FEDERAL UNIVERSITY OF JUIZ DE FORA

FACULTY OF PHARMACY

GRADUATE PROGRAM IN SCIENCE AND TECHNOLOGY OF MILK AND DAIRY PRODUCTS

Larissa de Souza Valladares

Selection and spray drying of lactic acid bacteria with potential industrial application

Juiz de Fora

2024

Larissa de Souza Valladares

Selection and spray drying of lactic acid bacteria with potential industrial application

Dissertation submitted to the Graduate Program in Science and Technology of Milk and Dairy Products of the Federal University of Juiz de Fora, in partial fulfillment of the requirements for the Master's degree in Science and Technology of Milk and Dairy Products.

Adviser: Prof. Ítalo Tuler Perrone

Co-adviser: Prof. João Batista Ribeiro

Juiz de Fora

2024

CATALOG RECORD

Ficha catalográfica elaborada através do programa de geração
automática da Biblioteca Universitária da UFJF,
com os dados fornecidos pelo(a) autor(a)

Valladares, Larissa de Souza.
Selection and spray drying of lactic acid bacteria with potential industrial application / Larissa de Souza Valladares. -- 2024.
55 p. : il.

Orientador: Ítalo Tuler Perrone
Coorientador: João Batista Ribeiro
Dissertação (mestrado profissional) - Universidade Federal de Juiz de Fora, Faculdade de Farmácia e Bioquímica. Programa de Pós-Graduação em Ciência e Tecnologia do Leite e Derivados, 2024.

1. Enterococcus durans. 2. Weissella paramesenteroides. 3. NSLAB. 4. Probiotic. 5. Spray drying. I. Perrone, Ítalo Tuler, orient. II. Ribeiro, João Batista, coorient. III. Título.

Larissa de Souza Valladares

Selection and spray drying of lactic acid bacteria with potential industrial application

Dissertação apresentada ao Programa de Pós-graduação em Ciência e Tecnologia do Leite e Derivados da Universidade Federal de Juiz de Fora como requisito parcial à obtenção do título de Mestre em Ciência e Tecnologia do Leite e Derivados. Área de concentração: Ciência e Tecnologia do Leite e Derivados.

Aprovada em 26 de março de 2024.

BANCA EXAMINADORA
Prof. Dr. Ítalo Tuler Perrone - Orientador - UFJF
Prof. Dr. João Batista Ribeiro - Coorientador – EMBRAPA Gado de leite
Profa. Dra. Carolina Carvalho Ramos Viana - EPAMIG/ILCT
Profa. Dra. Isis Rodrigues Toledo Renhe - EPAMIG/ILCT

Juiz de Fora, 19/07/2024.

Documento assinado eletronicamente por Italo Tuler Perroni, Professor(a), em 21/07/2024, às 21:42, conforme horário oficial de Brasília, com fundamento no § 3º do art. 4º do Decreto nº 10.543, de 13 de novembro de 2020.

Documento assinado eletronicamente por Carolina Carvalho Ramos Viana, Usuário Externo, em 24/07/2024, às 09:56, conforme horário oficial de Brasília, com fundamento no § 3º do art. 4º do Decreto nº 10.543, de 13 de novembro de 2020.

Documento assinado eletronicamente por Isis Rodrigues Toledo Renhe, Usuário Externo, em 24/07/2024, às 10:39, conforme horário oficial de Brasília, com fundamento no § 3º do art. 4º do Decreto nº 10.543, de 13 de novembro de 2020.

Documento assinado eletronicamente por João Batista Ribeiro, Usuário Externo, em 24/07/2024, às 11:27, conforme horário oficial de Brasília, com fundamento no § 3º do art. 4º do Decreto nº 10.543, de 13 de novembro de 2020.

A autenticidade deste documento pode ser conferida no Portal do SEI-Ufjf (www2.ufjf.br/SEI) através do ícone Conferência de Documentos, informando o código verificador 1874034 e o código CRC C236DE53.

