially diluted. The MRS agar (Difco, Detroit, MI, USA) was used for the

growth of *E. faecium* through the pour plate technique. The results were expressed in log CFU/mL. The pH of the media was determined at the end of the cultivation (24 h) using a pHmeter (Mettler-Toledo, Germany).

2.7 Antimicrobial activity

To evaluate the antimicrobial potential of the BLIS obtained from alternative culture media, the spot-on law technique was used (KUNIYOSHI et al., 2021; PEREIRA et al., 2022). The following indicator strains were used, *Listeria monocytogenes* CECT 934, *Salmonella enterica* serovar Chloreaesuis CECT 724 (both strains from the Spain Collection of Cultures, Spain) and *Salmonella enterica* serovar Typhimurium IOC 5551/16 (kindly donated by Fiocruz, Brazil). The strains were grown in BHI (Difco) broth, and their OD (optical density) was adjusted to 600 nm for *L. monocytogenes* and 300 nm to *S.* Chloreaesuis and *S.* Typhimurium. The indicators were added in a 0.75% (w/v) BHI agar, and once the agar was solid, 10 µL of *E. faecium* supernatant was added, and the plates were kept at 37º C for 18 hours. After this period, the inhibition halos were observed. The inhibitory activity was expressed in arbitrary units per mL (AU/mL) using the formula described below (1), in which π. R^2 is the area of the inhibition zone (cm^2) and V is the volume (mL) of BLIS used.

$$AU/mL = \frac{\pi.R2}{V} \qquad (1)$$

2.8 Pre-purification of BLIS

In order to concentrate the BLIS and reduce other compounds, the *E. faecium* cultured supernatant was passed through an Amicon tube (Merck-Millipore, USA) of 30 and 2 kDa, then the antimicrobial activity of the fractions was tested as described in topic 2.7. SDS gel Tris-Tricine was used to evaluate the size of the peptides present on the BLIS, using the fractions obtained through tangential filtration (SCHÄGGER, 2006).

2.9 Statistical analysis

Results were expressed as a mean ± standard deviation. The analyses were carried out in triplicate. The difference between the enumeration of viable cells and pH was submitted to analysis of variance (ANOVA) using the Statistica 12.0 software (TIBCO, USA). Mean values were compared using the Tukey's test ($p < 0.05$).

3. Results

3.1 Pre-treatment and detoxification

After the acid pre-treatment, the total sugars recovered (**Table 2**) from SCG was of 43.15 g/L and for CS was 9.67 g/L (data not shown). To increase the quantity of sugars, the hydrolysate was concentrated about 5 times and the total sugars raised to 27.61 g/L. Mannose was the monomers mostly present on SCG being 28 g/L for the raw hydrolysate, even though CS has a composition most equilibrated with similar quantities of xylose, galactose and mannose.

The process of detoxification reduced about 72% of total phenolic compounds from SCG, and 38% from CS (**Table 2**). However, CS has more than 5.0 g/L of phenolic compounds when compared with SCG, although raw SCG has more 5-HMF (0.53 g/L) and furfural (0.10 g/L) than raw CS 0.48 and 0.03 g/L, respectively, while CS has more than 1 g/L of 4-hydroxybenzoic acid compared to SCG. Besides the reduction of phenolic compounds, the detoxification also reduced by over 100 mg/L the primary amino acids present in the hydrolysate.

Table 2. Chemical composition of spent coffee grounds (SCG) and coffee silverskin (CS) hydrolysates raw, and after detoxification.

Compound	Concentration in the hydrolysate (g/L)			
	SCG hydrolysate		CS hydrolysate	
	Raw	Detoxicated	Raw (Conc. 5x)	Detoxicated
Cellobiose	0.211 ± 0.081	0.566 ± 0.285	0.310 ± 0.019	0.396 ± 0.013
Glucose	2.981 ± 0.458	0.962 ± 0.055	3.992 ± 0.394	nd
Xylose	nd	0.078 ± 0.038	6.615 ± 0.289	nd
Galactose	11.849 ± 0.075	11.447 ± 0.087	7.231 ± 0.021	9.277 ± 0.245
Arabinose	nd	nd	1.604 ± 0.276	nd
Mannose	28.322 ± 0.389	26.402 ± 0.094	8.172 ± 0.036	15.610 ± 0.326
5- HMF	0.527 ± 0.015	0.001 ± 0.000	0.481 ± 0.006	0.284 ± 0.087
Furfural	0.093 ± 0.020	nd	0.031 ± 0.007	0.018 ± 0.001
4-Hydroxybenzoic acid	0.228 ± 0.065	nd	1.236 ± 0.506	0.011 ± 0.001
Vanillic acid	0.139 ± 0.000	0.001 ± 0.000	0.157 ± 0.046	0.040 ± 0.010
Syringic acid	0.002 ± 0.001	nd	0.119 ± 0.006	0.033 ± 0.007
4-Hydroxybenzaldehyde	0.035 ± 0.002	nd	0.057 ± 0.002	0.030 ± 0.001
Coumaric acid	0.004 ± 0.001	nd	0.000 ± 0.000	0.000 ± 0.000
Vanillin	0.001 ± 0.000	nd	0.029 ± 0.003	0.001 ± 0.001
Syringaldehyde	0.015 ± 0.002	nd	0.018 ± 0.004	0.001 ± 0.001
Benzoic acid	nd	nd	nd	0.002 ± 0.000
Total phenolics	1.727 ± 0.057	0.473 ± 0.013	6.844 ± 0.011	4.235 ± 0.010
Total nitrogen (PAN) mg N/L	134.930 ± 0.427	26.294 ± 0.288	200.924 ± 0.367	99.943 ± 0.165

Nd = not detected.

LAB growth always involves the production of organic acids and *E. faecium*, being a facultative heterofermentative bacterium, is able to produce lactic and acetic acids (BLAJMAN et al., 2020). Following the counts of viable cells, the production of lactic acid (**Table 3**) on CS 25% was of 10.51 g/L and 8.26 g/L on SCG detox supplemented. However, the production of acetic acid was higher (8.24 g/L) in the CS detox supplemented, followed by 3.55 g/L on SCG detox supplemented and 2.91 g/L on CS 25%. In general, the cultives carried out on CS hydrolysates presented higher quantities

of organic acids when compared to SCG. The pH of the control, SCG detox supplement and CS 25% was about 4.6.

Table 3. Concentration (g/L) of organic acids and pH on the media based on the hydrolysates of SCG and CS, containing 0, 25, 50 and 100 % (v/v) of hydrolysate, and the detoxicated hydrolysate supplemented with nitrogen sources, after 24 hours of fermentation.

Elaborated media	Lactic acid (g/L)	Acetic acid (g/L)	Formic acid (g/L)	pH
Control	7.48 ± 0.62	3.70 ± 0.06	nd	4.69 ± 0.02
SCG				
0%	6.50 ± 0.03	2.70 ± 0.05	nd	4.71 ± 0.03
25%	6.80 ± 0.01	3.16 ± 0.08	nd	4.85 ± 0.02
50%	7.15 ± 0.05	2.78 ± 0.02	0.17 ± 0.01	4.85 ± 0.07
100%	6.91 ± 0.10	3.13 ± 0.07	0.37 ± 0.06	5.04 ± 0.03
Detox	0.29 ± 0.18	0.57 ± 0.00	0.32 ± 0.00	5.78 ± 0.01
Detox + Supl.	8.26 ± 0.05	3.55 ± 0.01	0.50 ± 0.08	4.67 ± 0.05
CS				
0%	7.46 ± 0.05	2.31 ± 0.01	nd	4.32 ± 0.02
25%	10.51 ± 0.01	2.91 ± 0.01	nd	4.66 ± 0.01
50%	9.62 ± 0.12	3.18 ± 0.11	nd	4.90 ± 0.06
100%	1.86 ± 0.05	5.84 ± 0.26	2.08 ± 0.07	5.88 ± 0.03
Detox	1.72 ± 0.03	5.86 ± 0.05	4.43 ± 0.02	5.86 ± 0.01
Detox + Supl.	7.85 ± 0.92	8.24 ± 1.11	3.49 ± 1.44	5.70 ± 0.02

Nd = not detected.

3.2 Antimicrobial activity

The BLIS obtained from the growth of *E. faecium* had a more effective action against *L. monocytogenes*, when compared with *Salmonellla*. BLIS was ineffective against *S.* Choleraesius (**Table 4**), even in the control group. The higher activities were CS 25 % with 481 AU/mL, followed by SCG detox supplemented with 429 AU/mL. Both groups had better antimicrobial activity than the control group (410 AU/mL).

Table 4. Quantification of antimicrobial activity (AU/mL) of BLIS produced by *Enterococcus faecium* 135 cultivated in elaborated media from SCG and CS hydrolysates, against *L. monocytogenes*, *S.* Typhimurium and *S.* Choleraesuis.

BLIS from *E. faecium*	**Antimicrobial activity (AU/mL)**		
	L. monocytogenes	*S.* Typhimurium	*S.* Choleraesuis
Control	410.58	8.71	-
SCG			-
0%	270.46	-	-
25%	74.21	-	-
50%	20.92	-	-
100%	1.99	0.10	-
Detox	-	-	-
Detox + Supl.	428.73	-	
			-
CS			-
0%	226.87	-	-
25%	480.86	-	-
50%	74.62	-	-
100%	0.11	-	-
Detox	-	-	-
Detox + Supl.	-	-	-

"-" no inhibition

After tangential filtration through 30 and 2 kDa pores, it was observed that the antimicrobial activity from SCG detox supplemented and control was able to doble (data not shown), mostly on the retendated from 30 kDa and 2 kDa (**Figure 2**). The BLIS obtained from CS 25% did not show any improvement in the antimicrobial activity.

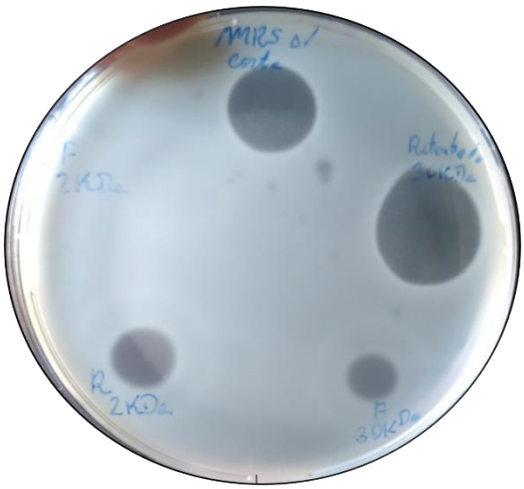

Figure 2. Antimicrobial activity against *Listeria monocytogenes* CECT 934 from the fractions of SCG detox supplemented BLIS.

A Tris-Tricine gel (**Figure 3**) was used to verify if a band with a similar weight of some enterocins would appear, since *E. faecium* 135 has enterocin genes. (Pereira, et al. 2022). The retendated from 30 kDa of control was the only condition that showed some visible bands present on the gel, one being close to the marker of 50 kDa and the other close to the 10 kDa marker. The fractions obtained from the elaborated media from SCG and CS did not show any bands on the gel.

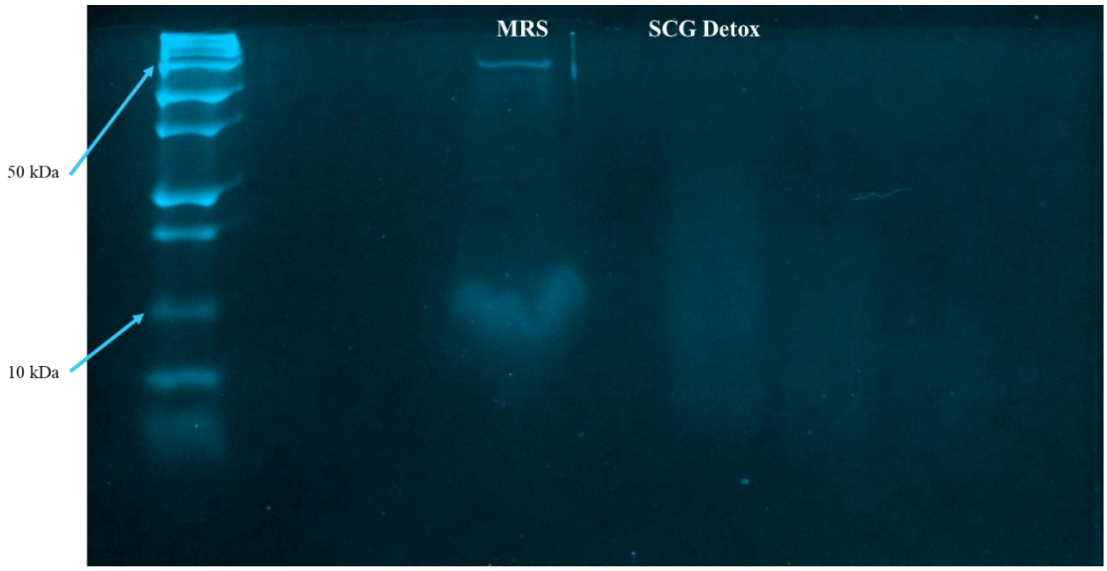

Figure 3. Screening for peptides in concentrated fractions (30 and 2 kDa) of BLIS from MRS (control) and SCG Detox supplemented.

4. Discussion

In this study, it was demonstrated that the residues originated from coffee processing, such as SCG and CS, were able to be used as carbon sources for *E. faecium* 135 growth and BLIS production. The recovered sugars obtained on this work were similar to results described by Mussatto, et al. (2011b), for example, the values of recovered galactose (11.24 g/L) were similar to this study, although the amounts of recovered mannose was (56%) lower on SCG. However, the polysaccharides present on coffee beans are mainly determined by the coffee species, growing conditions and, more importantly, the type of roasting applied to the grain (KLEINWÄCHTER; BYTOF; SELMAR, 2015; OLIVEIRA PETKOWICZ, 2015). In addition, hydrolysates obtained from SCG are expected to have a greater amount of available sugars, since the most important constituents in coffee beans are carbohydrates (MUSSATTO et al., 2011b), and CS is a pellicle and has more hemicellulose and cellulose, in its composition

(BALLESTEROS; TEIXEIRA; MUSSATTO, 2014; JIMÉNEZ-ZAMORA; PASTORIZA; RUFIÁN-HENARES, 2015; NARITA; INOUYE, 2014). The amount of phenolic compounds present on hydrolysate is related to the type of pre-treatment applied on the residue (MUSSATTO; ROBERTO, 2004). Mussatto et al. (2012) quantified about ~ 3g/L of phenolic compounds from SCG and CS hydrolysates, which is different from the current work that quantified 1.7 g/L for SCG and 6.8 g/L from CS concentrated. These differences are probably due to the fact that the authors used different pre-treatments.

Despite the negative influence of phenolic compounds on the growth of microorganisms (MACHADO et al., 2012; MUSSATTO; ROBERTO, 2004), the elaborated media CS 25% and SCG detox suplemented where it was able to provide good growth conditions for *E. faecium* and, consequently, production of BLIS, superior to the control group (MRS). The detoxification of the hydrolysate had a positive effect on the reduction of the phenolic compounds mostly on the SCG, which was proved by the superior number of viable cells on SCG detox supplemented medium. Although, when compared to CS 25%, the number of viable cells was similar.

CS and SCG had a variety of sugars, being galactose the most common in the hydrolysates, followed by mannose in SCG, both being hexoses that are easily consumed by *E. faecium* (PETRUT et al., 2019). However, CS 25% had the amount of sugar adjusted, so the medium would have the same amount as presented in CS 100%, while SCG detox had no sugar suplementation. The addition of synthetic sugars on CS 25% with the combination of reduction of phenolic compounds by dilution probably influenced the superior growth of *E. faecium*.

The production of lactic and acetic acids is proportional to the growth rate of LAB (PIAZENTIN et al., 2020). As *E. faecium* is a heterofermentative bacterium, it would produce both acids (BLAJMAN et al., 2020). Among the elaborated media, the one containing CS presented more organic acids in its composition when compared to SCG. The key difference is in the presence of acetic acid, which is already present in the hydrolysate and is part of the phenolic compounds formed during hydrolysis.

As said before about organic acids, BLIS production by LAB and, consequently, antimicrobial activity are associated to the growth rate of the strain (COTTER; HILL; ROSS, 2005). On **Table 4**, it is possible to observe that CS 25%, followed by SCG detox supplemented, had the highest antimicrobial activities. The presence of phenolic

compounds in CS 25% probably influenced the antimicrobial activity, since the supernatant had only the pH adjusted and was exposed to high temperatures to eliminate proteases. The other phenolic compounds, such as 5-HMF and furfural, are still present in the supernatant and may have an influence on antimicrobial activity (BONFIGLIO et al., 2021; JIMÉNEZ-ZAMORA; PASTORIZA; RUFIÁN-HENARES, 2015; MACHADO et al., 2012; MUSSATTO et al., 2011a, 2012).

After tangential filtration, we did not observe any increase in CS 25% activity. For SCG and MRS, we observed that the antimicrobial activity almost doubled, increasing on the retained of 30 kDa and 2 kDa for MRS and SCG BLIS. When evaluated in Tris-tricine gel, it was only possible to see a band at ~ 50 kDa, and between 2 and 10 kDa. The absence of bands from the BLIS fractions of SCG is probably due the low concentrations of the antimicrobial peptide present in the fractions. So, more research is needed to find out if there are antimicrobial peptides in the BLIS. This could be done by changing the concentration method or by purifying the BLIS by liquid chromatography.

Production of BLIS and bacteriocins using hemicellulose hydrolysate has already ben described on literature. Kuniyoshi et al. (2021) successfully used sugarcane bagasse hydrolysate to produce pediocin PA-1. However, the use of CS and SCG residues and hydrolysate to produce BLIS is innovative.

Conclusions

The coffee residues CS and SCG were a good carbon alternatives for the growth of *E. faecium* 135. The acid pretreatment provided the recuperation of monomers, such as mannose and galactose. Among the elaborated media, SCG detox supplemented, and CS 25% stand out, once on these media *E. faecium* 135 was able to growth almost 2 log CFU/mL exceeding the control. The production of organic acids and antimicrobial activity against *L. monocytogenes* were also superior. Either the presence of phenolic compounds was lower, or this factor had some influence on the growth and, consequently, production of biomolecules by *E. faecium* 135. After tangential filtration the antimicrobial activity increased, especially in the control group and using the Tris-tricine gel method, it was possible to confirm the presence of two bands in the control group. In conclusion, this study demonstrated the potential of CS and SCG residues to provide

carbon sources for alternative growth media, and the ability of *E. faecium* 135 to grow and produce antimicrobial compounds using these media.

References

BALLESTEROS, Lina F.; TEIXEIRA, José A.; MUSSATTO, Solange I. Chemical, Functional, and Structural Properties of Spent Coffee Grounds and Coffee Silverskin. **Food and Bioprocess Technology**, *[S. l.]*, v. 7, n. 12, p. 3493–3503, 2014. DOI: 10.1007/s11947-014-1349-z. Disponível em: http://link.springer.com/10.1007/s11947-014-1349-z.

BLAJMAN, J. E.; VINDEROLA, G.; PÁEZ, R. B.; SIGNORINI, M. L. The role of homofermentative and heterofermentative lactic acid bacteria for alfalfa silage: a meta-analysis. **The Journal of Agricultural Science**, *[S. l.]*, v. 158, n. 1–2, p. 107–118, 2020. DOI: 10.1017/S0021859620000386. Disponível em: https://www.cambridge.org/core/product/identifier/S0021859620000386/type/journal_article.

BONFIGLIO, Fernando; CAGNO, Matías; YAMAKAWA, Celina K.; MUSSATTO, Solange I. Production of xylitol and carotenoids from switchgrass and Eucalyptus globulus hydrolysates obtained by intensified steam explosion pretreatment. **Industrial Crops and Products**, *[S. l.]*, v. 170, n. July, p. 113800, 2021. DOI: 10.1016/j.indcrop.2021.113800. Disponível em: https://doi.org/10.1016/j.indcrop.2021.113800.

BORRELLI, Rosa Cinzia; ESPOSITO, Fabrizio; NAPOLITANO, Aurora; RITIENI, Alberto; FOGLIANO, Vincenzo. Characterization of a New Potential Functional Ingredient: Coffee Silverskin. **Journal of Agricultural and Food Chemistry**, *[S. l.]*, v. 52, n. 5, p. 1338–1343, 2004. DOI: 10.1021/jf034974x. Disponível em: https://pubs.acs.org/doi/10.1021/jf034974x.

COTTER, Paul D.; HILL, Colin; ROSS, R. Paul. Food microbiology: Bacteriocins: Developing innate immunity for food. **Nature Reviews Microbiology**, *[S. l.]*, v. 3, n. 10, p. 777–788, 2005. DOI: 10.1038/nrmicro1273.

DE VUYST, L. Screening for enterocins and detection of hemolysin and vancomycin resistance in enterococci of different origins. **International Journal of Food Microbiology**, *[S. l.]*, v. 84, n. 3, p. 299–318, 2003. DOI: 10.1016/S0168-1605(02)00425-7. Disponível em: https://linkinghub.elsevier.com/retrieve/pii/S0168160502004257. Acesso em: 8 jan. 2021.