RESUMO

Cem bactérias ácido láticas do território brasileiro foram submetidas a análises de estresse térmico, osmótico e oxidativo como método de triagem. Posteriormente, 15 cepas selecionadas passaram por análises de identificação em nível de espécie (MALDI-TOF). Destas, 14 passaram por análises de patogenicidade (DNase, gelatinase e hemólise) e análises de potencial tecnológico (proteolítico, lipolítico, amilolítico, fermentação de diferentes carboidratos, coagulação, acidificação, produção de diacetil, exopolissacarídeos e gás, sobrevivência em concentrações de NaCl, ambientes gástrico e pancreático simulados). Destas, 3 passaram por análise de atividade antibacteriana contra 4 patógenos (*Listeria monocytogenes* ATCC 5779, *Escherichia coli* IAL1848, *Staphylococcus aureus* ATCC 25923 e *Enterococcus faecalis* ATCC 29212) e susceptibilidade antimicrobiana com 9 antibióticos (ampicilina, penicilina, estreptomicina, gentamicina, eritromicina, tetraciclina, vancomicina, cloranfenicol e cotrimoxazol). As 3 cepas e um pool delas foram secas por pulverização, tiveram os pós analisados (atividade de água, microscopia eletrônica de varredura e umidade) e foram armazenadas por 30 dias a 35 °C ± 2 °C, 5 °C ± 2 °C e temperatura ambiente. As contagens após 30 dias foram acima de 8 log UFC/mL para todas as bactérias e condições de armazenamento. As bactérias não mostraram patogenicidade, foram susceptíveis a pelo menos 2 antibióticos (conforme recomendado pela ANVISA – Agência Nacional de Vigilância Sanitária) e inibiram os patógenos testados. Elas exibem características que se assemelham a bactérias láticas não iniciadoras (NSLAB) como culturas adjuntas, e os resultados inspiram a realização de testes adicionais para avaliar as características probióticas e as aplicações tecnológicas na indústria de laticínios.

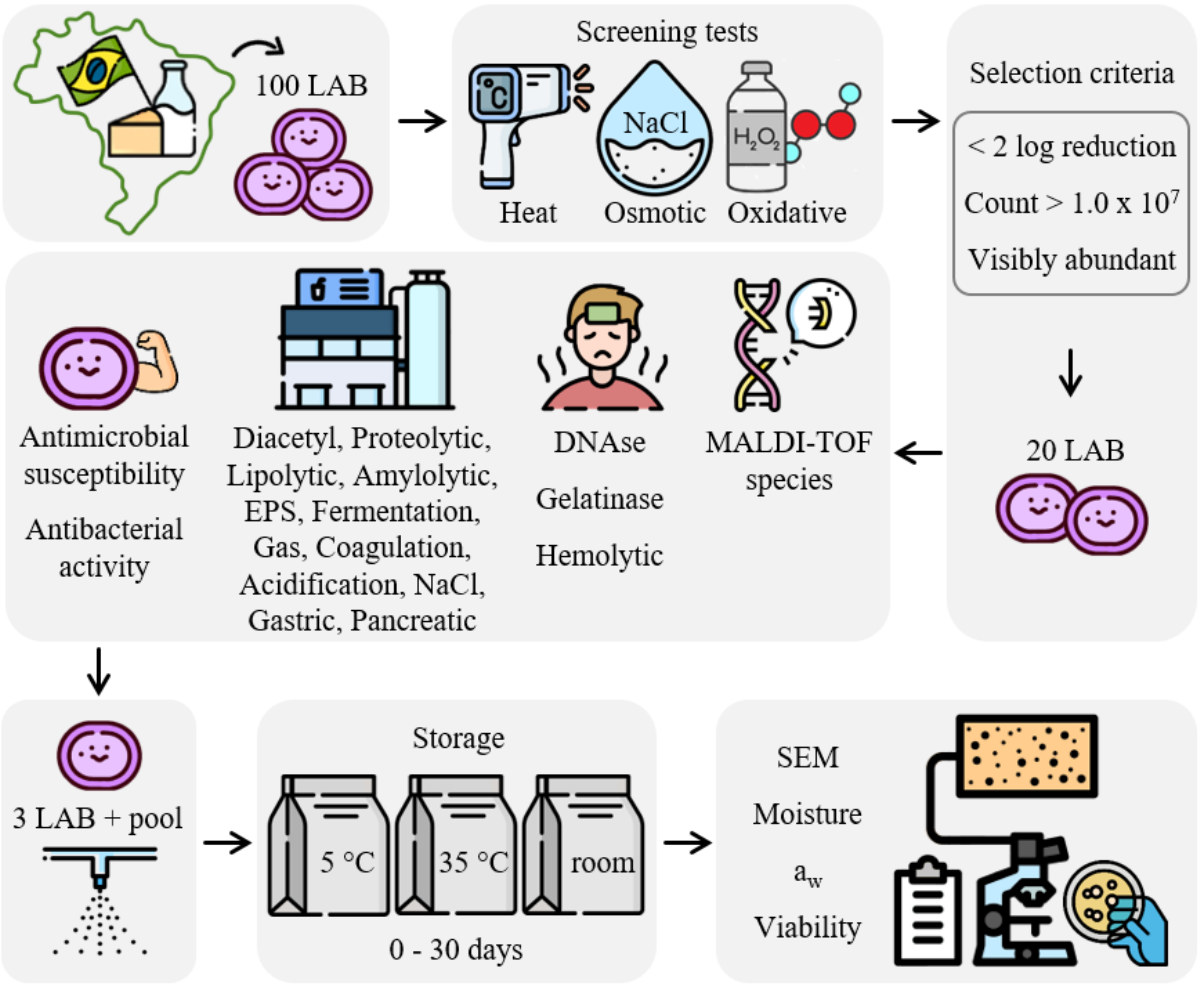
Palavras-chave: *Enterococcus durans*; *Weissella paramesenteroides*; NSLAB; probiótico; spray drying.