HENNING, Chris; GAUTAM, Dhiraj; MURIANA, Peter. Identification of Multiple Bacteriocins in Enterococcus spp. Using an Enterococcus-Specific Bacteriocin PCR

Array. **Microorganisms**, *[S. l.]*, v. 3, n. 1, p. 1–16, 2015. DOI: 10.3390/microorganisms3010001.

INTERNATIONAL COFFEE ORGANIZATION. **Trade Statistics Tables**. Disponível em: https://www.ico.org/trade_statistics.asp. Acessado em: 18/03/2022.

JIMÉNEZ-ZAMORA, Ana; PASTORIZA, Silvia; RUFIÁN-HENARES, José A. Revalorization of coffee by-products. Prebiotic, antimicrobial and antioxidant properties. **LWT - Food Science and Technology**, *[S. l.]*, v. 61, n. 1, p. 12–18, 2015. DOI: 10.1016/j.lwt.2014.11.031.

KHAN, Haider; FLINT, Steve; YU, Pak-Lam. Enterocins in food preservation. **International Journal of Food Microbiology**, *[S. l.]*, v. 141, n. 1–2, p. 1–10, 2010. DOI: 10.1016/j.ijfoodmicro.2010.03.005. Disponível em: https://linkinghub.elsevier.com/retrieve/pii/S0168160510001170. Acesso em: 25 nov. 2020.

KLEINWÄCHTER, Maik; BYTOF, Gerhard; SELMAR, Dirk. Coffee Beans and Processing. *In*: **Coffee in Health and Disease Prevention**. [s.l.] : Elsevier, 2015. p. 73–81. DOI: 10.1016/B978-0-12-409517-5.00009-7. Disponível em: http://dx.doi.org/10.1016/B978-0-12-409517-5.00009-7.

KUNIYOSHI, Taís Mayumi et al. Pediocin PA-1 production by Pediococcus pentosaceus ET34 using non-detoxified hemicellulose hydrolysate obtained from hydrothermal pretreatment of sugarcane bagasse. **Bioresource Technology**, *[S. l.]*, v. 338, n. May, p. 125565, 2021. DOI: 10.1016/j.biortech.2021.125565. Disponível em: https://linkinghub.elsevier.com/retrieve/pii/S0960852421009068.

LI, Ping; GU, Qing; WANG, Yuejiao; YU, Yue; YANG, Lanlan; CHEN, Jieyan V. Novel vitamin B12-producing Enterococcus spp. and preliminary in vitro evaluation of probiotic potentials. **Applied Microbiology and Biotechnology**, *[S. l.]*, v. 101, n. 15, p. 6155–6164, 2017. DOI: 10.1007/s00253-017-8373-7. Disponível em: http://link.springer.com/10.1007/s00253-017-8373-7.

LIU, Zhijia; NATALIZIO, Francesca; DRAGONE, Giuliano; MUSSATTO, Solange I. Maximizing the simultaneous production of lipids and carotenoids by Rhodosporidium toruloides from wheat straw hydrolysate and perspectives for large-scale implementation. **Bioresource Technology**, *[S. l.]*, v. 340, n. July, p. 125598, 2021. DOI: 10.1016/j.biortech.2021.125598. Disponível em: https://doi.org/10.1016/j.biortech.2021.125598.

MACHADO, Ercília M. S.; RODRIGUEZ-JASSO, Rosa M.; TEIXEIRA, José A.; MUSSATTO, Solange I. Growth of fungal strains on coffee industry residues with removal of polyphenolic compounds. **Biochemical Engineering Journal**, *[S. l.]*, v. 60, p. 87–90, 2012. DOI: 10.1016/j.bej.2011.10.007. Disponível em: http://dx.doi.org/10.1016/j.bej.2011.10.007.

MARTÍNEZ VIEDMA, Pilar; SOBRINO LÓPEZ, Angel; BEN OMAR, Nabil;

ABRIOUEL, Hikmate; LUCAS LÓPEZ, Rosario; VALDIVIA, Eva; MARTÍN BELLOSO, Olga; GÁLVEZ, Antonio. Enhanced bactericidal effect of enterocin AS-48 in combination with high-intensity pulsed-electric field treatment against Salmonella enterica in apple juice. **International Journal of Food Microbiology**, *[S. l.]*, v. 128, n. 2, p. 244–249, 2008. DOI: 10.1016/j.ijfoodmicro.2008.08.014. Disponível em: https://linkinghub.elsevier.com/retrieve/pii/S0168160508004765. Acesso em: 25 nov. 2020.

MATA, Teresa M.; MARTINS, António A.; CAETANO, Nídia S. Bio-refinery approach for spent coffee grounds valorization. **Bioresource Technology**, *[S. l.]*, v. 247, n. July 2017, p. 1077–1084, 2018. DOI: 10.1016/j.biortech.2017.09.106.

MCNUTT, Josiah; HE, Quan (Sophia). Spent coffee grounds: A review on current utilization. **Journal of Industrial and Engineering Chemistry**, *[S. l.]*, v. 71, p. 78–88, 2019. DOI: 10.1016/j.jiec.2018.11.054. Disponível em: https://doi.org/10.1016/j.jiec.2018.11.054.

MUSSATTO, Solange I.; BALLESTEROS, Lina F.; MARTINS, Silvia; TEIXEIRA, José A. Extraction of antioxidant phenolic compounds from spent coffee grounds. **Separation and Purification Technology**, *[S. l.]*, v. 83, n. 1, p. 173–179, 2011. a. DOI: 10.1016/j.seppur.2011.09.036. Disponível em: http://dx.doi.org/10.1016/j.seppur.2011.09.036.

MUSSATTO, Solange I.; CARNEIRO, Livia M.; SILVA, João P. A.; ROBERTO, Inês C.; TEIXEIRA, José A. A study on chemical constituents and sugars extraction from spent coffee grounds. **Carbohydrate Polymers**, *[S. l.]*, v. 83, n. 2, p. 368–374, 2011. b. DOI: 10.1016/j.carbpol.2010.07.063. Disponível em: http://dx.doi.org/10.1016/j.carbpol.2010.07.063.

MUSSATTO, Solange I.; MACHADO, Ercília M. S.; CARNEIRO, Lívia M.; TEIXEIRA, José A. Sugars metabolism and ethanol production by different yeast strains from coffee industry wastes hydrolysates. **Applied Energy**, *[S. l.]*, v. 92, p. 763–768, 2012. DOI: 10.1016/j.apenergy.2011.08.020. Disponível em: http://dx.doi.org/10.1016/j.apenergy.2011.08.020.

MUSSATTO, Solange Inês; ROBERTO, Inês Conceição. Alternatives for detoxification of diluted-acid lignocellulosic hydrolyzates for use in fermentative processes: A review. **Bioresource Technology**, *[S. l.]*, v. 93, n. 1, p. 1–10, 2004. DOI: 10.1016/j.biortech.2003.10.005.

NARITA, Yusaku; INOUYE, Kuniyo. Review on utilization and composition of coffee silverskin. **Food Research International**, *[S. l.]*, v. 61, p. 16–22, 2014. DOI: 10.1016/j.foodres.2014.01.023. Disponível em: http://dx.doi.org/10.1016/j.foodres.2014.01.023.

OLIVEIRA, A. C.; BETTCHER, L. Aspectos epidemiológicos da ocorrência do *Enterococcus* resistente a Vancomicina. **Revista da Escola de Enfermagem da USP**, v. 44, n. 3, p. 725-731, 2010.

OLIVEIRA PETKOWICZ, Carmen L. De. Polysaccharides in Coffee and Their Relationship to Health. *In*: **Coffee in Health and Disease Prevention**. [s.l.] : Elsevier, 2015. p. 163–172. DOI: 10.1016/B978-0-12-409517-5.00018-8. Disponível em: http://dx.doi.org/10.1016/B978-0-12-409517-5.00018-8.

PEREIRA, Wellison Amorim et al. Bacteriocinogenic probiotic bacteria isolated from an aquatic environment inhibit the growth of food and fish pathogens. **Scientific Reports**, *[S. l.]*, v. 12, n. 1, p. 5530, 2022. DOI: 10.1038/s41598-022-09263-0. Disponível em: https://doi.org/10.1038/s41598-022-09263-0.

PETRUT, Stefana; RUSU, Elena; TUDORACHE, Ioan Sorin; PELINESCU, Diana; SARBU, Ionela; STOICA, Ileana; VASSU, Tatiana. Influence of Various Carbon Sources on Growth and Biomass Accumulation of Some Lactic Acid Bacteria Strains. **Revista de Chimie**, *[S. l.]*, v. 70, n. 7, p. 2434–2438, 2019. DOI: 10.37358/RC.19.7.7356. Disponível em: https://revistadechimie.ro/Articles.asp?ID=7356.

PIAZENTIN, Anna Carolina Meireles; DA SILVA, Thamires Maria Simões; FLORENCE-FRANCO, Ana Carolina; BEDANI, Raquel; CONVERTI, Attilio; DE SOUZA OLIVEIRA, Ricardo Pinheiro. Soymilk fermentation: effect of cooling protocol on cell viability during storage and in vitro gastrointestinal stress. **Brazilian Journal of Microbiology**, *[S. l.]*, v. 51, n. 4, p. 1645–1654, 2020. DOI: 10.1007/s42770-020-00369-z. Disponível em: http://link.springer.com/10.1007/s42770-020-00369-z.

PIAZENTIN, Anna Carolina Meireles; MENDONÇA, Carlos Miguel Nóbrega; VALLEJO, Marisol; MUSSATTO, Solange I.; DE SOUZA OLIVEIRA, Ricardo Pinheiro. Bacteriocin-like inhibitory substances production by Enterococcus faecium 135 in co-culture with Ligilactobacillus salivarius and Limosilactobacillus reuteri. **Brazilian Journal of Microbiology**, *[S. l.]*, v. 53, n. 1, p. 131–141, 2022. DOI: 10.1007/s42770-021-00661-6. Disponível em: https://doi.org/10.1007/s42770-021-00661-6.

QIN, Chubin; ZHANG, Zhen; WANG, Yibing; LI, Shuning; RAN, Chao; HU, Jun; XIE, Yadong; LI, Weifen; ZHOU, Zhigang. EPSP of L. casei BL23 protected against the infection caused by Aeromonas veronii via enhancement of immune response in zebrafish. **Frontiers in Microbiology**, *[S. l.]*, v. 8, n. DEC, p. 1–13, 2017. DOI: 10.3389/fmicb.2017.02406.

SABO, Sabrina da Silva; LOPES, André Moreni; SANTOS-EBINUMA, Valéria de Carvalho; RANGEL-YAGUI, Carlota de Oliveira; OLIVEIRA, Ricardo Pinheiro de Souza. Bacteriocin partitioning from a clarified fermentation broth of Lactobacillus plantarum ST16Pa in aqueous two-phase systems with sodium sulfate and choline-based salts as additives. **Process Biochemistry**, *[S. l.]*, v. 66, p. 212–221, 2018. DOI: 10.1016/j.procbio.2017.11.018. Disponível em: https://linkinghub.elsevier.com/retrieve/pii/S1359511317311315. Acesso em: 25 nov. 2020.

SAENGER, M.; HARTGE, E. U.; WERTHER, J.; OGADA, T.; SIAGI, Z. Combustion of coffee husks. **Renewable Energy**, *[S. l.]*, v. 23, n. 1, p. 103–121, 2001. DOI:

10.1016/S0960-1481(00)00106-3.

SCHÄGGER, Hermann. Tricine–SDS-PAGE. **Nature Protocols**, *[S. l.]*, v. 1, n. 1, p. 16–22, 2006. DOI: 10.1038/nprot.2006.4. Disponível em: http://www.nature.com/articles/nprot.2006.4.

SINGHAL, Neelja; SINGH, Nambaram Somendro; MOHANTY, Shilpa; SINGH, Pooja; VIRDI, Jugsharan Singh. Evaluation of Probiotic Characteristics of Lactic Acid Bacteria Isolated from Two Commercial Preparations Available in Indian Market. **Indian Journal of Microbiology**, *[S. l.]*, v. 59, n. 1, p. 112–115, 2019. DOI: 10.1007/s12088-018-0762-9. Disponível em: https://doi.org/10.1007/s12088-018-0762-9.

WORAPRAYOTE, Weerapong; MALILA, Yuwares; SORAPUKDEE, Supaluk; SWETWIWATHANA, Adisorn; BENJAKUL, Soottawat; VISESSANGUAN, Wonnop. Bacteriocins from lactic acid bacteria and their applications in meat and meat products. **Meat Science**, *[S. l.]*, v. 120, p. 118–132, 2016. DOI: 10.1016/j.meatsci.2016.04.004. Disponível em: http://dx.doi.org/10.1016/j.meatsci.2016.04.004.

CHAPTER
.3.

Bacteriocinogenic probiotic bacteria isolated from an aquatic environment inhibit the growth of food and fish pathogens

Abstract

The conditions of aquatic environments have a great influence on the microbiota of several animals, many of which are a potential source of microorganisms with biotechnological interest. In this study, bacterial strains isolated from aquatic environments were bioprospected for their probiotic profile and antimicrobial effect against fish and food pathogens. Two isolates, identified via 16S rRNA sequencing as Lactococcus lactis (L1 and L2) and one as Enterococcus faecium 135 (EF), produced a bacteriocin-like antimicrobial substance (BLIS), active against Listeria monocytogenes, Salmonella Choleraesuis and Salmonella Typhimurium. Antimicrobial activity of BLIS was reduced when exposed to high temperatures and proteolytic enzymes (trypsin, pepsin, papain and pancreatin), indicative of their protein nature. All strains were sensitive to 7 types of antibiotics (vancomycin, clindamycin, streptomycin, gentamicin, chloramphenicol, rifampicin and ampicillin), had a high rate of adherence to Caco-2 cells and did not express hemolysin and gelatinase virulence factors. EF showed some resistance at pH 2.5 and 3.0 and L2 / EF showed good resistance to the action of bile salts. Finally, the presence of bacteriocin genes in the total DNA extracted from the isolates was evaluated, and Nisin (L1 and L2), Enterocin A, B, P, and Mundticin KS (EF) were detected. The molecular and physiological evidence indicates that all tested bacterial isolates could be used as natural antimicrobial agents and may be considered safe for probiotic application.

Keywords: aquatic environment, fish, bioprospection, bacteriocins, probiotic.

1. Introduction

Probiotics are defined as live microorganisms, which when administered in adequate amounts confer a health benefit on the host (HILL et al., 2014). However, to be considered a probiotic, these microorganisms must undergo experiments to attest safety for use in food. Probiotics isolated from aquatic animals are spread through water and via other living organisms, and once they reach the host's intestines, these microorganisms perform vital functions. Several anatomical structures are potential growth sites for microorganisms, such as the skin, gills and gastrointestinal tract (DAWOOD et al., 2019; VAN DOAN et al., 2020). Intestinal content is thus an important source of potential probiotic microorganisms that can subsequently be used as food supplements (RINGØ et al., 2018).

Proper nutrition is intrinsically associated with correct development and efficient immunological defenses. Thus, studies have shown that both in humans and animals, the microbiota plays an essential role in the proper development and defense against pathogens (DAWOOD et al., 2019). Probiotic use in feed improves the health of aquatic animals, without the presence of negative side-effects (PÉREZ-SÁNCHEZ; MORA-SÁNCHEZ; BALCÁZAR, 2018). Among the studies that have demonstrated the benefits of probiotic use, different mechanisms of action have been noted, that differ according to the species and environmental conditions that the microorganism encounters (QIN et al., 2017; SINGHAL et al., 2019). Probiotics used in aquaculture have included specific strains of yeasts and especially bacteria, including representatives of *Lactococcus* sp., *Enterococcus* sp., among others (GHEZIEL et al., 2019). Some species belonging to the lactic acid bacteria (LAB) are considered safe (GRAS, Generally Reported as Safe) (VILANDER; DEAN, 2019) and can be producers of natural antimicrobials, such as bacteriocins (SINGHAL et al., 2019).

LAB are commonly recommended for aquaculture, and dietary supplementation results in improved activity of digestive enzymes, immune response, development and even water quality (VAN DOAN et al., 2020; XIE et al., 2017). Stimulation of the production of digestive enzymes, such as amylase, protease, lipase and lysozyme, can be an important consequence of probiotic use (YOUSEFI et al., 2019). In healthy animals, these enzymes are intrinsically associated with improved digestibility, nutritional intake and weight gain (LIU et al., 2011; WANG et al., 2019). Colonization induction and

development of beneficial strains in the intestinal tract also leads to the production of other beneficial substances in addition to enzymes (DAWOOD et al., 2019).

As previously mentioned, an important characteristic of LAB is the ability to produce bacteriocins that play a key role in controlling pathogens (COTTER; ROSS; HILL, 2013). These are conceptualized as small, cationic, heterogeneous, hydrophobic antimicrobial peptides produced by different microorganisms, with high isoelectric points, an amphipathic character, and a variety of modes of action and biochemical properties (COTTER; ROSS; HILL, 2013; VERHEUL et al., 1997). Since 1925, with the discovery of colicin, research on bacteriocins has received great attention (MBANDLWA et al., 2022) and by 1995 more than a hundred different types of bacteriocins had been identified (CHEN; LUDESCHER; MONTVILLE, 1997). Bacteriocins provide an important competitive advantage for the species that produce them (YANG et al., 2014). Probiotics of interest can remain in the intestinal tract while producing bacteriocins, exercising synergistic effects, since they are not toxic to the host and the LAB exert various beneficial functions (KLAENHAMMER, 1993). Most of the bacteriocins that have been tested to date were isolated from LAB and are generally used in foods for their high antimicrobial potential (YANG et al., 2014).

The mode of action of bacteriocins is related to the need of the producing bacteria to survive competition for food and space in the microbiota (YANG et al., 2014). The antimicrobial effect of bacteriocins is related to their action on anionic lipids present in the membrane, which results in the formation of pores, errors in the events that lead to ATP synthesis and amino acid transport (OGAKI; FURLANETO; MAIA, 2015). For this reason, most studies which demonstrated good results with bacteriocins are carried out with Gram-positive bacteria, as they have membranes that are richer in anionic lipids. The same effect can be observed in Gran-negatives, however bacteriocin needs to cross the complex structure of the outer membrane (YANG et al., 2014). An example is the bacteriocin microcin C7-C51 which has already been described as effective against strains of the genus *Escherichia*, *Enterobacteria*, *Klebsiella*, *Salmonella*, *Shigella*, *Proteus*, among others (COTTER; ROSS; HILL, 2013). Studies also point to the possibility of using bacteriocins as an alternative to combat antibiotic-resistant microorganisms, since the mode of action of both is different (OGAKI; FURLANETO; MAIA, 2015).

Bacterial diseases can affect various sectors such as food production and fish farming. In this sense, some of the pathogens of interest belong to the genus

Streptococcus, *Staphylococcus*, *Listeria* and *Salmonella*. Streptococcosis is a disease that causes bacteria of the genus *Streptococcus* due to stress and high density in fish production sites, which can lead to considerable production losses (XU; MING, 2018). Staphylococcal outbreaks is food poisoning caused by *Staphylococcus aureus*, a producer of enterotoxins. Despite not being part of the microbiota of aquatic animals, its presence may indicate the presence of diseases (ABDELFATAH; MAHBOUB, 2018). Thus, bacteria of the *Salmonella* genus are important pathogens known in the literature for their dissemination via water and/or contaminated food and difficult control (ROGERS; TSOLIS; BÄUMLER, 2021). And finally, *Listeria monocytogenes* is a pathogen with a high incidence in fish processing, it is difficult to control and has been shown to be resistant to several antimicrobials (SKOWRON et al., 2018).

Experiments with aquatic animals have yielded promising results and feed supplementation effectiveness can be optimized if different approaches for the use of probiotics are tested (GUERREIRO; OLIVA-TELES; ENES, 2018). Recent studies have shown that the future of probiotic research in aquaculture lies in the use of new supplementation techniques, such as the mixing of two or more strains. Indeed, mixing different probiotic microorganisms increases the product efficacy, which opens up the possibility of researching new lines aimed at investigating the interaction of these microorganisms as well as their joint action for the benefit of animal health. But as few examples have been analyzed in detail, specific studies are needed to test each of the strains used and their impact on individual animal models (DAWOOD et al., 2019).

Therefore, the aim of this study was to evaluate the probiotic and bacteriocinogenic potential of bacteria isolated from an aquatic environment and their antimicrobial potential against important fish and food pathogens.

2. Materials and methods

2.1. Sampling and ethical aspects

Samples were obtained by field collection carried out at the Salmoniculture Experimental Station of the São Paulo Fishing Institute (Campos do Jordão, Brazil). Rainbow trout (*Oncorhynchus mykiss*), approximately 16 weeks old, were selected for

the start of bioprospecting. After capture, the animals were sacrificed respecting biosafety and anesthesia rules validated by the institutions themselves, and then, under aseptic conditions, the cecum was removed, stored in a sterile flask in thermal boxes (~ 4º C), and transported to the laboratory for immediate analysis. This study was analyzed and approved by Ethics Committee of São Paulo Fishing Institute (registration number 07/2020). For fish anesthesia, an aqueous solution of benzocaine (100 mg/L^{-1}) was used until the loss of balance and reduction of opercular movements. Testing was done respecting all the relevant guidelines and regulations.

EF was obtained from the collection belonging to the Laboratory of Bacterial Biotechnology (Universidad Nacional de la Patagonia, Argentina). The strain was isolated from starfish (order *Forcipulatida*) in Playa Unión, Rawson-Chubut (Patagonia, Argentina) and donated by Prof. Marisol Vallejo, National University of Patagonia San Juan Bosco (Argentina).

2.2. Bioprospecting and identification by biochemical tests and MALDI-TOF

The protocols described below were used for the isolation and identification of samples present in the cecum content of rainbow trout and starfish. The isolation was carried out according to the methods described by Schirru, *et al.* (2012) with minor modifications. Samples of 25 g of excrement were homogenized in 225 mL of peptone water in a Stomacher. Serial dilutions were performed and cultivated in Man, Rogosa and Sharpe (MRS) and M17 media (BD Difco, New Jersey, USA) with cycloheximide (0.1 g/L). The plates were incubated under different temperatures (15, 25, 32 and 37º C), for up to 48 hours in anaerobic and aerobic conditions. After this period, approximately 300 CFUs were randomly chosen on each plate and replicated in the same culture medium and conditions. Then, biochemical tests were carried out for the classification of isolated microorganisms, such as Gram test (Gram method), production of Catalase (addition of hydrogen peroxide), and analysis by MALDI-TOF (Optical Microscopy and Ionization Mass Spectrometry by Laser Desorption Matrix assisted with flight time analyzer). For MALDI-TOF analysis, isolates defined as Gram-positive, Catalase negative and with morphology corresponding to cocci and/or bacilli were selected. The protocol described by Alves *et al.* (2016) was used for this test.

Therefore, the isolated strains were grown according to their isolation conditions previously described in plates with 1.5% MRS / M17 medium for 24 hours. After the period, isolates and 200 μL of sterile distilled water were added to a 1.5 mL microtube, being homogenized for 1 minute using a vortex device. 900 μL of ethanol were transferred to the tubes, being centrifuged at 12,000 g for a period of 5 minutes. The supernatant was discarded, and the samples were dried at room temperature for the loss of alcohol residues. 50 μL of formic acid (70%) and 50 μL of acetonitrile were added to the tubes, with a vortex homogenization. Subsequently, a matrix of α-cyano-4-hydroxycinnamic acid was prepared as a solution saturated in 50% acetonitrile and 2.5% trifluoroacetic acid. In a steel target plate, 1μL of treated samples and 1μL of matrix solution were added for drying at room temperature. Finally, the selected strains were cryopreserved in glycerol (20% v / v) at -80 ° C. For identification by mass spectrometry, the ItrafeXtreme MALDI-TOF equipment (Bruker Daltonics, Germany) was used, operating in the positive linear ion mode. The mass spectra were acquired in a mass range of 2 to 20 kDa with ions formed by intelligent beam radiation using a frequency of 2000 Hz, PIE 100ns, 7kV lens. The voltages for the first and second ion sources were 25 kV and 3kV, respectively. The bacteria were identified using the Biotyper 3.1 database. Cut-off values greater than 2 and 1.7 were used to identify species and genera, respectively (ALVES et al., 2016).

16S rRNA sequencing

For the identification of species at the molecular level, isolates L1, L2 and EF were subjected to partial sequencing of the 16S gene (rRNA) using the following primers: (PLB16) AGAGTTTGATCCTGGCTCAG and (MLB16) GGCTGCTGGCACGTAGTTAG. Genomic DNA was extracted using the PrepMan Ultra® kit protocol (Applied Biosystems, Carlsbad, CA, USA), following manufacturer's instructions. The DNA was quantified using a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific, Wilmington, DE, USA) and used for amplification reactions with PCR Master Mix (Promega, San Luis Obispo, CA, USA) under the following thermal cycling conditions: 94 °C for 5 min, followed by 35 cycles at 94 °C for 1 min, 55 °C and 72 °C for 1 min, followed by a final extension of 7 min at 72 °C. PCR products were purified with a QIAquick PCR Purification kit (Qiagen, Hilden, Germany) and sequenced in both directions using a Big Dye® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems). After contig assembly and edition, 16S sequences were used to

conduct BLAST search analysis for species identification. All sequences generated in this study were deposited in the GenBank database (Table 1).

Table 1. Molecular identification (16S rRNA) and screening for presence of bacteriocin genes in *L. lactis* (L1 and L2) and *E. faecium* strains (EF).

Strains	Molecular Identification	Accession number	Bacteriocin genes	Results	Reference
L1	*Lactococcus lactis*	MZ926851	Nisin	+	(ALEGRÍA et al., 2010)
			Lacticin 3147	-	(ALEGRÍA et al., 2010)
			Lacticin 481	-	(ALEGRÍA et al., 2010)
			Lactococcin 972	-	(MARTÍNEZ et al., 1999)
			Lactococcin A, B, M	-	(ALEGRÍA et al., 2010)
			Lactococcin G and Q	-	(ALEGRÍA et al., 2010)
L2	*Lactococcus lactis*	MZ926852	Nisin	+	(ALEGRÍA et al., 2010)
			Lacticin 3147	-	(ALEGRÍA et al., 2010)
			Lacticin 481	-	(ALEGRÍA et al., 2010)
			Lactococcin 972	-	(ALEGRÍA et al., 2010)
			Lactococcin A, B, M	-	(ALEGRÍA et al., 2010)
			Lactococcin G and Q	-	(ALEGRÍA et al., 2010)
EF	*Enterococcus faecium*	MZ735396	Enterocin A	+	(DE VUYST, 2003)
			Enterocin B	+	(DE VUYST, 2003)
			Enterocin P	+	(DE VUYST, 2003)
			Enterocin LB50A	-	(DE VUYST, 2003)
			Enterocin LB50B	-	(DE VUYST, 2003)
			Enterocin 96	-	(HENNING; GAUTAM; MURIANA, 2015)
			Enterocin 31	-	(HENNING; GAUTAM; MURIANA, 2015)
			Enterocin 1071	-	(MARTÍN et al., 2006)
			Enterocin Q	-	(BELGACEM et al., 2010)
			Mundticin KS	+	(ALMEIDA et al., 2011)
			Hiracin JM79	-	(ALMEIDA et al., 2011)

+ target gene detected; - target gene not detected.

2.3. Screening for presence of bacteriocin genes

To assess the presence of bacteriocin-specific genes in L1, L2 and EF, a PCR reaction was performed targeting genes encoding for nisin, lacticin, lactococcin,

enterocin, mundticin, and hiracin (Table 1). Amplification reactions were performed with PCR Master Mix (Promega, San Luis Obispo, CA, USA) and the same thermal cycling conditions as described above, modifying the annealing temperature when appropriate. The amplified PCR products were analyzed by 1.2% agarose gel electrophoresis at 100 V for 50 minutes and bands were visualized with UV light equipment.

2.4. Agar diffusion: evaluation of the antimicrobial effect of BLIS

To assess the potential antimicrobial effect of BLIS from probiotic strains and its possible ability to produce antimicrobial peptides, such as bacteriocins, BLIS sensitivity tests against important bioindicator strains was performed using the agar diffusion test. FIOCRUZ (Rio de Janeiro, RJ, Brazil) provided the pathogen *S.* Typhimurium 5551/16, the Fishing Institute of São Paulo (São Paulo, SP, Brazil) provided the pathogen *S. agalactiae*, whilst the strains *L. monocytogenes CECT 934, S. aureus CECT 237 and S.* Choleraesuis CECT 724 were acquired from the Spanish Type Culture Collection (CECT) (Valencia, Spain). All isolates were reactivated 24 hours before the start of the experiments, followed by pre-inoculum preparation. The optical density (OD_{600nm} 0.8) was determined, the inoculum diluted 100 times (~10^6 CFU/mL) and then incubated according to the initially described growth conditions. After a period of 24 hours, the samples were centrifuged at 4,470 g at 4º C for 15 minutes, with 10 mL of the supernatant being removed for subsequent filtration through a 25 μm hydrophilic PVDF membrane (Filtrilo, Colombo, Brazil). The product resulted from this process was the BLIS.

Before testing for antimicrobial activity, the pH of BLIS was adjusted to ~ 6 using NaOH (1 M) and exposed to high temperatures (80ºC / 10 minutes) in order to stabilize the substance and inactivate possible acids present in the sample. For the agar diffusion test, 1 mL of the inoculum of the pathogens *S.* Choleraesuis and *S.* Typhimurium was added to Petri dishes (90 x 15 mm) containing 10 ml of TSB (Difco, Michigan, USA) and 1 mL of *L. monocytogenes*, *S. aureus* or *S. agalactiae* on BHI agar (Difco, Michigan, USA) in a semi-solid state (supplemented with 0.75% agar). After solidification, 10 µL of the BLIS were pipetted onto the agar, and the plates were incubated for 18 hours at 37º C. Subsequently, inhibition halos were measured with the aid of digital calipers. Antimicrobial activity was expressed as arbitrary units per milliliter (AU/mL) using the

formula described below (1), in which $\pi.R^2$ is the area of the inhibition zone (cm^2) and V is the volume (mL) of BLIS used (KUNIYOSHI et al., 2021; SABO et al., 2018).

$$AU/mL = \frac{\pi.R^2}{V} \qquad (1)$$

2.5. Absorbance microplate reader

An absorbance microplate reader (BioTech, Vermont, USA) was used to assess the mode of action of BLIS against the pathogens tested at different stages of bacterial growth. For this, the BLIS and pathogens were prepared according to the pre-established conditions and incubated in a Microplate Reader (Bioteck Instruments, Vermont, USA) at 37º C. The OD_{600nm} was determined automatically every hour for 24 hours. From this experiment, it was possible not only to confirm the results obtained in the agar diffusion test, but also to determine the stages of bacterial growth that BLIS interfere with. Subsequently, in a sterile 96-well plate (TPP, Trasadingen, Switzerland) all combinations of variables necessary for this analysis were considered, such as positive (BLIS) and negative controls (saline 0.85%), and associations between the BLIS and different pathogens (CABO et al., 1999; SABO et al., 2018).

2.6. Tolerance of isolates to bile salts and low pH

The tolerance to acid pH and bile salts was evaluated based on the methodology described by Tan *et al*. (2013). L1, L2, and EF previously grown in MRS broth (~10^8 CFU/mL), were centrifuged (4,470 *g*), washed and resuspended in MRS with pH adjusted to 2, 2.5, 3 and 6 (negative control) with sterile 1N HCl (Labsynth, Diadema, Brazil). The samples were then incubated at 37ºC, and 1 mL aliquots were taken after 0, 1, 2 and 3 hours for CFU counting on MRS 1.5% (w/v) agar.

To evaluate the effect of bile salts, LAB were grown in MRS broth and incubated with bile salts (Sigma-Aldrich, Missouri, USA) at different concentrations (0.1%, 0.2%, 0.3% and the control, without addition) at 37º C. Aliquots (1 mL) were taken at 0, 2, 4 and 6 hours for CFU counting on MRS 1.5% (w/v) agar plates.

2.7. Tolerance of BLIS to low pH, high temperatures and proteolytic enzymes.

To verify the stability of BLIS against different temperatures and pH, the method described by Todorov and Dicks (2006) was used. To this end, BLIS were subjected to heat treatments (30, 50, 70 or 90 ºC for 1 hour; 121ºC for 15 minutes) and pH treatments adjusted to pH 2, 4, 6, 8 or 10 with 1N NaOH and HCl; Labsynth, Diadema, Brazil) at 30ºC for 1 h. To evaluate the proteinaceous nature of BLIS, samples were subjected to 1% (w/v) trypsin, pepsin, papain or pancreatin (Inlab, Alamar Tecno Científica Ltda, São Paulo, Brazil) and incubated at 30ºC for 2 hours. After this period, the stability of BLIS was verified using the diffusion agar technique against *L. monocytogenes*.

2.8. Hemolytic activity

The production capacity of the extracellular protein hemolysin was evaluated in Petri dishes containing BHI agar supplemented with 5% sheep's blood. After preparing the inoculum, the isolates were spread on the surface of the sheep's blood agar and incubated according to the pre-established growth conditions. The activity of hemolytic hemolysin protein was confirmed by the formation of different types of halos, whose interpretation was performed by their coloring: α-hemolysin when there were greenish areas around the colonies, β-hemolysin when the zones were light colored, and γ-hemolysin in the absence of such zones (SABO et al., 2020).

2.9. Gelatinase production

For the gelatinase production test, the inoculum was cultivated on the surface of Petri dishes containing BHI supplemented with skimmed milk (1.5%) and incubated according to the respective growth conditions described above. According to Tan *et al.* (2013), a clear halo around the colony indicates a positive result for gelatinase production.

2.10. Coexistence Test

This test investigates the possibility of co-cultivation between the three probiotic bacteria evaluated in this study. The tests were carried out according to method described by Guo *et al*. (2009). Specifically, the bacteria were grown in their respective growth conditions for 24 hours, and then samples were streaked perpendicularly to each other on

the surface of plates containing 1.5% MRS (w/v) agar. After a 24-hour incubation period, plates were examined for possible antagonistic effects.

2.11. Antibiotic resistance

Antibiotics of clinical importance were used, including vancomycin (30 μg), clindamycin (2 μg), streptomycin (10 μg), gentamicin (30 μg), chloramphenicol (30 μg), rifampicin (5 μg) and ampicillin (10 μg) (all provided by LABORCLIN, São Paulo, Brazil) loaded onto disks. Therefore, isolates were reactivated in the conditions mentioned above and, after 24 hours of cultivation, bacterial growth at OD_{600nm} was determined and adjusted to 0.8. Finally, the samples were streaked on the surface of a Petri dish containing Mueller Hinton agar (Difco, Michigan, USA) and, after drying, the antibiotic-containing disks were added to the plates. Following an incubation at 37º C for 24 hours, the presence or absence of inhibition halos around the disks was interpreted (BIEMER, 1973).

2.12. Adherence to intestinal epithelial cells

The method described by Jensen *et al.* (2012) was used, with minor changes. For this, DMEM medium (Vitrocell Embriolife, Campinas, Brazil) was added to 24-well culture plates with 2,105 human colon adenocarcinoma cells (Caco-2; ATCC HTB-37, Manassas, USA) with low content glucose, 20% (v/v) fetal bovine serum (Vitrocell Embriolife, Campinas, Brazil) and 100 U/mL antibiotic (penicillin/streptomycin) (Sigma-Aldrich, St. Louis, USA). Then, the plates were incubated at 37º C (humidified atmosphere, 5% CO_2 and 95% air) for three days, until the appropriate growth point was reached. To perform the adhesion test, the isolated bacteria were grown for 24 hours in suitable conditions and centrifuged (10,000 g for 10 minutes), and the pellet was resuspended in DMSO medium (without antibiotics). The monolayer formed by the growth of Caco-2 cells was washed twice with PBS prior to the start of the adhesion test, so that there was complete removal of the antibiotic used in the cell growth medium.

Thus, 1 mL of each bacterial culture (10^7 CFU/mL) was transferred individually to the wells and the plates were incubated at 37º C for 1, 2 or 4 hours, in order to optimize the assay. Subsequently, the cell monolayers were washed twice (PBS) to remove bacteria

that were unable to adhere, and lysis of the monolayer was performed by adding PBS with 0.1% Triton-X100 (Sigma-Aldrich, St. Louis, USA). The resulting suspension (viable adherent bacteria) was diluted in different concentrations and incubated in MRS medium (pouring plate method) for 48 hours. At the end of the experiment, the number of CFU/mL was determined, and results expressed as a percentage. Additionally, the ratio between the number of bacterial cells that remained adhered to the monolayer and the total number of bacterial cells added was measured.

2.13. Statistical analysis

The mean and standard deviation were used to express the results. The counts of viable bacteria were transformed into log values. The values in the tolerance test were compared using the software Statistica 12.0 (TIBCO, Palo Alto, CA, USA) applying the Tukey test with a level of significance $p < 0.05$.

3. Results

3.1. Isolation and identification by MALDI-TOF and 16S rRNA sequencing

A substantial number of CFU isolated from the cecum content of rainbow trout (*Oncorhynchus mykiss*) and starfish (order *Forcipulatida*) was observed. Subsequently, the isolated bacteria were collected and used in biochemical and morphological identification tests. All isolates belonging to the LAB group were selected for the next stages of this study and the bacteriocin-like inhibitory substances (BLIS) of each one were evaluated for their antimicrobial effect against important pathogens of fish and food. From rainbow trout samples, two isolates identified via MALDI-TOF as *Lactococcus lactis* (L1) and another as *Lactococcus garvieae* (L2), and one isolate from starfish identified as *Enterococcus faecium* 135 (EF) were selected for further molecular identification. The results obtained using the 16S rRNA method confirmed the previous data obtained by MALDI-TOF for isolates L1 and EF; however, molecular analysis indicated that isolate L2 is actually *L. lactis*. Sequences generated in this study were deposited at GenBank (NCBI) under accession numbers MZ926851, MZ735396 and MZ926852, respectively.

3.2. Tolerance of the isolates to low pH and bile salts

In order to assess the resistance of the isolates to environments that reflect the adverse conditions of the gastrointestinal tract, they were exposed to different pH and concentrations of bile salts (Figure 1). In the test of tolerance to different pH (Table S1), L1 was able to grow only in control conditions (pH = 6). The same behavior was observed in the test with bile salts, where after 1 hour of incubation there was no growth of L1 in any of the concentrations tested. Therefore, L1 was sensitive to low pH and high concentrations of bile salts, indicating that it must be protected by, for example, microencapsulation, if it is to be used as a probiotic. In contrast, the L2 isolate grew until 1 hour of incubation at pH 3, and no negative effect was observed in the test with bile salts, with good growth observed in all concentrations tested. Finally, EF was resistant to low pH and bile salts during all evaluated periods (Table S2), with the test data with 0.3% bile similar to the results in control conditions.

3.3. Hemolysin and gelatinase virulence factors

The capacity of the isolates to produce the extracellular proteins gelatinase and hemolysin was evaluated. None showed α or β-hemolytic profiles and there was also no gelatinolytic activity, since physical properties of the agar remained unchanged.

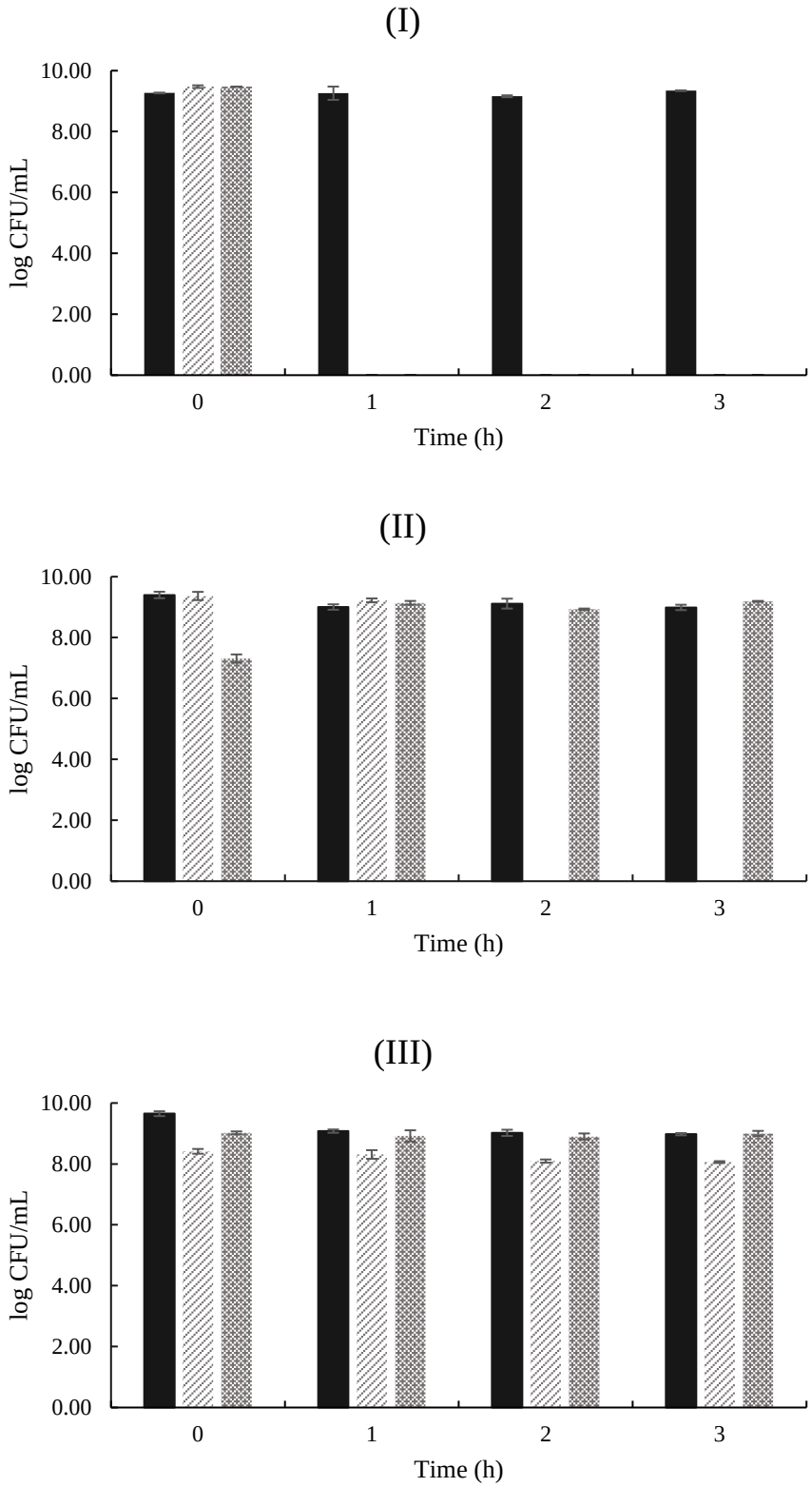

Figure 1. Tolerance of L1 (I), L2 (II) and EF (III), to pH 3 (▨) and 0.3% bile salts (▩. Strains without treatment of acid and bile salts were used as controls (■). Bars represent means ± standard deviation, n =3.