ABSTRACT

One hundred lactic acid bacteria from the Brazilian territory underwent thermal, osmotic, and oxidative stress analyses as a screening method. Subsequently, 15 selected strains underwent species-level identification analysis (MALDI-TOF). Of these, 14 underwent pathogenicity analyzes (DNase, gelatinase and hemolytic) and technological potential analyzes (proteolytic, lipolytic, amylolytic, fermentation of different carbohydrates, coagulation, acidification, production of diacetyl, exopolysaccharides and gas, survival in NaCl concentrations, simulated gastric and pancreatic environments). From these, 3 underwent antibacterial activity against 4 pathogens (*Listeria monocytogenes* ATCC 5779, *Escherichia coli* IAL1848, *Staphylococcus aureus* ATCC 25923 and *Enterococcus faecalis* ATCC 29212) and antimicrobial susceptibility with 9 antibiotics (ampicillin, penicillin, streptomycin, gentamicin, erythromycin, tetracycline, vancomycin, chloramphenicol and cotrimoxazole). The 3 strains and a pool of them were spray-dried, had the powders analyzed (water activity, scanning electron microscopy and moisture content) and were stored for 30 days at 35 °C ± 2 °C, 5 °C ± 2 °C and room temperature. The counts after 30 days were above 8 log CFU/mL for all bacteria and storage conditions. The bacteria showed no pathogenicity, were susceptible to at least 2 antibiotics (as recommended by ANVISA – Brazilian National Health Surveillance Agency), and inhibited the tested pathogens. They exhibit characteristics that resemble non-starter lactic acid bacteria (NSLAB) as adjunct cultures, and the results inspire carrying out additional tests to evaluate the probiotic features and technological applications in dairy industries.

Keywords: *Enterococcus durans*; *Weissella paramesenteroides*; NSLAB; probiotic; spray drying.

GRAPHICAL ABSTRACT



Source: created by the author (2024).

SUMMARY

1 INTRODUCTION

Lactic acid bacteria (LAB) have received prominence in the scientific scene due to their applicability in the pharmaceutical and food areas (Hao et al., 2021).

These bacteria are capable of producing biomolecules that can be used in the manufacture of repellents, cosmetics, antimicrobials, dairy products, meat and fermented vegetables, for example (Moreira, Martins, Perrone, Freitas, et al., 2021). In addition to the use of the microorganism itself in human and animal food (Vieco-Saiz et al., 2019).

The use of LAB as bioprotectors in foods has been studied, as they have inhibitory activity on pathogenic and spoilage microorganisms (Cosentino et al., 2018).

To be applied as a probiotic, that is, living organisms capable of exerting beneficial effects on those who consume them in adequate quantities, they must have generally recognized as safe (GRAS) status, resist adverse conditions in the gastrointestinal tract (GIT), mainly stomach acidity and the presence of bile salts in the intestine, have the ability to adhere to the intestinal mucosa and competitively exclude pathogenic microorganisms. (Guidelines for the Evaluation of Probiotics in Food, 2002).

Thus, in 2023 the probiotics market reached more than 70 billion dollars and is expected to expand at a compound annual growth rate of 10.3% until 2028 (RESEARCH AND MARKETS, 2023).

There is a significant dependence in Brazil on technology related to microorganisms and bioproducts coming from other countries, despite Brazil being a country of continental dimensions, which presents great biodiversity and also research institutions that operate in these fields. Studies show the Brazilian potential for the development of adjunct cultures with balanced properties for the production of low-cost, high-quality, safe, and value-added Brazilian functional dairy products (Campagnollo et al., 2018; Margalho et al., 2020; Margalho, Jorge, et al., 2021).

As an example of bacteria that can be used as adjunct cultures, there are the non-starter lactic acid bacteria (NSLAB). These bacteria are known in the literature for their poor acidifying activity but with interesting technological or functional properties, that can be used combined with more acidifying bacteria in cheese manufacturing (Tsigkrimani et al., 2022). They guarantee quality and improve sensory properties, since potent NSLAB strains tend to inhibit the accidental growth of undesirable NSLAB that generate inconsistency in the characteristics of the products (Martley & Crow, 1993; Meng et al., 2018).