3.4. Antibiotic susceptibility testing

The susceptibility of isolates to the main antimicrobials of clinical interest was evaluated. In this sense, the three isolates possessed different sensitivity profiles, as observed from the measurement of inhibition halos when cultivated in the different antibiotics tested. Of note is that L1 was especially sensitive to ampicillin and clindamycin, and L2 and EF to clindamycin and rifampicin. When gentamicin was tested against EF, it was observed that the isolate is not very sensitive; however, its degree of resistance is considered low (CHARTERIS et al., 1998), so it could not be defined as resistant (Table 2).

Table 2. Sensitivity of isolates to antibiotics by diffusion in agar.

Isolated Probiotic Strains	**Antibiotic**			
	Name	**Disc concentration (µg)**	**Inhibition zone (mm)**	**Results ***
L1	Ampicillin	10	64.30	S
	Vancomycin	30	39.92	S
	Streptomycin	10	32.88	S
	Gentamicin	10	45.35	S
	Rifampicin	5	18.21	S
	Chloramphenicol	30	52.75	S
	Clindamycin	2	60.03	S
L2	Ampicillin	10	37.08	S
	Vancomycin	30	35.42	S
	Streptomycin	10	15.54	S
	Gentamicin	10	24.60	S
	Rifampicin	5	46.48	S
	Chloramphenicol	30	44.46	S
	Clindamycin	2	48.84	S
EF	Ampicillin	10	25.50	S
	Vancomycin	30	23.00	S
	Streptomycin	10	8.50	R
	Gentamicin	10	12.50	MS
	Rifampicin	5	30.50	S
	Chloramphenicol	30	29.50	S
	Clindamycin	2	31.50	S

S: susceptible; R: resistant; MS: mostly resistant. * Charteris *et* al. 1998.

3.5. Adhesion test to intestinal cells

All three isolates adhered to Caco-2 cells (Figure 2). After the first hour of the experiment, L2 presented adhesion of 94.2%, L1 77.1% and EF 65.6%. In the second hour, the adhesion percentages of L2 and EF were statistically similar (83%, P > 0.005), whilst L1 adhesion fell only marginally (76.6%, P > 0.005) compared to the first hour. It was observed that after the fourth hour of the experiment, all isolates tested suffered a reduction in adherence, ranging from 67.9% (L2) to 76.2% (EF). L1 possessed the most stable adherence over the time course of the assay. For L1 and L2, only one hour was necessary for the cells to adhere to the Caco-2 cells, whilst the best adherence of EF was obtained after 2 hours. With the high percentages of adherent cells, we conclude that if these isolates were administered to a host, they would probably adhere to intestinal cells, and exert a probiotic effect.

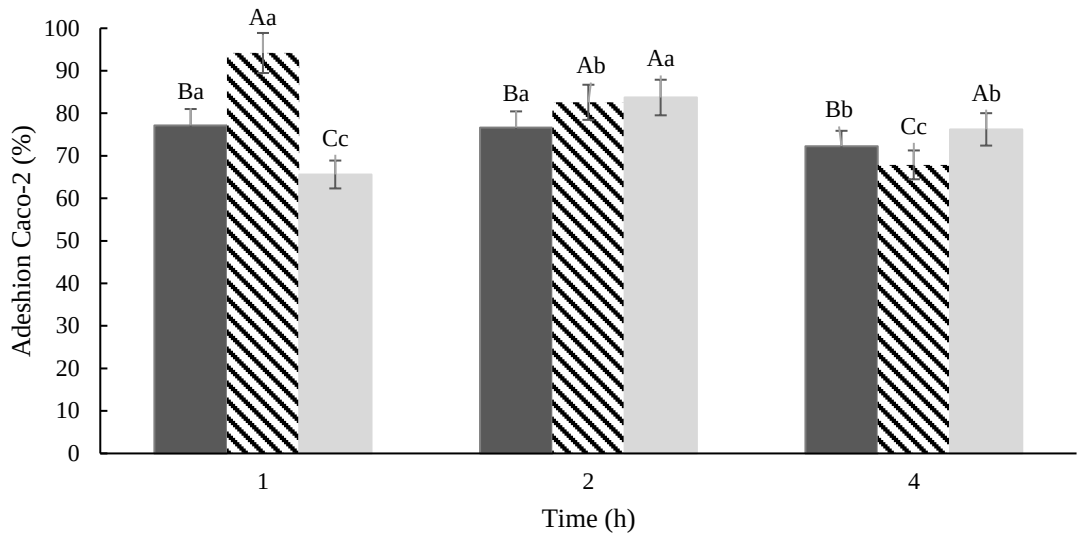

Figure 2. Adhesion (%) of L1 (■), L2 (▩) and EF (■) to Caco-2 cells, after 1, 2 and 4 hours of incubation. Different uppercase letters indicate statistically significant differences for all cultures taken at the same time (P < 0.005). Different lowercase letters indicate statistically significant differences for the same strain at different timepoints (P < 0.005). Bars represent means ± standard deviation, n = 3.

3.6. Coexistence test

After plating L1, L2 and EF in crossed lines, plates were incubated for 48 hours at 37º C. At the end of the experiment, it was observed that there was a substantial growth of all isolates tested and no antagonistic effects were evident (Figure 3).

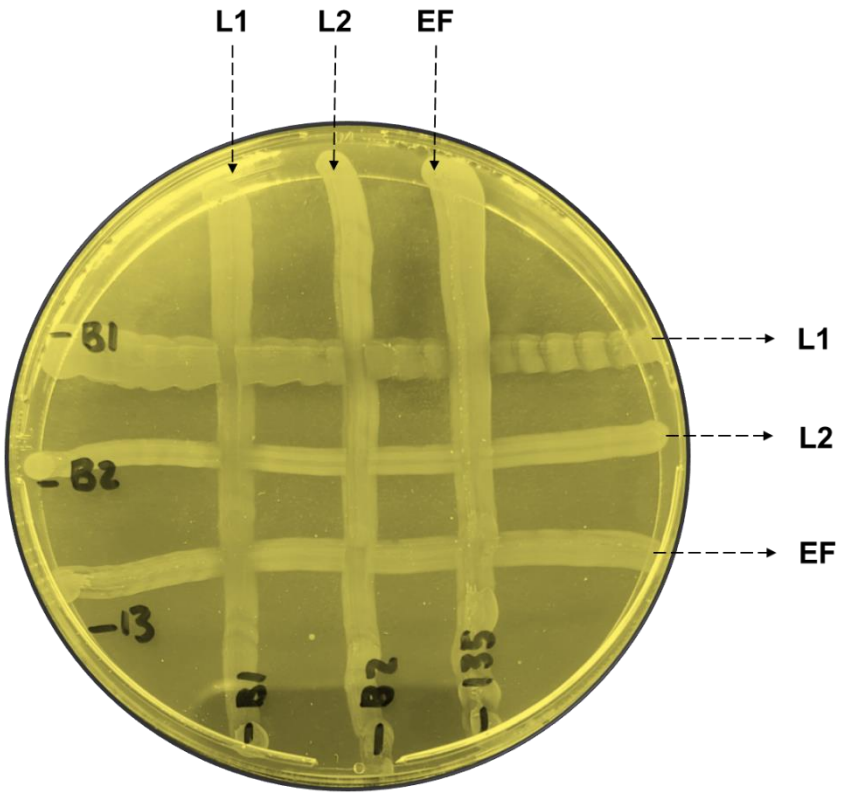

Figure 3. Coexistence test between isolates *L. lactis* (L1 and L2) and *E. faecium* (EF). No antagonist effects were observed.

3.7. Bacteriostatic effect of BLIS and interference with different growth stages

To assess the antimicrobial potential of BLIS produced by isolates and their possible capacity to produce bacteriocins, BLIS sensitivity tests against important pathogens were performed. After the incubation period, the formation of inhibition halos was observed. These were measured, and the antimicrobial effect of BLIS was determined by quantifying the area of the halo, considering the amount of BLIS used (Table 3). The BLIS of L1 had a good inhibitory effect against *Listeria monocytogenes* and *Staphylococcus aureus*, L2 against *L. monocytogenes*, *S. agalactiae*, *S. aureus* and

Salmonella Choleraesuis and EF against *L. monocytogenes and S.* Choleraesuis. Furthermore, the quantification of BLIS produced by the isolates revealed that L2 was the largest producer, particularly inhibiting the pathogens *L. monocytogenes* and *S. aureus*. None of the three isolates was able to inhibit the growth of *Salmonella* Typhimurium in this agar diffusion test.

Table 3. Average diameter (cm) and quantification (AU / mL) of the BLIS inhibition halos against pathogens.

Bioindicator strains	**BLIS of L1**		**BLIS of L2**		**BLIS of EF**	
	Inhibition zone (cm)	**Quant. (AU/mL)**	**Inhibition zone (cm)**	**Quant. (AU/mL)**	**Inhibition zone (cm)**	**Quant. (AU/mL)**
S. agalactiae	1.300	132.660	1.460	167.420	—	—
L. monocytogenes	1.035	162.338	1.629	255.596	2.282	408.790
S. aureus	1.025	160.768	1.014	159.198	—	—
S. Choleraesuis	—	—	0.898	140.986	1.263	125.220
S. Typhimurium	—	—	—	—	—	—

"—"; no inhibition.

These preliminary findings were corroborated by using a microplate reader, as a means of assessing BLIS mode of action against the tested pathogens. From this experiment, it was possible not only to confirm the positive results obtained in the agar diffusion test, but also to pinpoint the specific bacterial growth stage that was affected by BLIS. In general, it was observed that there was interference by the BLIS of all isolates on all growth phases of the pathogens, especially in the delay of the LAG phase and the early stages of the LOG phase, equivalent to the full exponential multiplication phase of microorganisms. In this experiment, *L. monocytogenes* was the most sensitive pathogen and the BLIS produced by L2 was the most potent (Figure 4).

In the test with *L. monocytogenes* (Figure 4A), BLIS of all isolates delayed the initial growth phases. Notably, the BLIS of EF and L2 delayed the end of the LAG phase of *L. monocytogenes* for up to 13h/OD_{600nm} 0.07 and 12h/OD_{600nm} 0.06, respectively, longer than the control (2h50/OD_{600nm} 0.08). In the group treated with the BLIS of EF, *L. monocytogenes* reached the beginning of the stationary phase at 21h50/OD_{600nm} 0.45 compared to 5h50/OD_{600nm} 0.70 in the control group. When general pathogen growth data were compared with those of the control, it was noted that the BLIS of the isolates

effectively slowed pathogen growth, an important indication of their potential use and of the possible presence of molecules with antimicrobial effects similar to bacteriocins.

Challenges with *S.* Choleraesuis and *S.* Typhimurium had similar results in this test. When exposed to BLIS of L1 and L2, the time needed by *S.* Choleraesuis to reach the end of the LAG and LOG phase increased (Figure 4B). The BLIS of EF influenced growth of both pathogens similar to the control, but it was able to maintain an OD_{600nm} of 1.0. However, the BLIS of L2 had the most potent antimicrobial effect, maintaining not only the microbial population at levels below the control amounts, but also delaying the end of the LOG phase from 5h/OD_{600nm} 1.4 to 16h/OD_{600nm} 0.71 in the treated group.

Unlike the agar diffusion test results, BLIS derived from the three isolates inhibited *S.* Typhimurium growth (Figure 4C). EF was able to reduce the OD_{600nm} by half when compared to the control, and L1 and L2 had similar effects of prolonging the LAG phase. Once again, a significant reduction in absorbance and an increase in the time of the LAG and LOG phase of bacterial growth, compared to the control, was observed. L2 proved to be the most potent; in the treated group, the stationary phase was reached at 6h/OD_{600nm} 0.58 versus 9h/OD_{600nm} 1.4 in the control group. Despite this, BLIS of L2 exerted its bacteriostatic effect throughout the period, limiting growth to just half of the OD_{600nm} seen in the control group.

The interference in the microbial growth phases occurred in a different way in tests with *S. aureus* (Figure 4D). When exposed to BLIS, especially from L1, more time was required for *S. aureus* to reach the end of the LAG phase (9h/OD_{600nm} 0.09, against 3h/OD_{600nm} 0.12 in the control). However, growth superior to that of the control was observed, a finding repeated in multiple independent assays. This may be because after the delay in the start of the exponential phase, there may have been an increase in the consumption of substrates present in the medium; alternatively, BLIS might boost growth when these biomolecules lose their inhibitory effect. Further investigations are thus needed to clarify the causes.

Regarding the test with *S. agalactiae* (Figure 4E), all the different BLIS used were able to prolong the LAG phase. Of special note is that the bactericidal effect of the BLIS of L2 prevented pathogen growth, as observed by the maintenance of OD_{600nm} below 0.1 throughout the experiment.

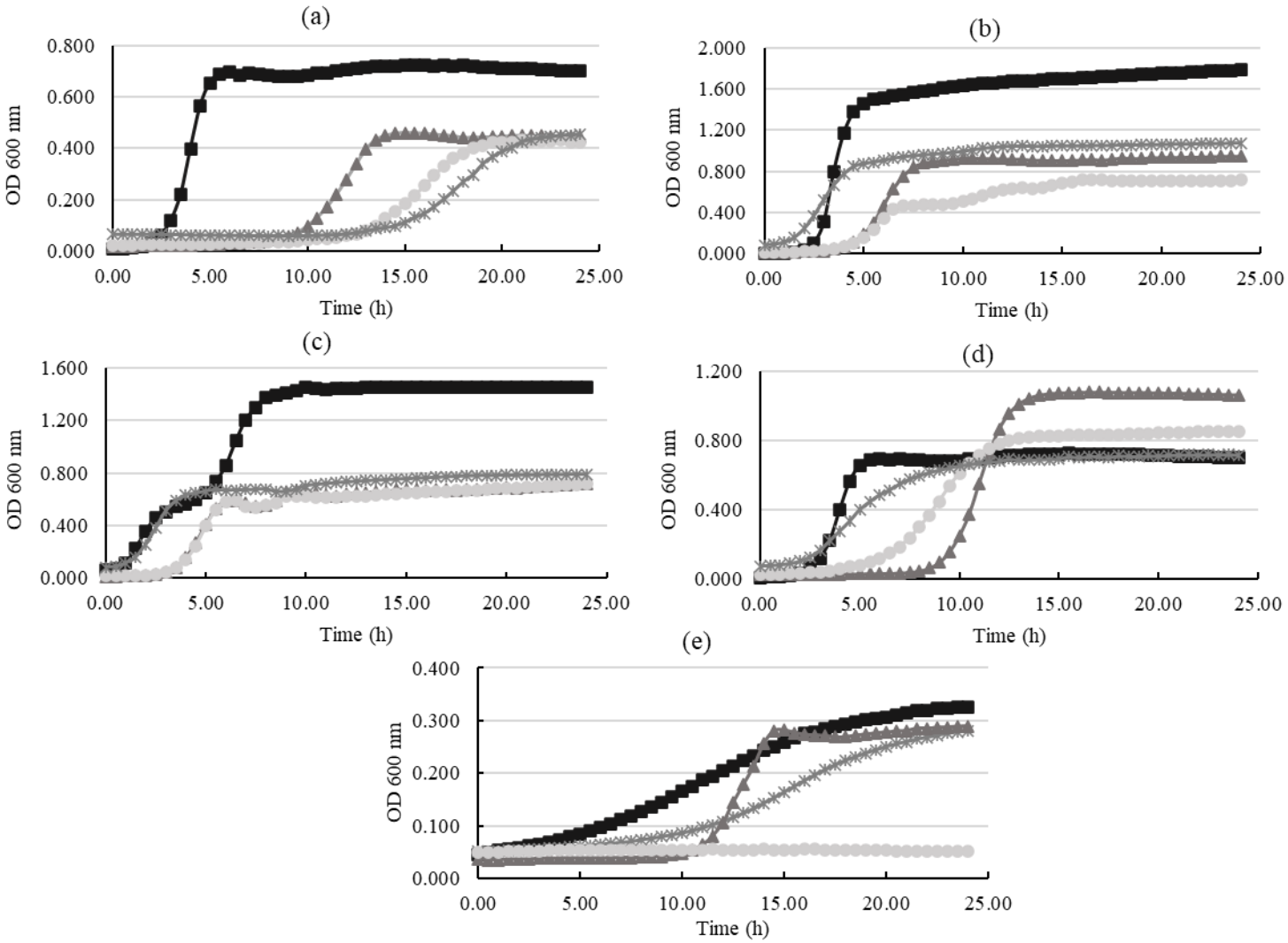

Figure 4. Antimicrobial activity of BLIS produced by L1 (▲), L2 (●), and EF (×) against the pathogens *L. monocytogenes* (a), *S.* Choleraesnius (b), *S.* Typhimurium (c), S. *aureus* (d), and S. *agalactae* (e). Assays performed with positive controls (■). The results are represented as an average of three readings.

3.8. Tolerance of BLIS to low pH and high temperatures

The tolerance of BLIS to low pH and high temperatures was also investigated. In this sense, the cell-free supernatant of the isolates was recovered and subjected to different pH (2, 4, 6, 8 and 10) and temperature (30, 50, 70, 90 and 120º C) treatments and then tested against *L. monocytogenes* (Figure S1 and S2). It was observed that the BLIS of L1 and L2 maintained their activity against the pathogen up to 70º C, while EF

maintained its activity up to 90º C. In the exposure test to different pH, none of the BLIS lost activity at any of the different pH values tested.

3.9. Assessment of the protein nature of BLIS

An important step in the characterization of BLIS is the use of proteolytic enzymes to assess their possible protein nature. As already described, since bacteriocins are characterized as antimicrobial peptides, it is expected that there is a loss of antimicrobial activity after treatment with enzymes such as trypsin, pancreatin, papain and pepsin (Figure S3). In such assays, when compared to the control group, EF BLIS had a total loss of inhibitory activity after incubation with all enzymes tested (Table 4). In turn, L1 and L2 BLIS had a considerable loss of inhibitory activity after treatment with all 4 enzymes. These data strongly suggest the presence of protein molecules with antimicrobial activity in BLIS from all three aquatic isolates.

Table 4. Effect of enzymatic treatment, pH and temperature on the stability of the BLIS produced by *L. lactis* (L1 and L2), and *E. faecium* 135 (EF).

Treatment	**Inhibition zone ***		
	L1	L2	EF
Control	+++	+++	+++
Enzymatic treatment:			
Trypsin	++	++	-
Pepsin	+	+	-
Papain	+	+	-
Pancreatin	+	+	-
pH resistance:			
2, 4, 6, 8 and 10 for 1 hour	+++	+++	+++
Heat treatment:			
30, 50, 70 or 90 ºC for 1 hour	+++	+++	+++
120 ºC for 15 minutes	-	-	-

* (+++) > 12 millimeters, (++) 10 – 11.99 millimeters, (+) 8 – 9.99 millimeters, and (-) did not show inhibition zone. The bioindicator strain used to evaluate antimicrobial activity was *Listeria monocytogenes* CECT 934. Control: BLIS without any treatment. The concentration of the enzymes used in the experiments was 1% (w/v).

3.10. Presence of genes for different bacteriocins

As a preliminary approach, a study was carried out to detect the main bacteriocins that have been described in the literature in recent years for bacteria of the genus *Lactococcus* and *Enterococcus*. Primers were designed and synthesized to amplify the most well-studied bacteriocins of these genera, which were subsequently used for amplification in PCR reactions. The PCR amplicons were analyzed, revealing the presence of promising amplicons (Figure S4) for Nisin in L1 and L2 and for different Enterocins A, B and P, and for Mundticin KS in EF (Table 1).

4. Discussion

In this study, it was demonstrated that the intestinal tracts of two animals belonging to aquatic environments are an important source of probiotic bacteria with bacteriocinogenic potential, data that demonstrates the importance of this environment for the isolation of new probiotic strains with biotechnological potential (HUSAIN et al., 2022). These results corroborate the data of Sarika *et al.* (2012), where the authors report that a strain of *L. lactis* PSY2 isolated from marine perch (*Perca flavescens*) had a bacteriocinogenic profile and a significant antimicrobial effect against several Gram-negative and Gram-positive bacteria, such as *L. monocytogenes* and *S. aureus*. The authors also emphasize that such a bacteriocinogenic profile can assist in food preservation; in tests carried out with the strain there was an increase of more than 21 days of shelf-life, useful for the preservation of high-value sea foods. Thus, in relation to our study, it is important to emphasize that once the antimicrobial potential of the BLIS identified in the three isolates has been demonstrated, specific studies will be carried out in order to evaluate their possible use in seafood preservation.

After confirming the presence of genes for bacteriocins in all isolates (such as Nisin and Enterocin) and the loss of BLIS activity after enzymatic treatment, their bacteriocinogenic potential should be evaluated further. Indeed, the preliminary tests demonstrated that the L2 and EF isolates from rainbow trout and starfish, respectively, are not only bacteriocin producers, but also have substantial probiotic potential, as they can resist pH 3 and various concentrations of bile salts. In this study, among the pathogens analyzed, *L. monocytogenes*, *S.* Choleraesuis and *S.* Typhimurium were the most sensitive

to the bacteriostatic effect of the isolates. The BLIS of L2 had the best results in the inhibition tests, including a bacteriostatic effect against *S. agalactiae*.

The *Salmonella* pathogen is a major concern for the food industry, as it is transmitted through contaminated food and water. In recent years, probiotic bacteria have been studied for the control of the pathogen with promising results (LIU et al., 2022). The preliminary inhibition results observed in our study need to be further evaluated. Nevertheless, they are promising, as they indicate that bacteriocins could be used as a possible non-chemical containment strategy for these pathogens. In a similar survey, Sahnouni *et al.* (2014) investigated the antagonistic effect of 38 LAB isolates against several pathogens, including *Salmonella* sp. The BLIS tested were found to be ineffective against Gram-negative bacteria such as *Salmonella* sp. and *Escherichia coli*, compared to the others. However, in an *in vivo* study, Mulaw *et al.* (2020) observed a different result. These authors tested a mix of probiotic bacteria (*Lactobacillus plantarum* K132, *Lactobacillus paracasei* K114 and *L. lactis* E124) against infection by *S.* Typhimurium DT104 in mice. They observed that, compared to the control group, treatment with a mix of probiotics led to a reduction in *S.* Typhimurium DT104 counts in feces and the survival rate was significantly higher.

In the test of tolerance to low pH and different concentrations of bile salts, isolates EF and L2 had the best results, with EF resisting all ranges of pH and bile salts tested. As in our study, Yerlikaya (2019) evaluated isolated probiotic bacteria in order to select strains for the production of functional foods. During the characterization phase of isolated *L. lactis* strains, the researchers evaluated their ability to resist bile salts and found that none of the tested strains managed to grow in their presence, an important indicator of the high sensitivity of the genus *Lactococcus* to such substances. In turn, Jawan *et al*. [43] also evaluated the susceptibility of *L. lactis* Gh1 to these factors and found that the strain was tolerant to pH 3 and bile salts at a concentration of 0.3%, indicating that resistance against these factors is strain-specific. Moreover, Dowdell *et al*. (2020) demonstrated the ability of *E. faecium* and *L. lactis* to survive a simulation of adverse conditions in the gastrointestinal tract. The results were similar to those present in our study, and the authors also demonstrated the superior ability of EF to survive acidic environments when compared to *L. lactis*.

An important step for the development of products with new probiotic strains is the characterization study. In this study, it is possible to simulate different conditions that

the probiotic will find in the body, such as those of the gastrointestinal tract, especially the high acidity and the action of bile salts. By demonstrating resistance in these tests, the probiotic strain becomes an important candidate for more specific tests, since resisting these tests is an important indication that the potential of the probiotic observed in in vivo studies will be maintained in in vivo studies (CERDÁ-BERNAD et al., 2022). Thus, Fahim *et al*. (2016) state that a viable alternative would be the use of microencapsulation to increase cell viability. According to the authors, the use of microencapsulation with alginate in association with chitosan offers protection to both the probiotic and biomolecules in the passage through the gastrointestinal tract. Other studies, such as those of Rodklongtan *et al*. (2014), Song *et al*. (2013) and Zohri *et al*. (2010), also report increased cell viability after using different microencapsulation techniques.

Considering that one of the longer term objectives of the present work is the biotechnological application of isolated bacteria and their biomolecules in the formulation of, for example, functional foods, the expression of hemolysin and gelatinase virulence factors in the isolates needed to be investigated. This is because the presence of microorganisms with such characteristics in food matrices is a problem, as these virulence factors may be associated with the development of serious diseases and death (SABO et al., 2020; TAN et al., 2013). Therefore, the absence of expression of such virulence factors in this study is encouraging, although the presence of other virulence genes also needs to be evaluated before performing experiments *in vivo*.

In the same sense, one of the most undesirable characteristics of a probiotic microorganism is the ability to withstand exposure to antibiotics. In our study, none of the isolates showed resistance to the antibiotics tested, all of which are of clinical importance. Therefore, our results are of great importance and reflect what has also been previously described by other studies with LAB (GHARBI et al., 2019; PACHECO DA SILVA et al., 2016; SABO et al., 2020).

After evaluating the expression of these important virulence factors, future work should focus on the possibility of using the isolates in a probiotic mixture. Indeed, Mariam *et al.* (2014) isolated probiotic strains belonging to the LAB group and, after several tests, raised an important issue. Specifically, according to the authors, co-culture in mixtures was not only possible but also increased BLIS antimicrobial action, which started to inhibit pathogenic bacteria such as *L. monocytogenes* (a microorganism that can resist common food preservation methods) more effectively, thus reducing cell count to much

lower levels than the control group. For this reason, the authors encourage studies with new probiotic strains to assess their interaction in mixed cultures.

In the experiment with Caco-2 cells, the percentage of adherence was high for all isolates tested (>70%), a finding which is encouraging for future *in vivo* studies. Although promising, the high adhesion potential of *L. lactis* and *E. faecium* are well described in the literature. Nascimento *et al*. (2019) and Downdell *et al*. (2020) carried out similar studies and obtained good adherence percentages, but lower than those observed in our study. Vasiee (2019), in turn, evaluated the adherence potential of the recombinant strain *L. lactis* NZ1330 to Caco-2 cells and its antagonistic effect on *E. coli*. In the end, a good adhesion potential and ability to compete and prevent the adhesion of *E. coli* to Caco-2 cells was observed. Furthermore, He *et al.* (2019) demonstrated the ability of *E. faecium* WEFA23 to compete and inhibit (>50%) the adherence of *L. monocytogenes* and *S.* Typhimurium to Caco-2 cells.

The promising results observed in this study indicate the possibility of using bacteriocinogenic probiotic strains as a food supplement. Because they are bacteriocin-producing strains, the effects on pathogen control may be even greater. The increase in recently published studies in the area demonstrating the beneficial effects on health and disease resistance after supplementation with probiotics reveals the great scientific potential of this segment (DENG et al., 2022; IORIZZO et al., 2022).

In summary, from the promising results obtained in this study, a bacteriocin purification study will be carried out, in addition to the evaluation of the protective potential of microencapsulation on the isolates and the individual and concomitant (mix) probiotic effect in an *in vivo* test.

5. Conclusions

The aquatic environment proved to be an important source of bacteriocinogenic probiotic bacteria. All isolates evaluated in this study harbor genes for bacteriocins, showed antimicrobial activity against important fish and food pathogens, were sensitive to all antibiotics tested, had a high rate of adherence to Caco-2 cells and did not express hemolysin and gelatinase virulence factors. It was shown that isolates L1 and L2 from rainbow trout were not able to resist low pH. However, isolates L2 and EF (from starfish)

demonstrated good resistance to the action of bile salts, and EF was also resistant to pH 2.5 and 3. For this reason, future tests to evaluate the protective effect of microencapsulation on the viability of the isolates and their effect on an animal model will be carried out. There is no doubt that the new discoveries in the field of probiotics will bring countless changes in this area of study, which will result in ever higher quality foods and consumer health, whilst lowering impacts on nature. One of the main advances brought about by research with individual and mixtures of probiotics, is the gradual replacement of antibiotics, decreased new episodes of microbial resistance and better responses to production diseases, commonly treated with chemicals or antibiotics.

References

ABDELFATAH, Eman N.; MAHBOUB, Heba Hassan H. Studies on the effect of Lactococcus garvieae of dairy origin on both cheese and Nile tilapia (O. niloticus). **International Journal of Veterinary Science and Medicine**, *[S. l.]*, v. 6, n. 2, p. 201–207, 2018. DOI: 10.1016/j.ijvsm.2018.11.002. Disponível em: https://doi.org/10.1016/j.ijvsm.2018.11.002.

ALEGRÍA, Ángel; DELGADO, Susana; ROCES, Clara; LÓPEZ, Belén; MAYO, Baltasar. Bacteriocins produced by wild Lactococcus lactis strains isolated from traditional, starter-free cheeses made of raw milk. **International Journal of Food Microbiology**, *[S. l.]*, v. 143, n. 1–2, p. 61–66, 2010. DOI: 10.1016/j.ijfoodmicro.2010.07.029.

ALMEIDA, Tereza et al. Identification of bacteriocin genes in enterococci isolated from game animals and saltwater fish. **Journal of Food Protection**, *[S. l.]*, v. 74, n. 8, p. 1252–1260, 2011. DOI: 10.4315/0362-028X.JFP-11-016.

ALVES, Levy Anderson César; SOUZA, Rafael Celestino; DA SILVA, Taciana Mara Couto; WATANABE, Andreia; DIAS, Meriellen; MENDES, Maria Anita; CIAMPONI, Ana Lídia. Identification of microorganisms in biofluids of individuals with periodontitis and chronic kidney disease using matrix-assisted laser desorption/ionization time-of-flight mass spectrometry. **Rapid Communications in Mass Spectrometry**, *[S. l.]*, v. 30, n. 10, p. 1228–1232, 2016. DOI: 10.1002/rcm.7555. Disponível em: https://onlinelibrary.wiley.com/doi/10.1002/rcm.7555.

BELGACEM, Zouhaier Ben; ABRIOUEL, Hikmate; OMAR, Nabil Ben; LUCAS, Rosario; MARTÍNEZ-CANAMERO, Magdalena; GÁLVEZ, Antonio; MANAI, Mohamed. Antimicrobial activity, safety aspects, and some technological properties of bacteriocinogenic Enterococcus faecium from artisanal Tunisian fermented meat. **Food Control**, *[S. l.]*, v. 21, n. 4, p. 462–470, 2010. DOI: 10.1016/j.foodcont.2009.07.007.

Disponível em: https://linkinghub.elsevier.com/retrieve/pii/S0956713509002230. Acesso em: 8 jan. 2021.

BIEMER, J. J. Antimicrobial susceptibility testing by the Kirby-Bauer disc diffusion method. **Annals of clinical laboratory science**, *[S. l.]*, v. 3, n. 2, p. 135–140, 1973. DOI: 10.1128/aem.63.12.4770-4777.1997. Disponível em: http://www.ncbi.nlm.nih.gov/pubmed/4575155.

CABO, M. L.; MURADO, M. A.; GONZALEZ, M. P.; PASTORIZA, L. A method for bacteriocin quantification. **Journal of Applied Microbiology**, *[S. l.]*, v. 87, n. 6, p. 907–914, 1999. DOI: 10.1046/j.1365-2672.1999.00942.x. Disponível em: http://doi.wiley.com/10.1046/j.1365-2672.1999.00942.x.

CERDÁ-BERNAD, Débora; VALERO-CASES, Estefanía; PASTOR, Joaquín-Julián; FRUTOS, María José; PÉREZ-LLAMAS, Francisca. Probiotic red quinoa drinks for celiacs and lactose intolerant people: study of functional, physicochemical and probiotic properties during fermentation and gastrointestinal digestion. **International Journal of Food Sciences and Nutrition**, *[S. l.]*, v. 73, n. 1, p. 49–59, 2022. DOI: 10.1080/09637486.2021.1921707. Disponível em: https://doi.org/10.1080/09637486.2021.1921707.

CHARTERIS, WILLIAM P.; KELLY, PHILLIP M.; MORELLI, LORENZO; COLLINS, J. KEVIN. Antibiotic Susceptibility of Potentially Probiotic Lactobacillus Species. **Journal of Food Protection**, *[S. l.]*, v. 61, n. 12, p. 1636–1643, 1998. DOI: 10.4315/0362-028X-61.12.1636. Disponível em: https://meridian.allenpress.com/jfp/article/61/12/1636/167794/Antibiotic-Susceptibility-of-Potentially-Probiotic.

CHEN, Yuhuan; LUDESCHER, Richard D.; MONTVILLE, Thomas J. Electrostatic interactions, but not the YGNGV consensus motif, govern the binding of pediocin PA-1 and its fragments to phospholipid vesicles. **Applied and Environmental Microbiology**, *[S. l.]*, v. 63, n. 12, p. 4770–4777, 1997. DOI: 10.1128/aem.63.12.4770-4777.1997. Disponível em: https://journals.asm.org/doi/10.1128/aem.63.12.4770-4777.1997.

COTTER, Paul D.; ROSS, R. Paul; HILL, Colin. Bacteriocins-a viable alternative to antibiotics? **Nature Reviews Microbiology**, *[S. l.]*, v. 11, n. 2, p. 95–105, 2013. DOI: 10.1038/nrmicro2937.

DAWOOD, Mahmoud A. O.; KOSHIO, Shunsuke; ABDEL-DAIM, Mohamed M.; VAN DOAN, Hien. Probiotic application for sustainable aquaculture. **Reviews in Aquaculture**, *[S. l.]*, v. 11, n. 3, p. 907–924, 2019. DOI: 10.1111/raq.12272. Disponível em: https://onlinelibrary.wiley.com/doi/10.1111/raq.12272.

DE VUYST, L. Screening for enterocins and detection of hemolysin and vancomycin resistance in enterococci of different origins. **International Journal of Food Microbiology**, *[S. l.]*, v. 84, n. 3, p. 299–318, 2003. DOI: 10.1016/S0168-1605(02)00425-7. Disponível em:

https://linkinghub.elsevier.com/retrieve/pii/S0168160502004257. Acesso em: 8 jan. 2021.

DENG, Yale; VERDEGEM, Marc C. J.; EDING, Ep; KOKOU, Fotini. Effect of rearing systems and dietary probiotic supplementation on the growth and gut microbiota of Nile tilapia (Oreochromis niloticus) larvae. **Aquaculture**, *[S. l.]*, v. 546, n. May 2021, p. 737297, 2022. DOI: 10.1016/j.aquaculture.2021.737297. Disponível em: https://doi.org/10.1016/j.aquaculture.2021.737297.

DOWDELL, Panya; CHANKHAMHAENGDECHA, Surang; PANBANGRED, Watanalai; JANVILISRI, Tavan; AROONNUAL, Amornrat. Probiotic Activity of Enterococcus faecium and Lactococcus lactis Isolated from Thai Fermented Sausages and Their Protective Effect Against Clostridium difficile. **Probiotics and Antimicrobial Proteins**, *[S. l.]*, v. 12, n. 2, p. 641–648, 2020. DOI: 10.1007/s12602-019-09536-7.

FAHIM, Hazem A.; KHAIRALLA, Ahmed S.; EL-GENDY, Ahmed O. Nanotechnology: A valuable strategy to improve bacteriocin formulations. **Frontiers in Microbiology**, *[S. l.]*, v. 7, n. SEP, 2016. DOI: 10.3389/fmicb.2016.01385.

GHARBI, Yosra; FHOULA, Imene; RUAS-MADIEDO, Patricia; AFEF, Najjari; BOUDABOUS, Abdellatif; GUEIMONDE, Miguel; OUZARI, Hadda-Imene. In-vitro characterization of potentially probiotic Lactobacillus strains isolated from human microbiota: interaction with pathogenic bacteria and the enteric cell line HT29. **Annals of Microbiology**, *[S. l.]*, v. 69, n. 1, p. 61–72, 2019. DOI: 10.1007/s13213-018-1396-1. Disponível em: http://link.springer.com/10.1007/s13213-018-1396-1.

GHEZIEL, Chahira et al. Evaluating the Probiotic Potential of Lactobacillus plantarum Strains from Algerian Infant Feces: Towards the Design of Probiotic Starter Cultures Tailored for Developing Countries. **Probiotics and Antimicrobial Proteins**, *[S. l.]*, v. 11, n. 1, p. 113–123, 2019. DOI: 10.1007/s12602-018-9396-9.

GUERREIRO, Inês; OLIVA-TELES, Aires; ENES, Paula. Prebiotics as functional ingredients: focus on Mediterranean fish aquaculture. **Reviews in Aquaculture**, *[S. l.]*, v. 10, n. 4, p. 800–832, 2018. DOI: 10.1111/raq.12201.

GUO, Zhuang; WANG, Jicheng; YAN, Liya; CHEN, Wei; LIU, Xiao ming; ZHANG, He ping. In vitro comparison of probiotic properties of Lactobacillus casei Zhang, a potential new probiotic, with selected probiotic strains. **LWT - Food Science and Technology**, *[S. l.]*, v. 42, n. 10, p. 1640–1646, 2009. DOI: 10.1016/j.lwt.2009.05.025. Disponível em: http://dx.doi.org/10.1016/j.lwt.2009.05.025.

HE, Yao; XU, Xiongpeng; ZHANG, Fen; XU, Di; LIU, Zhengqi; TAO, Xueying; WEI, Hua. Anti-adhesion of probiotic Enterococcus faecium WEFA23 against five pathogens and the beneficial effect of its S-layer proteins against Listeria monocytogenes. **Canadian Journal of Microbiology**, *[S. l.]*, v. 65, n. 3, p. 175–184, 2019. DOI: 10.1139/cjm-2018-0031. Disponível em: http://www.nrcresearchpress.com/doi/10.1139/cjm-2018-0031.

HENNING, Chris; GAUTAM, Dhiraj; MURIANA, Peter. Identification of Multiple Bacteriocins in Enterococcus spp. Using an Enterococcus-Specific Bacteriocin PCR Array. **Microorganisms**, *[S. l.]*, v. 3, n. 1, p. 1–16, 2015. DOI: 10.3390/microorganisms3010001.

HILL, Colin et al. The International Scientific Association for Probiotics and Prebiotics consensus statement on the scope and appropriate use of the term probiotic. **Nature Reviews Gastroenterology & Hepatology**, *[S. l.]*, v. 11, n. 8, p. 506–514, 2014. DOI: 10.1038/nrgastro.2014.66. Disponível em: http://www.nature.com/articles/nrgastro.2014.66.

HUSAIN, Fazal; DURAISAMY, Senbagam; BALAKRISHNAN, Senthilkumar; RANJITH, Sukumar; CHIDAMBARAM, Prahalathan; KUMARASAMY, Anbarasu. Phenotypic assessment of safety and probiotic potential of native isolates from marine fish Moolgarda seheli towards sustainable aquaculture. **Biologia**, *[S. l.]*, v. 77, n. 3, p. 775–790, 2022. DOI: 10.1007/s11756-021-00957-w. Disponível em: https://doi.org/10.1007/s11756-021-00957-w.

IORIZZO, Massimo et al. Probiotic Potentiality from Versatile Lactiplantibacillus plantarum Strains as Resource to Enhance Freshwater Fish Health. **Microorganisms**, *[S. l.]*, v. 10, n. 2, p. 463, 2022. DOI: 10.3390/microorganisms10020463.

JENSEN, Hanne; GRIMMER, Stine; NATERSTAD, Kristine; AXELSSON, Lars. In vitro testing of commercial and potential probiotic lactic acid bacteria. **International Journal of Food Microbiology**, *[S. l.]*, v. 153, n. 1–2, p. 216–222, 2012. DOI: 10.1016/j.ijfoodmicro.2011.11.020. Disponível em: http://dx.doi.org/10.1016/j.ijfoodmicro.2011.11.020.

KLAENHAMMER, Todd R. Genetics of bacteriocins produced by lactic acid bacteria. **FEMS Microbiology Reviews**, *[S. l.]*, v. 12, n. 1–3, p. 39–85, 1993. DOI: 10.1016/0168-6445(93)90057-G.

KUNIYOSHI, Taís Mayumi et al. Pediocin PA-1 production by Pediococcus pentosaceus ET34 using non-detoxified hemicellulose hydrolysate obtained from hydrothermal pretreatment of sugarcane bagasse. **Bioresource Technology**, *[S. l.]*, v. 338, n. May, p. 125565, 2021. DOI: 10.1016/j.biortech.2021.125565. Disponível em: https://linkinghub.elsevier.com/retrieve/pii/S0960852421009068.

LIU, Guorong; GRIFFITHS, Mansel W.; WU, Pengpeng; WANG, Haifeng; ZHANG, Xiangmei; LI, Pinglan. Enterococcus faecium LM-2, a multi-bacteriocinogenic strain naturally occurring in “Byaslag”, a traditional cheese of Inner Mongolia in China. **Food Control**, *[S. l.]*, v. 22, n. 2, p. 283–289, 2011. DOI: 10.1016/j.foodcont.2010.07.023. Disponível em: https://linkinghub.elsevier.com/retrieve/pii/S0956713510002446. Acesso em: 11 jan. 2021.

LIU, Junsheng; LI, Xiangfei; SONG, Fanfen; CUI, Shumao; LU, Wenwei; ZHAO, Jianxin; ZHANG, Hao; GU, Zhennan; CHEN, Wei. Dietary supplementation with low-dose xylooligosaccharide promotes the anti-Salmonella activity of probiotic

Lactiplantibacillus plantarum ZS2058 in a murine model. **Food Research International**, *[S. l.]*, v. 151, n. December 2021, p. 110858, 2022. DOI: 10.1016/j.foodres.2021.110858. Disponível em: https://doi.org/10.1016/j.foodres.2021.110858.

MARIAM, SH; ZEGEYE, N.; TARIKU, T. Potential of cell-free supernatants from cultures of selected lactic acid bacteria and yeast obtained from local fermented foods as inhibitors of Listeria. **Bmc**, *[S. l.]*, p. 1–9, 2014. Disponível em: http://bmcresnotes.biomedcentral.com/articles/10.1186/1756-0500-7-606.

MARTÍN, María; GUTIÉRREZ, Jorge; CRIADO, Raquel; HERRANZ, Carmen; CINTAS, Luis M.; HERNÁNDEZ, Pablo E. Genes encoding bacteriocins and their expression and potential virulence factors of enterococci isolated from wood pigeons (Columba palumbus). **Journal of Food Protection**, *[S. l.]*, v. 69, n. 3, p. 520–531, 2006. DOI: 10.4315/0362-028X-69.3.520.

MARTÍNEZ, Beatriz; FERNÁNDEZ, María; SUÁREZ, Juan E.; RODRÍGUEZ, Ana. Synthesis of lactococcin 972, a bacteriocin produced by Lactococcus lactis IPLA 972, depends on the expression of a plasmid-encoded bicistronic operon. **Microbiology**, *[S. l.]*, v. 145, n. 11, p. 3155–3161, 1999. DOI: 10.1099/00221287-145-11-3155.

MBANDLWA, Philiswa; DOYLE, Natasha; HILL, Colin; STANTON, Catherine; ROSS, R. Paul. **Bacteriocins: Novel Applications in Food, and Human and Animal Health**. Third Edition ed. [s.l.] : Elsevier, 2022. v. 4 DOI: 10.1016/b978-0-08-100596-5.23030-8. Disponível em: http://dx.doi.org/10.1016/B978-0-08-100596-5.23030-8.

MULAW, Guesh; MULETA, Diriba; TESFAYE, Anteneh; SISAY, Tesfaye. Protective Effect of Potential Probiotic Strains from Fermented Ethiopian Food against Salmonella Typhimurium DT104 in Mice. **International Journal of Microbiology**, *[S. l.]*, v. 2020, p. 1–8, 2020. DOI: 10.1155/2020/7523629. Disponível em: https://www.hindawi.com/journals/ijmicro/2020/7523629/.

NASCIMENTO, Liane Caroline Sousa; CASAROTTI, Sabrina Neves; TODOROV, Svetoslav Dimitrov; PENNA, Ana Lúcia Barretto. Probiotic potential and safety of enterococci strains. **Annals of Microbiology**, *[S. l.]*, v. 69, n. 3, p. 241–252, 2019. DOI: 10.1007/s13213-018-1412-5. Disponível em: http://link.springer.com/10.1007/s13213-018-1412-5.

OGAKI, Mayara Baptistucci; FURLANETO, Márcia Cristina; MAIA, Luciana Furlaneto. Revisão: Aspectos gerais das bacteriocinas. **Brazilian Journal of Food Technology**, *[S. l.]*, v. 18, n. 4, p. 267–276, 2015. DOI: 10.1590/1981-6723.2215. Disponível em: http://www.scielo.br/scielo.php?script=sci_arttext&pid=S1981-67232015000400267&lng=pt&tlng=pt.

PACHECO DA SILVA, Fabiana Fernanda; BISCOLA, Vanessa; LEBLANC, Jean Guy; GOMBOSSY DE MELO FRANCO, Bernadette Dora. Effect of indigenous lactic acid bacteria isolated from goat milk and cheeses on folate and riboflavin content of fermented goat milk. **LWT - Food Science and Technology**, *[S. l.]*, v. 71, p. 155–161, 2016. DOI: 10.1016/j.lwt.2016.03.033. Disponível em: http://dx.doi.org/10.1016/j.lwt.2016.03.033.

PÉREZ-SÁNCHEZ, Tania; MORA-SÁNCHEZ, Brenda; BALCÁZAR, José Luis. Biological Approaches for Disease Control in Aquaculture: Advantages, Limitations and Challenges. **Trends in Microbiology**, *[S. l.]*, v. 26, n. 11, p. 896–903, 2018. DOI: 10.1016/j.tim.2018.05.002. Disponível em: http://dx.doi.org/10.1016/j.tim.2018.05.002.

QIN, Chubin; ZHANG, Zhen; WANG, Yibing; LI, Shuning; RAN, Chao; HU, Jun; XIE, Yadong; LI, Weifen; ZHOU, Zhigang. EPSP of L. casei BL23 protected against the infection caused by Aeromonas veronii via enhancement of immune response in zebrafish. **Frontiers in Microbiology**, *[S. l.]*, v. 8, n. DEC, p. 1–13, 2017. DOI: 10.3389/fmicb.2017.02406.

RINGØ, Einar; HOSEINIFAR, Seyed Hossein; GHOSH, Koushik; DOAN, Hien Van; BECK, Bo Ram; SONG, Seong Kyu. Lactic acid bacteria in finfish-An update. **Frontiers in Microbiology**, *[S. l.]*, v. 9, n. AUG, p. 1–37, 2018. DOI: 10.3389/fmicb.2018.01818.

RODKLONGTAN, A.; LA-ONGKHAM, O.; NITISINPRASERT, S.; CHITPRASERT, P. Enhancement of Lactobacillus reuteri KUB-AC5 survival in broiler gastrointestinal tract by microencapsulation with alginate-chitosan semi-interpenetrating polymer networks. **Journal of Applied Microbiology**, *[S. l.]*, v. 117, n. 1, p. 227–238, 2014. DOI: 10.1111/jam.12517.

ROGERS, Andrew W. L.; TSOLIS, Renée M.; BÄUMLER, Andreas J. Salmonella versus the Microbiome. **Microbiology and molecular biology reviews : MMBR**, *[S. l.]*, v. 85, n. 1, 2021. DOI: 10.1128/MMBR.00027-19. Disponível em: https://journals.asm.org/doi/10.1128/MMBR.00027-19.

SABO, Sabrina da Silva et al. Bioprospecting of probiotics with antimicrobial activities against Salmonella Heidelberg and that produce B-complex vitamins as potential supplements in poultry nutrition. **Scientific Reports**, *[S. l.]*, v. 10, n. 1, p. 1–14, 2020. DOI: 10.1038/s41598-020-64038-9.

SABO, Sabrina da Silva; LOPES, André Moreni; SANTOS-EBINUMA, Valéria de Carvalho; RANGEL-YAGUI, Carlota de Oliveira; OLIVEIRA, Ricardo Pinheiro de Souza. Bacteriocin partitioning from a clarified fermentation broth of Lactobacillus plantarum ST16Pa in aqueous two-phase systems with sodium sulfate and choline-based salts as additives. **Process Biochemistry**, *[S. l.]*, v. 66, p. 212–221, 2018. DOI: 10.1016/j.procbio.2017.11.018. Disponível em: https://linkinghub.elsevier.com/retrieve/pii/S1359511317311315. Acesso em: 25 nov. 2020.

SAHNOUNI, Fatima; MATALLAH-BOUTIBA, Amaria; ZITOUNI, Boutiba. Characterization of bacteriocin produced by Lactococcus lactis ssp. lactis strains isolated from marine fish caught in the Algerian west coast. **Türk Tarım ve Doğa Bilimleri Dergisi-Turkish Journal of Agricultural and Natural Sciences**, *[S. l.]*, v. 1, n. Özel Sayı-2, p. 1838–1843, 2014.

SARIKA, A. R.; LIPTON, A. P.; AISHWARYA, M. S.; DHIVYA, R. S. Isolation of a bacteriocin-producing lactococcus lactis and application of its bacteriocin to manage

spoilage bacteria in high-value marine fish under different storage temperatures. **Applied Biochemistry and Biotechnology**, *[S. l.]*, v. 167, n. 5, p. 1280–1289, 2012. DOI: 10.1007/s12010-012-9701-0.

SCHIRRU, Stefano; TODOROV, Svetoslav Dimitrov; FAVARO, Lorenzo; MANGIA, Nicoletta Pasqualina; BASAGLIA, Marina; CASELLA, Sergio; COMUNIAN, Roberta; FRANCO, Bernadette Dora Gombossy de Melo; DEIANA, Pietrino. Sardinian goat's milk as source of bacteriocinogenic potential protective cultures. **Food Control**, *[S. l.]*, v. 25, n. 1, p. 309–320, 2012. DOI: 10.1016/j.foodcont.2011.10.060. Disponível em: http://dx.doi.org/10.1016/j.foodcont.2011.10.060.

SINGHAL, Neelja; SINGH, Nambaram Somendro; MOHANTY, Shilpa; SINGH, Pooja; VIRDI, Jugsharan Singh. Evaluation of Probiotic Characteristics of Lactic Acid Bacteria Isolated from Two Commercial Preparations Available in Indian Market. **Indian Journal of Microbiology**, *[S. l.]*, v. 59, n. 1, p. 112–115, 2019. DOI: 10.1007/s12088-018-0762-9. Disponível em: https://doi.org/10.1007/s12088-018-0762-9.

SKOWRON, Krzysztof; KWIECIŃSKA-PIRÓG, Joanna; GRUDLEWSKA, Katarzyna; ŚWIECA, Agnieszka; PALUSZAK, Zbigniew; BAUZA-KASZEWSKA, Justyna; WAŁECKA-ZACHARSKA, Ewa; GOSPODAREK-KOMKOWSKA, Eugenia. The occurrence, transmission, virulence and antibiotic resistance of Listeria monocytogenes in fish processing plant. **International Journal of Food Microbiology**, *[S. l.]*, v. 282, n. February, p. 71–83, 2018. DOI: 10.1016/j.ijfoodmicro.2018.06.011. Disponível em: https://linkinghub.elsevier.com/retrieve/pii/S0168160518303167.

SONG, Huiyi; YU, Weiting; GAO, Meng; LIU, Xiudong; MA, Xiaojun. Microencapsulated probiotics using emulsification technique coupled with internal or external gelation process. **Carbohydrate Polymers**, *[S. l.]*, v. 96, n. 1, p. 181–189, 2013. DOI: 10.1016/j.carbpol.2013.03.068. Disponível em: http://dx.doi.org/10.1016/j.carbpol.2013.03.068.

TAN, Qianglai et al. Safety Assessment and Probiotic Evaluation of Enterococcus Faecium YF5 Isolated from Sourdough. **Journal of Food Science**, *[S. l.]*, v. 78, n. 4, p. M587–M593, 2013. DOI: 10.1111/1750-3841.12079. Disponível em: https://onlinelibrary.wiley.com/doi/10.1111/1750-3841.12079.

TODOROV, S. D.; DICKS, L. M. T. Screening for bacteriocin-producing lactic acid bacteria from boza, a traditional cereal beverage from Bulgaria. **Process Biochemistry**, *[S. l.]*, v. 41, n. 1, p. 11–19, 2006. DOI: 10.1016/j.procbio.2005.01.026. Disponível em: https://linkinghub.elsevier.com/retrieve/pii/S1359511305002424. Acesso em: 25 nov. 2020.

VAN DOAN, Hien; HOSEINIFAR, Seyed Hossein; RINGØ, Einar; ÁNGELES ESTEBAN, Maria; DADAR, Maryam; DAWOOD, Mahmoud A. O.; FAGGIO, Caterina. Host-Associated Probiotics: A Key Factor in Sustainable Aquaculture. **Reviews in Fisheries Science and Aquaculture**, *[S. l.]*, v. 28, n. 1, p. 16–42, 2020. DOI:

10.1080/23308249.2019.1643288. Disponível em: https://doi.org/10.1080/23308249.2019.1643288.

VASIEE, Alireza; MORTAZAVI, Seyed Ali; SANKIAN, Mojtaba; YAZDI, Farideh Tabatabaee; MAHMOUDI, Mahmoud; SHAHIDI, Fakhri. Antagonistic activity of recombinant Lactococcus lactis NZ1330 on the adhesion properties of Escherichia coli causing urinary tract infection. **Microbial Pathogenesis**, *[S. l.]*, v. 133, n. April, p. 103547, 2019. DOI: 10.1016/j.micpath.2019.103547. Disponível em: https://doi.org/10.1016/j.micpath.2019.103547.

VERHEUL, Annette; RUSSELL, Nicholas J.; VAN'T HOF, R.; ROMBOUTS, Frans M.; ABEE, Tjakko. Modifications of membrane phospholipid composition in nisin-resistant Listeria monocytogenes Scott A. **Applied and Environmental Microbiology**, *[S. l.]*, v. 63, n. 9, p. 3451–3457, 1997. DOI: 10.1128/aem.63.9.3451-3457.1997. Disponível em: https://journals.asm.org/doi/10.1128/aem.63.9.3451-3457.1997.

VILANDER; DEAN. Adjuvant Strategies for Lactic Acid Bacterial Mucosal Vaccines. **Vaccines**, *[S. l.]*, v. 7, n. 4, p. 150, 2019. DOI: 10.3390/vaccines7040150. Disponível em: https://www.mdpi.com/2076-393X/7/4/150.

WANG, Anran et al. Use of probiotics in aquaculture of China—a review of the past decade. **Fish & Shellfish Immunology**, *[S. l.]*, v. 86, n. December 2018, p. 734–755, 2019. DOI: 10.1016/j.fsi.2018.12.026. Disponível em: https://doi.org/10.1016/j.fsi.2018.12.026.

XIE, Fengxing; ZHANG, Fengfeng; ZHOU, Ke; ZHAO, Yujie; ZHAO, Qiong; SUN, Haibo. [Isolation, identification and fermentation optimization of lactic acid bacteria for aquaculture water purification]. **Wei sheng wu xue bao = Acta microbiologica Sinica**, China, v. 57, n. 2, p. 304–14, 2017. Disponível em: http://www.ncbi.nlm.nih.gov/pubmed/29750493.

XU, Pao; MING, Junchao. Status and Trends of the Tilapia Farming Industry Development. *In*: **Aquaculture in China**. Chichester, UK: John Wiley & Sons Ltd, 2018. p. 404–420. DOI: 10.1002/9781119120759.ch4_4. Disponível em: https://onlinelibrary.wiley.com/doi/10.1002/9781119120759.ch4_4.

YANG, Shih-Chun; LIN, Chih-Hung; SUNG, Calvin T.; FANG, Jia-You. Antibacterial activities of bacteriocins: application in foods and pharmaceuticals. **Frontiers in Microbiology**, *[S. l.]*, v. 5, n. MAY, p. 1–10, 2014. DOI: 10.3389/fmicb.2014.00241. Disponível em: http://journal.frontiersin.org/article/10.3389/fmicb.2014.00241/abstract.

YERLIKAYA, Oktay. Probiotic potential and biochemical and technological properties of Lactococcus lactis ssp. lactis strains isolated from raw milk and kefir grains. **Journal of Dairy Science**, *[S. l.]*, v. 102, n. 1, p. 124–134, 2019. DOI: 10.3168/jds.2018-14983. Disponível em: http://dx.doi.org/10.3168/jds.2018-14983.

YOUSEFI, Bahman; ESLAMI, Majid; GHASEMIAN, Abdolmajid; KOKHAEI, Parviz; SALEK FARROKHI, Amir; DARABI, Narges. Probiotics importance and their

immunomodulatory properties. **Journal of Cellular Physiology**, *[S. l.]*, v. 234, n. 6, p. 8008–8018, 2019. DOI: 10.1002/jcp.27559. Disponível em: https://onlinelibrary.wiley.com/doi/10.1002/jcp.27559.

ZOHRI, Maryam; ALAVIDJEH, Mohammad Shafiee; HARIRIAN, Ismaeil; ARDESTANI, Mehdi Shafiee; EBRAHIMI, Seyed Esmaeil Sadat; SANI, Hadi Tarighati; SADJADI, Seyed Kazem. A Comparative Study Between the Antibacterial Effect of Nisin and Nisin-Loaded Chitosan/Alginate Nanoparticles on the Growth of Staphylococcus aureus in Raw and Pasteurized Milk Samples. **Probiotics and Antimicrobial Proteins**, *[S. l.]*, v. 2, n. 4, p. 258–266, 2010. DOI: 10.1007/s12602-010-9047-2. Disponível em: http://link.springer.com/10.1007/s12602-010-9047-2

GENERAL CONCLUSIONS

In this study was observed that the BLIS produced by *E. faecium* 135 have a good antimicrobial activity against *L. monocytogenes*, an important food pathogen. It was also observed that *E. faecium* 135 was capable to grow and produce BLIS when an alternative growth media elaborated with the detoxified hydrolysate of SCG and 25% (w/w) diluted CS hydrolysate, bout supplemented with nitrogen sources was used. A characterization study was carried out and revealed that *E. faecium* 135 was resistant to low pH conditions (from 2.5 to 3.0) and different concentrations of bile salts (3%), the strain was able to adhere to Caco-2 cells and in the tests of expression of virulence/resistance factors, such as hemolysin and resistance to antibiotics, had negative results, an important demonstration of its safety and probiotic potential. In conclusion, *E. faecium* 135 is a promising LAB as it can grow in an alternative growth media based on coffee residues, produce BLIS with antimicrobial activity against *L. monocytogenes* and had good results and the study of probiotic characterization. However, a further investigation is necessary to confirm the presence of bacteriocins on the BLIS produced by *E. faecium* 135.

ATTACHMENTS

ATTACHAMENT 1

Brazilian Journal of Microbiology (2022) 53:131–141
https://doi.org/10.1007/s42770-021-00661-6

BIOTECHNOLOGY AND INDUSTRIAL MICROBIOLOGY - RESEARCH PAPER

Bacteriocin-like inhibitory substances production by *Enterococcus faecium* 135 in co-culture with *Ligilactobacillus salivarius* and *Limosilactobacillus reuteri*

Anna Carolina Meireles Piazentin[1] · Carlos Miguel Nóbrega Mendonça[1] · Marisol Vallejo[2] · Solange I. Mussatto[3] · Ricardo Pinheiro de Souza Oliveira[1]

Received: 19 April 2021 / Accepted: 16 November 2021 / Published online: 6 January 2022

Abstract
The use of lactic acid bacteria (LAB) and probiotic cultures in the breeding of animals such as poultry and swine are quite common. It is known that those strains can produce bacteriocins when grown in pure culture. However, the production of bacteriocin using co-culture of microorganisms has not been much studied so far. The present study contributes with innovation in this area by embracing the production of bacteriocin-like inhibitory substances (BLIS) by a newly isolated strain of *Enterococcus faecium* 135. Additionally, the co-cultivation of this strain with *Ligilactobacillus salivarius* and *Limosilactobacillus reuteri* was also investigated. The antimicrobial activity of the produced BLIS was evaluated against *Listeria monocytogenes*, *Listeria innocua*, *Salmonella enterica*, and *Salmonella enterica* serovar Typhimurium using two methods: turbidimetric and agar diffusion. In addition, the presence of enterocin genes was also evaluated. The BLIS produced showed a bacteriostatic effect against the bio-indicator strains, and the highest antimicrobial activities expressed by arbitrary units per mL (AU/mL) were obtained against *L. monocytogenes* in monoculture (12,800 AU/mL), followed by the co-culture of *E. faecium* with *Limosilactobacillus reuteri* (400 AU/mL). After concentration with ammonium sulfate, the antimicrobial activity raised to 25,600 AU/mL. Assays to determine the proteinaceous nature of the BLIS showed susceptibility to trypsin and antimicrobial activity until 90 °C. Finally, analysis of the presence of structural genes of enterocins revealed that four enterocin genes were present in *E. faecium* 135. These results suggest that BLIS produced by *E. faecium* 135 has potential to be a bacteriocin and, after purification, could potentially be used as an antimicrobial agent in animal breeding.

Keywords *Enterococcus faecium* · Antimicrobial Activity · Co-culture · Foodborne pathogens

ATTACHAMENT 2

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scientific reports

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OPEN

Bacteriocinogenic probiotic bacteria isolated from an aquatic environment inhibit the growth of food and fish pathogens

Wellison Amorim Pereira[1,8], Anna Carolina M. Piazentin[1,8], Rodrigo Cardoso de Oliveira[1,8], Carlos Miguel N. Mendonça[1,8], Yara Aiko Tabata[2], Maria Anita Mendes[3], Ricardo Ambrósio Fock[4], Edson Naoto Makiyama[4], Benedito Corrêa[5], Marisol Vallejo[6], Elias Figueroa Villalobos[7] & Ricardo Pinheiro de S. Oliveira[1]✉

The conditions of aquatic environments have a great influence on the microbiota of several animals, many of which are a potential source of microorganisms of biotechnological interest. In this study, bacterial strains isolated from aquatic environments were bioprospected to determine their probiotic profile and antimicrobial effect against fish and food pathogens. Two isolates, identified via 16S rRNA sequencing as *Lactococcus lactis* (L1 and L2) and one as *Enterococcus faecium* 135 (EF), produced a bacteriocin-like antimicrobial substance (BLIS), active against *Listeria monocytogenes*, *Salmonella* Choleraesuis and *Salmonella* Typhimurium. Antimicrobial activity of BLIS was reduced when exposed to high temperatures and proteolytic enzymes (trypsin, pepsin, papain and pancreatin). All strains were sensitive to 7 types of antibiotics (vancomycin, clindamycin, streptomycin, gentamicin, chloramphenicol, rifampicin and ampicillin), exhibited a high rate of adherence to Caco-2 cells and expressed no hemolysin and gelatinase virulence factors. EF showed some resistance at pH 2.5 and 3.0, and L2/EF showed higher resistance to the action of bile salts. Finally, the presence of bacteriocin

ATTACHAMENT 3

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Other titles: Lactic acid bacteria (Albuquerque)
Description: Boca Raton : CRC Press, Taylor & Francis Group, [2020] | Includes bibliographical references and index. | Summary: "The book deals with advances made in the functionalities of lactic acid bacteria (LAB) such as, their effect on vitamin D receptor expression, impact on neurodegeneratives pathologies, production of B-vitamins for food bio-enrichment, production of bacteriocins to improve gut microbiota dysbiosis, production of metabolites from polyphenols and their effects on human health, effect on reducing the immunoreaction of food allergens, as biological system using time-temperature to improve food safety, and the use of probiotic in animal feed. The book also reviews the use of LAB and probiotics technologies to develop new functional foods and functional pharmaceutical"-- Provided by publisher.
Identifiers: LCCN 2019056212 | ISBN 9781138391635 (hardback)
Subjects: MESH: Lactobacillales--physiology | Probiotics--therapeutic use | Nutritive Value | Lactobacillales--metabolism | Functional Food--microbiology
Classification: LCC QR82.L3 | NLM QU 145.5 | DDC 579.3/7--dc23
LC record available at https://lccn.loc.gov/2019056212

15

Impact of Probiotics on Animal Health

Sabrina da Silva Sabo,[1] *Elías Figueroa Villalobos,*[2] *Anna Carolina Meireles Piazentin,*[1] *André Moreni Lopes*[3] and *Ricardo Pinheiro de Souza Oliveira*[1,*]

Introduction

The concept of probiotics is very well known and their effects on human's health have been extensively reported along the last years. Meanwhile, the application of probiotics in feed nutrition are far less explored and documented. Probiotics started to be described in 1974, when Parker stated that probiotics are "organisms and substances which contribute to intestinal microbial balance" thus including both living organisms and non-living substances. Later, Fuller (1989) defined probiotics as "a live microbial

ATTACHAMENT 4

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Buffalo milk increases viability and resistance of probiotic bacteria in dairy beverages under in vitro simulated gastrointestinal conditions

Thamires Maria Simões da Silva,[1] Anna Carolina Meirelles Piazentin,[1] Carlos Miguel Nóbrega Mendonça,[1] Attilio Converti,[2] Cristina Stewart Bittencourt Bogsan,[1] Diego Mora,[3] and Ricardo Pinheiro de Souza Oliveira[1]*

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ATTACHAMENT 5

International Journal of Biological Macromolecules 186 (2021) 788–799

Contents lists available at ScienceDirect

International Journal of Biological Macromolecules

journal homepage: www.elsevier.com/locate/ijbiomac

Characterization of levan produced by a *Paenibacillus* sp. isolated from Brazilian crude oil

Carlos M.N. Mendonça [a,b], Rodrigo C. Oliveira [a], Rominne K.B. Freire [a], Anna C.M. Piazentin [a], Wellison A. Pereira [a], Eduardo J. Gudiña [c], Dmitry V. Evtuguin [b], Attilio Converti [d], João H.P. M. Santos [a], Cláudia Nunes [b], Lígia R. Rodrigues [c,1], Ricardo P.S. Oliveira [a,*,1]

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[b] *CICECO, Aveiro Institute of Materials, Department of Chemistry, University of Aveiro, 3810-193 Aveiro, Portugal*
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[d] *Department of Civil, Chemical and Environmental Engineering, Pole of Chemical Engineering, University of Genoa, Via Opera Pia 15, 16145 Genoa, Italy*

ATTACHAMENT 6

Veterinary Microbiology 269 (2022) 109431

Contents lists available at ScienceDirect

Veterinary Microbiology

journal homepage: www.elsevier.com/locate/vetmic

Beneficial effects of probiotics on the pig production cycle: An overview of clinical impacts and performance

Wellison A. Pereira [a,1], Sara M. Franco [a,1], Iara L. Reis [a], Carlos M.N. Mendonça [a], Anna C.M. Piazentin [a], Pamela O.S. Azevedo [a], Marcos L.P. Tse [b], Elaine C.P. De Martinis [c], Martin Gierus [d], Ricardo P.S. Oliveira [a,*]

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[b] Department of Animal Production and Preventive Veterinary Medicine, Faculty of Veterinary Medicine and Animal Science, São Paulo State University, Distrito de Rubião Junior, S/N, Rubião Júnior, 18618970 Botucatu, SP, Brazil

[c] Department of Clinical Analysis, Toxicology and Food Science, Faculty of Pharmaceutical Sciences at Ribeirão Preto, University of São Paulo, Ribeirão Preto, São Paulo, Brazil

[d] Institute of Animal Nutrition, Livestock Products, and Nutrition Physiology, Department of Agrobiotechnology, IFA-Tulln, University of Natural Resources and Life Sciences, Vienna, Austria

ATTACHAMENT 7

Supplementary material from chapter 3

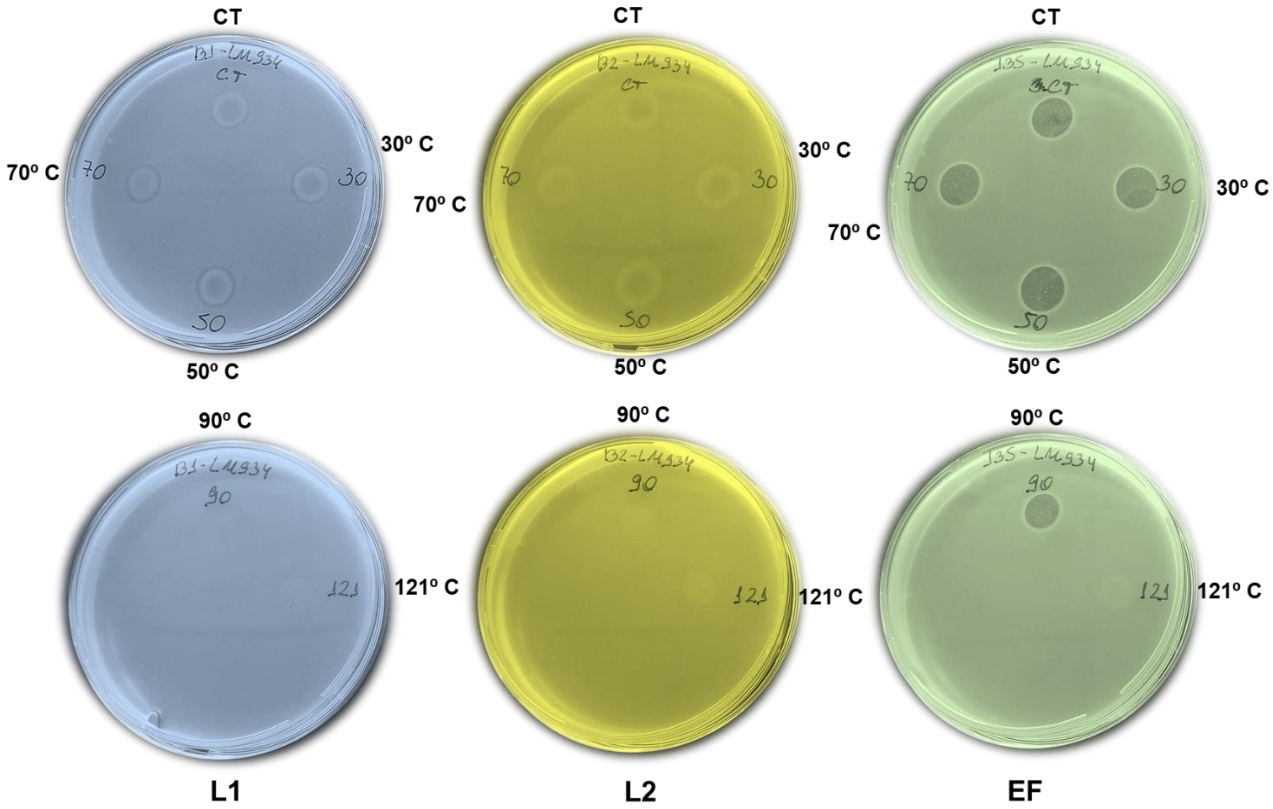


Figure S1. Exposure of BLIS produced by isolates to different temperatures. CT= control (BLIS without the temperature treatment applied in the other samples).

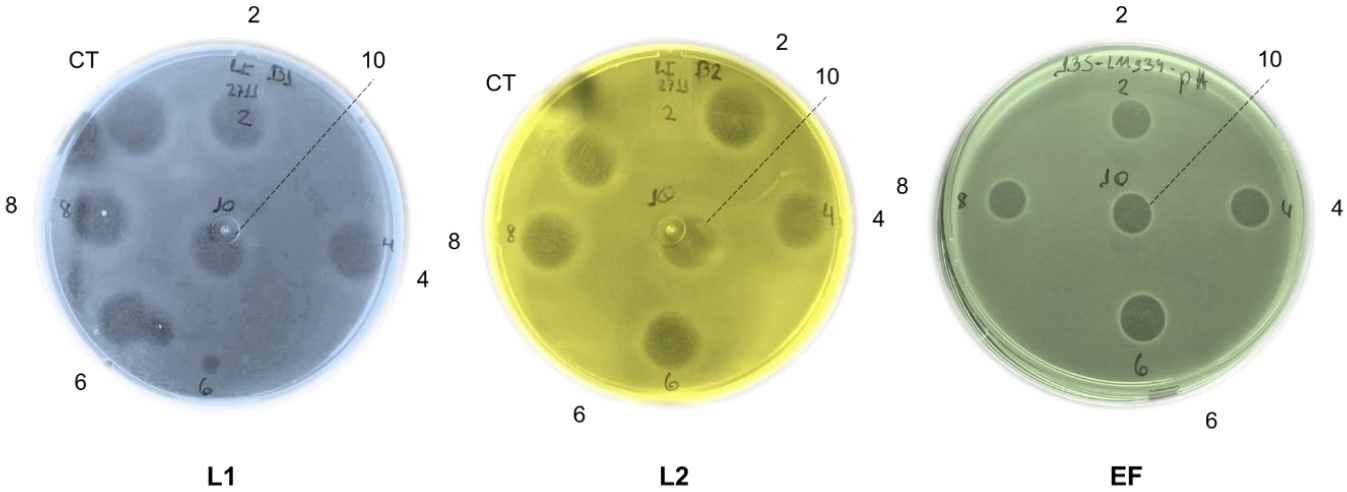


Figure S2. Exposure of BLIS produced by isolates at different pHs. CT= control (BLIS without the pH treatment).

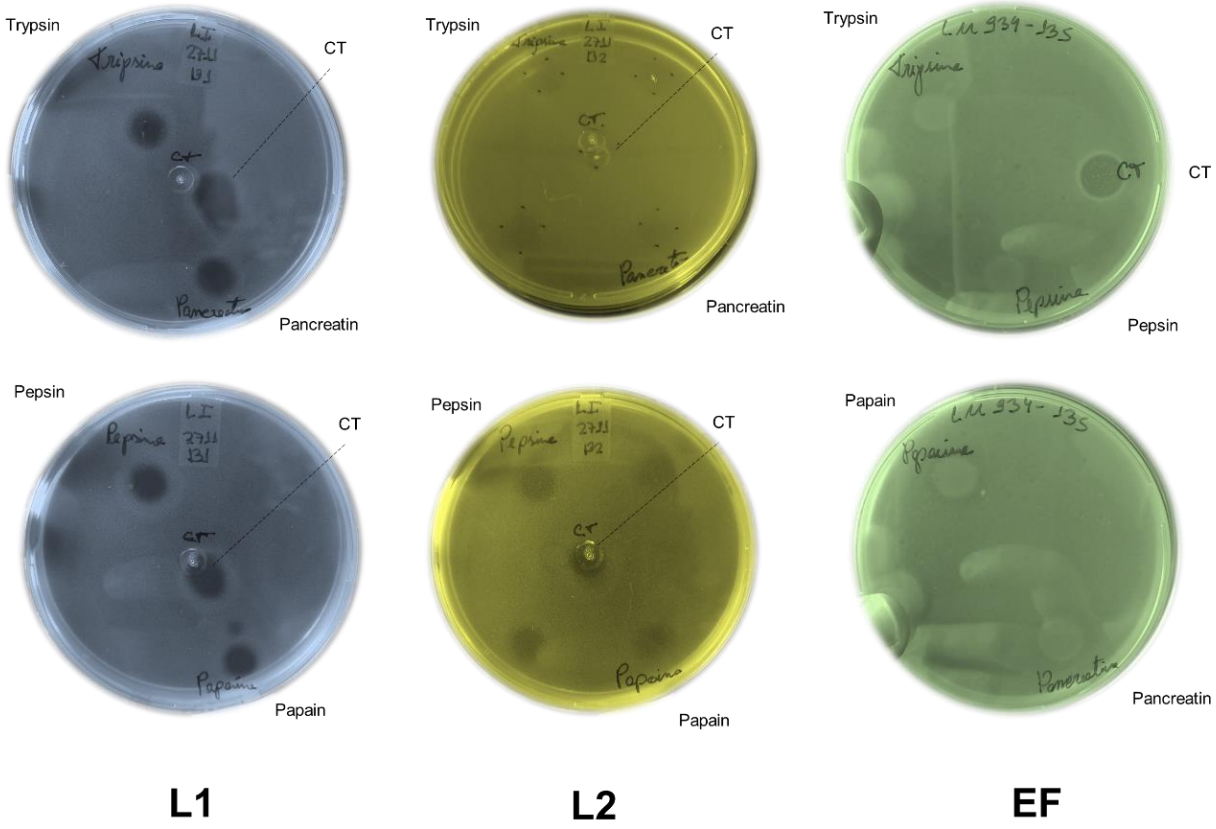


Figure S3. Exposure of BLIS produced by isolates to different proteolytic enzymes. CT= control (BLIS without the enzymatic treatment).

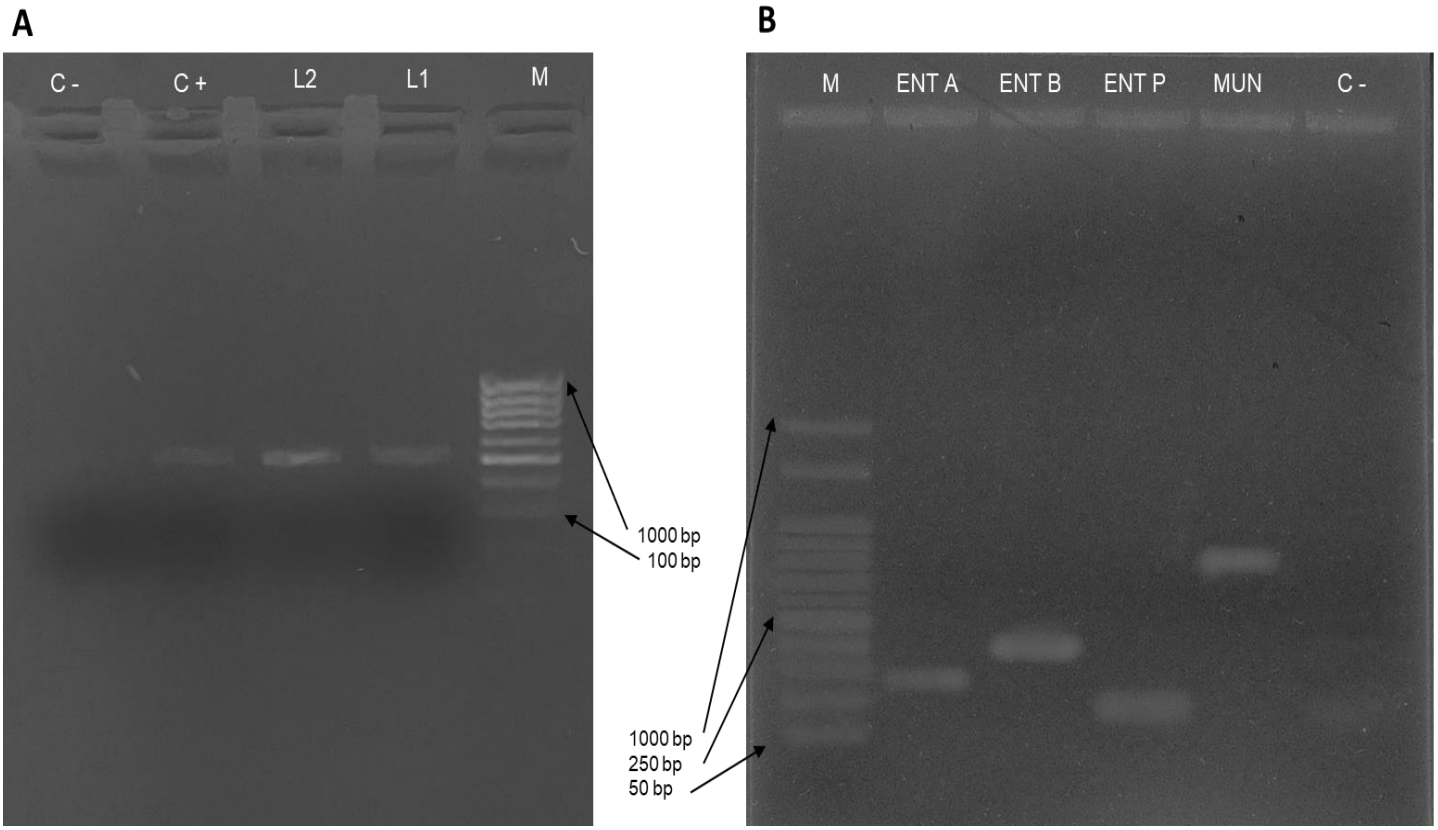


Figure S4. Screening for presence of bacteriocin genes in *L. lactis* L1 – L2 (A) and *E, faecium* strains (B). Lane M: 1 kb DNA markers; Lane C + and C -: products of positive and negative control PCR reactions, respectively. Lane L2 and L1: PCR amplification products of the nisin gene of *L. lactis* L1 and L2; Lane ENT A, ENT B, ENT P, and MUN: PCR amplification products of bacteriocin genes of *E. faecium*.

Table S1. Effect of acids (pH 2, 2.5 and 3) on the viability (log CFU/mL) of *L. lactis* (L1 and L2) and *E. faecium* (EF).

Strain	pH	Time			
		0h	1h	2h	3h
L1	Control	9.27 ± 0.01 ^{Aa}	9.26 ± 0.22 ^{Aa}	9.15 ± 0.03 ^{Aa}	9.34 ± 0.00 ^{Aa}
	pH 2	7.45 ± 0.15 ^{D}	-	-	-
	pH 2.5	8.78 ± 0.18 ^{C}	-	-	-
	pH 3	9.47 ± 0.04 ^{A}	-	-	-
L2	Control	9.15 ± 0.11 ^{Aa}	9.09 ± 0.09 ^{Aa}	9.12 ± 0.16 ^{Aa}	8.99 ± 0.09 ^{Ba}
	pH 2	7.95 ± 0.05 ^{E}	-	-	-
	pH 2.5	9.14 ± 0.06 ^{A}	-	-	-
	pH 3	9.31 ± 0.14 ^{Aa}	9.14 ± 0.06 ^{Aa}	-	-
EF	Control	8.45 ± 0.08 ^{Ba}	8.25 ± 0.05 ^{Bb}	8.46 ± 0.10 ^{Ba}	8.59 ± 0.04 ^{Ca}
	pH 2	8.59 ± 0.19 ^{BC}	-	-	-
	pH 2.5	8.69 ± 0.09 ^{BCa}	6.78 ± 0.35 ^{Cb}	5.03 ± 0.10 ^{Dc}	3.69 ± 0.20 ^{Ed}
	pH 3	8.41 ± 0.08 ^{Ba}	8.31 ± 0.14 ^{Ba}	8.09 ± 0.05 ^{Cb}	8.06 ± 0.03 ^{Db}

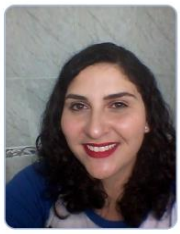
The results are expressed as means ± standard deviations, n = 3; (-) indicates that the counts were < 100 CFU/mL. Different uppercase letters in the same column mean statistically different values according to the Tukey's test ($P < 0.05$). Different lowercase letters in the same row mean statistically different values according to the Tukey's test ($P < 0.05$).

Table S2. Effect of bile salts (0.1, 0.2, and 0.3%) on the viability (log CFU/mL) of *L. lactis* (L1 and L2) and *E. faecium* (EF).

Strain	Bile (%)	Time			
		0h	2h	4h	6h
L1	Control	9.48 ± 0.08 ^{ABa}	9.37 ± 0.16 ^{Aa}	8.00 ± 0.00 ^{Cb}	7.54 ± 0.06 ^{Dc}
	0.1	9.27 ± 0.27 ^{ABC}	-	-	-
	0.2	6.78 ± 0.18 ^{E}	-	-	-
	0.3	6.00 ± 0.00 ^{F}	-	-	-
L2	Control	9.15 ± 0.11 ^{ABCa}	9.09 ± 0.09 ^{ABa}	9.10 ± 0.02 ^{ABa}	8.19 ± 0.04 ^{Cb}
	0.1	8.95 ± 0.05 ^{Ca}	8.59 ± 0.59 ^{Ba}	9.09 ± 0.09 ^{ABa}	9.05 ± 0.10 ^{ABa}
	0.2	7.14 ± 0.06 ^{DEb}	9.02 ± 0.06 ^{ABa}	8.99 ± 0.09 ^{ABa}	9.08 ± 0.04 ^{ABa}
	0.3	7.31 ± 0.14 ^{Dc}	9.14 ± 0.06 ^{Aa}	8.93 ± 0.03 ^{Bb}	9.19 ± 0.01 ^{Aa}
EF	Control	9.65 ± 0.29 ^{Aa}	9.08 ± 0.04 ^{ABb}	9.02 ± 0.10 ^{ABb}	8.98 ± 0.05 ^{ABb}
	0.1	9.08 ± 0.07 ^{BCa}	9.10 ± 0.07 ^{ABa}	9.13 ± 0.05 ^{Aa}	8.90 ± 0.04 ^{Bb}
	0.2	9.00 ± 0.08 ^{Ca}	8.95 ± 0.07 ^{ABa}	9.00 ± 0.08 ^{ABa}	8.83 ± 0.20 ^{Ba}
	0.3	9.02 ± 0.05 ^{Ca}	8.92 ± 0.18 ^{ABa}	8.90 ± 0.10 ^{Ba}	9.00 ± 0.09 ^{ABa}

The results are expressed as means ± standard deviations n = 3; (-) indicates that the counts were < 100 CFU/mL. Different uppercase letters in the same column mean statistically different values according to the Tukey's test ($p < 0.05$). Different lowercase letters in the same row mean statistically different values according to the Tukey's test ($p < 0.05$).

ATTACHAMENT 8



Anna Carolina Meireles Piazentin

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Última atualização do currículo em 02/05/2022

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Identificação

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Formação acadêmica/titulação

2017	Doutorado em andamento em Programa de Pós-Graduação em Tecnologia Bioquímico-Farmacêutica. Universidade de São Paulo, USP, Brasil. com **período sanduíche** em Technical University of Denmark (Orientador: Solange Inês Mussatto). Título: Cultivo de bactérias ácido-láticas em meio contendo resíduos de café e obtenção de compostos antimicrobianos de interesse alimentar e farmacêutico, Orientador: Ricardo Pinheiro de Souza Oliveira. Bolsista do(a): Conselho Nacional de Desenvolvimento Científico e Tecnológico, CNPq, Brasil. Palavras-chave: BLIs; Probióticos; Resíduos agroindustriais do café; Campylobacter jejuni; Listeria monocytogenes; Salmonella enterica. Grande área: Ciências da Saúde
2014 - 2016	Mestrado em Tecnologia Bioquímico-Farmacêutica. Universidade de São Paulo, USP, Brasil. Título: Efeito de culturas probióticas em produto a base de soja: resistência ao armazenamento refrigerado, ao estresse in vitro gastrointestinal e atividade antimicrobiana.,Ano de Obtenção: 2016. Orientador: Ricardo Pinheiro de Souza Oliveira. Bolsista do(a): Coordenação de Aperfeiçoamento de Pessoal de Nível Superior, CAPES, Brasil.
2009 - 2012	Graduação em Farmácia. Universidade Cruzeiro do Sul, UNICSUL, Brasil. Título: O uso de Synadenium grantii Hook f. no tratamento de Diabetes Mellitus. Orientador: Roberto Adati Tsuyoshi.
2008 - 2009	Curso técnico/profissionalizante em Técnico em Química. ETEC Getúlio Vargas, ETEC GV, Brasil.

Formação Complementar

Atuação Profissional

Universidade de São Paulo, USP, Brasil.

Vínculo institucional

2018 - 2018 Vínculo: Estágiário, Enquadramento Funcional: Monitor, Carga horária: 6

Atividades

07/2018 - 12/2018 Estágios , Faculdade de Ciências Farmacêuticas.
Estágio realizado
Programa de Aperfeiçoamento de Ensino (PAE), realizado na Faculdade de Ciências Farmacêuticas/ USP, para o curso de Farmácia dentro da disciplina de Biotecnologia-Farmacêutica.

Hospital das Clínicas da Faculdade de Medicina da USP, HCFMUSP, Brasil.

Vínculo institucional

2012 - 2014 Vínculo: Servidor Público, Enquadramento Funcional: Técnico de Laboratório, Carga horária: 20

Outras informações Atuação na divisão de farmácia, unidade de farmacotécnica, setor de acabamento, relativo à unitarização. Utilização de maquinário industrial, para a emblistamento de medicamentos que são produzidos pelo hospital, como os que são comprados pelo mesmo. Controle microbiológico dos principais produtos produzidos na unidade.

Bem Emergências Médicas, BEM, Brasil.

Vínculo institucional

2012 - 2012 Vínculo: Funcionário, Enquadramento Funcional: Auxiliar de almoxarifado, Carga horária: 40

Outras informações Organização de almoxarifado médico-hospitalar, montagem de kits para as viaturas, assim como a verificação de aparelhos médicos, tais, como oxímetros, desfibriladores, respiradores e incubadores.

Associação Paulista para o Desenvolvimento da Medicina - Hospital São Paulo, SPDM/HSP, Brasil.

Vínculo institucional

2010 - 2011 Vínculo: Funcionário, Enquadramento Funcional: Auxiliar de farmácia, Carga horária: 30

Outras informações Dispensação de medicamentos e materiais médico-hospitalares, organização de almoxarifado médico e atendimento ao público.

Revisor de periódico

2015 - 2015 Periódico: International Journal of Food Sciences and Nutrition

Áreas de atuação

1. Grande área: Ciências da Saúde / Área: Farmácia.
2. Grande área: Ciências Biológicas / Área: Biotecnologia.
3. Grande área: Ciências Biológicas / Área: Microbiologia / Subárea: Fermentações láticas.

Idiomas

Inglês Compreende Bem, Fala Razoavelmente, Lê Bem, Escreve Razoavelmente.

Prêmios e títulos

2021 Menção Honrosa pelo trabalho "Use of spent coffee grounds as alternative media for growth and production of antimicrobial compounds by Enterococcus faecium 135", XXIV

Semana Farmacêutica de Ciência e, Faculdade de Ciências Farmacêuticas da Universidade de São Paulo.

Produções

Produção bibliográfica

Artigos completos publicados em periódicos

Ordenar por

Ordem Cronológica

1. **PIAZENTIN, ANNA CAROLINA MEIRELES**; MENDONÇA, CARLOS MIGUEL NÓBREGA ; VALLEJO, MARISOL ; MUSSATTO, SOLANGE I. ; DE SOUZA OLIVEIRA, RICARDO PINHEIRO . Bacteriocin-like inhibitory substances production by Enterococcus faecium 135 in co-culture with Ligilactobacillus salivarius and Limosilactobacillus reuteri. BRAZILIAN JOURNAL OF MICROBIOLOGY (ONLINE) JCR, v. 1, p. 1, 2022.
2. PEREIRA, WELLISON AMORIM ; **PIAZENTIN, ANNA CAROLINA M.** ; DE OLIVEIRA, RODRIGO CARDOSO ; MENDONÇA, CARLOS MIGUEL N. ; TABATA, YARA AIKO ; MENDES, MARIA ANITA ; FOCK, RICARDO AMBRÓSIO ; MAKIYAMA, EDSON NAOTO ; CORRÊA, BENEDITO ; VALLEJO, MARISOL ; VILLALOBOS, ELIAS FIGUEROA ; DE S. OLIVEIRA, RICARDO PINHEIRO . Bacteriocinogenic probiotic bacteria isolated from an aquatic environment inhibit the growth of food and fish pathogens. Scientific Reports JCR, v. 12, p. 5530, 2022.
3. PEREIRA, WELLISON A. ; FRANCO, SARA M. ; REIS, IARA L. ; MENDONÇA, CARLOS M.N. ; **PIAZENTIN, ANNA C.M.** ; AZEVEDO, PAMELA O.S. ; TSE, MARCOS L.P. ; DE MARTINIS, ELAINE C.P. ; GIERUS, MARTIN ; OLIVEIRA, RICARDO P.S. . Beneficial effects of probiotics on the pig production cycle: An overview of clinical impacts and performance. VETERINARY MICROBIOLOGY JCR, v. 269, p. 109431, 2022.
4. MENDONCA, C. M. N. ; OLIVEIRA, R. C. ; FRREIRE, R. K. ; **PIAZENTIN, A. C. M.** ; PEREIRA, W. A. ; GUDINA, E. J. ; EVTUGUIN, D. V. ; CONVERTI, ATTILIO ; SANTOS, J. H. ; NUNES, C. ; RODRIGUES, L. R. ; OLIVEIRA, R. P. S. . Characterization of levan produced by a Paenibacillus sp. isolated from Brazilian crude oil. INTERNATIONAL JOURNAL OF BIOLOGICAL MACROMOLECULES JCR, v. 186, p. 788-799, 2021.

   Citações: WEB OF SCIENCE ™ 1
5. SIMÕES DA SILVA, THAMIRES MARIA ; **PIAZENTIN, ANNA CAROLINA MEIRELLES** ; MENDONÇA, CARLOS MIGUEL NÓBREGA ; CONVERTI, ATTILIO ; BOGSAN, CRISTINA STEWART BITTENCOURT ; MORA, DIEGO ; DE SOUZA OLIVEIRA, RICARDO PINHEIRO . Buffalo milk increases viability and resistance of probiotic bacteria in dairy beverages under in vitro simulated gastrointestinal conditions. JOURNAL OF DAIRY SCIENCE JCR, v. 103, p. 7890-7897, 2020.

   Citações: WEB OF SCIENCE ™ 7
6. **PIAZENTIN, ANNA CAROLINA MEIRELES**; DA SILVA, THAMIRES MARIA SIMÕES ; FLORENCE-FRANCO, ANA CAROLINA ; BEDANI, RAQUEL ; CONVERTI, ATTILIO ; DE SOUZA OLIVEIRA, RICARDO PINHEIRO . Soymilk fermentation: effect of cooling protocol on cell viability during storage and in vitro gastrointestinal stress. BRAZILIAN JOURNAL OF MICROBIOLOGY (ONLINE) JCR, v. 51, p. 1645-1654, 2020.

Capítulos de livros publicados

1. SABO, S. S. ; VILLALOBOS, E. F. ; **PIAZENTIN, A. C. M.** ; LOPES, A. M. ; OLIVEIRA, R. P. S. . Impact of Probiotics on Animal Health. In: Marcela Albuquerque Cavalcanti de Albuquerque; Alejandra de Moreno de LeBlanc; Jean Guy LeBlanc; Raquel Bedani. (Org.). Lactic Acid Bacteria: A Functional Approach. 1ed.Boca Raton: CRC Press, 2020, v. , p. 261-290.

Resumos publicados em anais de congressos

1. **PIAZENTIN, A. C. M.**; Mussatto, S. I. ; OLIVEIRA, R. P. S. . Use of spent coffee grounds as alternative media for growth and production of antimicrobial compounds by Enterococcus faecium 135. In: 31º Congresso Brasileiro de Microbiologia, 2021. Anais do 31º Congreso Brasileiro de Microbiologia 2021, 2021.
2. **PIAZENTIN, A. C. M.**; SILVA, T. M. S. ; ALBUQUERQUE, M. A. A. ; FREITAS, T. ; OLIVEIRA, M. N. . Efeito da adição de inulina sobre o crescimento de streptococcus thermophilus e Bifidobacterium animalis subsp. lactis BB-12 em leites fermentados simbióticos. In: II Congresso Brasileiro de Pre, Pro e Simbióticos, 2015, São Paulo. Nutrire. São Paulo: Editora Cubo, 2015. v. 40. p. 208-208.
3. **PIAZENTIN, A. C. M.**; ALBUQUERQUE, M. A. A. ; FREITAS, T. ; SILVA, T. M. S. ; PESANHA, M. ; AUAD, I. ; OLIVEIRA, M. N. . Effect of inulin suplementation in the growth of Streptococcus thermophillus and Bifidobacterium animalis subsp. lactis BB-12 in fermented milks. In: XX Pharmaceutical Science and Techonology Meeting of the Faculty of Pharmaceutical Sciences, University of São Paulo, 2015, São Paulo. Brazilian Journal of Pharmaceutical Scienes, 2015. v. 51. p. 24-24.
4. **PIAZENTIN, A. C. M.**; SILVA, T. M. S. ; OLIVEIRA, R. P. S. . Influence of fermentation temperature on viability of yoghurt cultures and Lactobacillus paracasei Lpc-37 using soy extract medium. In: 28º Congresso Brasileiro de Microbiologia, 2015, Florianópolis. ANAIS DO 28º CBM 2015, 2015.

5. FARINHA, L. R. L. ; **PIAZENTIN, A. C. M.** ; AZEVEDO, P. O. S. ; OLIVEIRA, R. P. S. . Acidification kinectic and growth of Streptococcus thermophilus TA040 and Lactococcus lactis CECT4434 from whey. In: 28º Congresso Brasileiro de Microbiologia, 2015, Florianópolis. ANAIS DO 28º CBM 2015, 2015.
6. PORTO, M. C. W. ; AZEVEDO, P. O. S. ; **PIAZENTIN, A. C. M.** ; OLIVEIRA, R. P. S. . EVALUATION OF Pediococcus pentosaceus ATCC 43200 GROWTH AND BIOPRODUCTION OF ANTIMICROBIAL COMPOUND IN DIFFERENTS GROWTHS SUPPLEMENTED BY POLYDEXTROSE, CARBON SOURCE SYNTHETIC WITH PREBIOTIC EFFECT. In: 28º Congresso Brasileiro de Microbiologia, 2015, Florianópolis. ANAIS DO 28º CBM 2015, 2015.

Apresentações de Trabalho

1. **PIAZENTIN, A. C. M.**; Mussatto, S. I. ; OLIVEIRA, R. P. S. . Use of spent coffee grounds as alternative media for growth and production of antimicrobial compounds by Enterococcus faecium 135. 2021. (Apresentação de Trabalho/Outra).
2. **PIAZENTIN, A. C. M.**; OLIVEIRA, R. P. S. . Produção de biomoléculas antimicrobianas por bactérias ácidos láticas contra Salmonella spp. Listeria monocytogenes. 2019. (Apresentação de Trabalho/Congresso).
3. **PIAZENTIN, A. C. M.**; SILVA, T. M. S. ; OLIVEIRA, R. P. S. . Antimicrobial activity of two biomolecules produced by two lactic acid bacteria and their action against Listeria monocytogenes and Salmonella spp.. 2019. (Apresentação de Trabalho/Congresso).
4. **PIAZENTIN, A. C. M.**; OLIVEIRA, R. P. S. . BLIs PRODUCTION BY PROBIOTIC CULTURES: ACTIVITY AGAINST Listeria innocua. 2018. (Apresentação de Trabalho/Outra).
5. **PIAZENTIN, A. C. M.**; SILVA, T. M. S. ; OLIVEIRA, R. P. S. . RESISTANCE OF Lactobacillus paracasei LPC-37 AND YOGHURT CULTURES TO IN VITRO GASTROINTESTINAL STRESS IN FERMENTED SOY POWDER. 2017. (Apresentação de Trabalho/Outra).
6. **PIAZENTIN, A. C. M.**; OLIVEIRA, R. P. S. . INFLUENCE OF COOLING TEMPERATURE ON VIABILITY AND POST-ACIDIFICATION OF YOGHURT CULTURES AND LACTOBACILLUS PARACASEI LPC-37 IN FERMENTEND SOY POWDER. 2017. (Apresentação de Trabalho/Congresso).
7. AZEVEDO, P. O. S. ; **PIAZENTIN, A. C. M.** ; OLIVEIRA, R. P. S. . DISCONTINUOUS FERMENTATION PROCESS BY PROBIOTIC BACTERIUM TO PRODUCE ANTIMICROBIAL PEPTIDE WITH POTENTIAL APPLICATION AS FOOD PRESERVATIVE. 2017. (Apresentação de Trabalho/Congresso).
8. **PIAZENTIN, A. C. M.**; SILVA, T. M. S. ; OLIVEIRA, R. P. S. . Influence of fermentation temperature on viability of yoghurt cultures and Lactobacillus paracasei Lpc-37 using soy exctract medium. 2015. (Apresentação de Trabalho/Congresso).
9. FARINHA, L. R. L. ; **PIAZENTIN, A. C. M.** ; AZEVEDO, P. O. S. ; OLIVEIRA, R. P. S. . Acidificaton kinetic and growth of Streptococcus thermophilus TA 040 and Lactococcus lactis CECT 4434 from whey. 2015. (Apresentação de Trabalho/Congresso).
10. PORTO, M. C. W. ; AZEVEDO, P. O. S. ; **PIAZENTIN, A. C. M.** ; OLIVEIRA, R. P. S. . Evaluation of Pediococcus pentosaceus ATCC 43200 growth and bioproduction of antimicrobial compound in diferents growths supplemented by polydextrose, carbon source synthetic with prebiotic effect. 2015. (Apresentação de Trabalho/Congresso).
11. **PIAZENTIN, A. C. M.**; ALBUQUERQUE, M. A. A. ; FREITAS, T. ; SILVA, T. M. S. ; PESANHA, M. ; AUAD, I. ; OLIVEIRA, M. N. . Effect of inulin suplementation in the growth of Streptococcus thermephilus and Bifidobacterium animalis subsp. lactis BB-12 in fermented milks. 2015. (Apresentação de Trabalho/Outra).
12. **PIAZENTIN, A. C. M.**; SILVA, T. M. S. ; ALBUQUERQUE, M. A. A. ; FREITAS, T. ; OLIVEIRA, M. N. . Efeito da adição de inulina sobre o crescimento de Streptococcus thermpphilus e Bifidobacterium animalis subsp. lactis BB-12 em leites fermentados simbióticos. 2015. (Apresentação de Trabalho/Congresso).

Demais tipos de produção técnica

1. **PIAZENTIN, A. C. M.**. Biprocessos, Controle de Qualidade e Atividade Prática de Antimicrobianos. 2019. (Curso de curta duração ministrado/Outra).
2. **PIAZENTIN, A. C. M.**. Tecnologia de Iogurtes e elaboração de novos produtos. 2015. (Curso de curta duração ministrado/Outra).
3. SILVA, T. M. S. ; **PIAZENTIN, A. C. M.** . Tecnologia de iogurtes e elaboração de novos produtos. 2015. (Curso de curta duração ministrado/Outra).

Bancas

Participação em bancas de comissões julgadoras

Outras participações

1. **PIAZENTIN, A. C. M.**. Participou como membro da Comissão de Avaliação dos trabalhos apresentados na 19ª Feira Brasileira de Ciência e Engenharia. 2021. Universidade de São Paulo.
2. **PIAZENTIN, A. C. M.**. Participou como membro da Comissão de Avaliação dos trabalhos apresentados na 18ª Feira Brasileira de Ciência e Engenharia. 2020. Universidade de São Paulo.
3. **PIAZENTIN, A. C. M.**. Participou como membro da Comissão de Avaliação dos trabalhos apresentados na 17ª Feira Brasileira de Ciência e Engenharia. 2019. Universidade de São Paulo.

Apresentações de Trabalho

1. **PIAZENTIN, A. C. M.**; OLIVEIRA, R. P. S. . Produção de biomoléculas antimicrobianas por bactérias ácidos láticas contra Salmonella spp. Listeria monocytogenes. 2019. (Apresentação de Trabalho/Congresso).
2. **PIAZENTIN, A. C. M.**; SILVA, T. M. S. ; OLIVEIRA, R. P. S. . Antimicrobial activity of two biomolecules produced by two lactic acid bacteria and their action against Listeria monocytogenes and Salmonella spp.. 2019. (Apresentação de Trabalho/Congresso).

Cursos de curta duração ministrados

1. **PIAZENTIN, A. C. M.**. Tecnologia de Iogurtes e elaboração de novos produtos. 2015. (Curso de curta duração ministrado/Outra).
2. **PIAZENTIN, A. C. M.**. Biprocessos, Controle de Qualidade e Atividade Prática de Antimicrobianos. 2019. (Curso de curta duração ministrado/Outra).

Organização de eventos, congressos, exposições e feiras

1. MONTEIRO, G. ; TAVARES, L. C. ; OROZCO, A. ; SILVA, A. R. S. ; **PIAZENTIN, A. C. M.** ; MENDONCA, C. M. N. ; KLEINGESINDS, E. K. ; FREITAS, F. P. P. ; CHAVES, F. S. ; SILVA, R. R. O. . IV Curso de Inverno. 2019. (Outro).