With the growing demand for these bacteria, manufacturing through spray drying allows a higher yield compared to manufacturing through freeze drying (Vera-Peña et al., 2019). The specific comparison with the lyophilization technique arises due to the fact that the majority of products requiring post-drying bacterial cell viability are prepared using this method (Paéz et al., 2012).

Many products have the disadvantage of requiring a cold chain in order to extend their shelf life and stabilize them for storage or transport (Schuck et al., 2012). In the case of developing dry powders containing BAL, the costs of maintaining a cold chain could be greatly reduced or even eliminated, while also potentially providing better stability/viability of the cells compared to traditionally marketed products. (Coghetto et al., 2016).

In both cases, bacterial cells undergo stress and experience losses. One alternative found is to use protective materials against high or low temperatures (El-Salam & El-Shibiny, 2015; G. Dos Santos et al., 2018; Z. Zhang et al., 2020).

Focusing on the spray drying process, which is a microencapsulation method, the equipment is fed with a liquid mixture composed of BAL cells combined with the appropriate protector, which is sprayed into fine droplets of micron size and mixed with a hot air flow to achieve rapid dehydration. (N. Wang et al., 2022). Bacterial cells can reach temperatures of approximately 60 °C for a few seconds after the powder particle is dried (Moreira, Martins, Perrone, de Freitas, et al., 2021).

Milk has proven to be the best protein matrix for drying so far, followed by untreated sweet whey obtained from coagulated cheeses, which can be a way to reduce costs as it is a by-product, with associated advantages also related to the potential good quality of the final powder, such as solubility, flowability, dispersibility, for example, when compared to casein-based products (Blajman et al., 2020; Huang, 2020; N. Wang et al., 2022).

Supplementation with mono- and disaccharides of low molecular weight and high glass transition temperature, such as lactose and trehalose, has also been shown to be ideal for drying heat-sensitive bacteria, reducing the loss of cell viability, where the protective effect is related to the stabilization of the membrane and macromolecules by hydrogen bonds previously occupied by water (Rudolph & Crowe, 1985), and also by the ability to cover bacterial cells (Martins, Cnossen, Silva, Vakarelova, et al., 2019).

The use of calcium and magnesium for maintaining cell viability has also been effective, as they promote the activation of heat shock proteins through autophosphorylation, that is, they have intracellular action (N. Wang et al., 2022).

On the other hand, the use of antioxidants was inconclusive for bacterial survival, as well as the use of lipids (Huang, 2020; Martins, Cnossen, Silva, Vakarelova, et al., 2019; N. Wang et al., 2022).

Mucilage and soluble seed proteins were also able to protect bacteria in the drying process, as well as other non-dairy materials such as gum arabic and fruit juices, for example (J. Barbosa & Teixeira, 2017; Kavitake et al., 2018; Reyes et al., 2018).

In addition to the use of protective matrices, there is also the configuration of the drying equipment, such as air inlet temperature, air and product inlet rate, which can be optimized for each microorganism (Agudelo et al., 2017; Çabuk & Harsa, 2015; Eratte et al., 2016; Moayyedi et al., 2018; R. C. S. dos Santos et al., 2014; Shu et al., 2020; Souza et al., 2020; Tang et al., 2020; Tantratian et al., 2018; Umashankar et al., 2019; Vivek et al., 2021; Y. Zhang et al., 2016).

Protection strategies involve not only the development of protective formulations and the optimization of drying processes but also the adjustment of cultivation conditions and pre-adaptation of LAB (Desmond et al., 2002; Paéz et al., 2012; Y. Zhang et al., 2016).

Rehydration is another critical parameter to consider, as described by J. Barbosa & Teixeira (2017), since it consists of another osmotic stress. The solution used, the time and temperature of rehydration affects the recovery of spray dried probiotics. The bacterial behavior during rehydration is strain-dependent, therefore instructions for this step should be described on the product packaging, thus ensuring higher survival rates (J. Barbosa & Teixeira, 2017).

As demonstrated by J. I. B. Barbosa et al. (2015), bacterial resistance to stresses is strain-dependent. Therefore, a resistance test can be conducted to preselect bacteria with higher expression capacity of these genes. Subsequently, they can be subjected to sublethal stimulation, growing under stressful conditions before drying to ensure continuous expression of these genes encoding proteins called chaperones. These chaperones contribute to resistance against various adverse conditions such as temperature variations, changes in osmotic pressure, pH variations, and exposure to bile salts (Sugimoto et al., 2008). These proteins are being sought for use as biomarkers, facilitating their detection in multi-omic analyses for faster screening of bacteria with higher stress tolerance (Aakko et al., 2014; Adu et al., 2018; Palud et al., 2018; Pan et al., 2021).

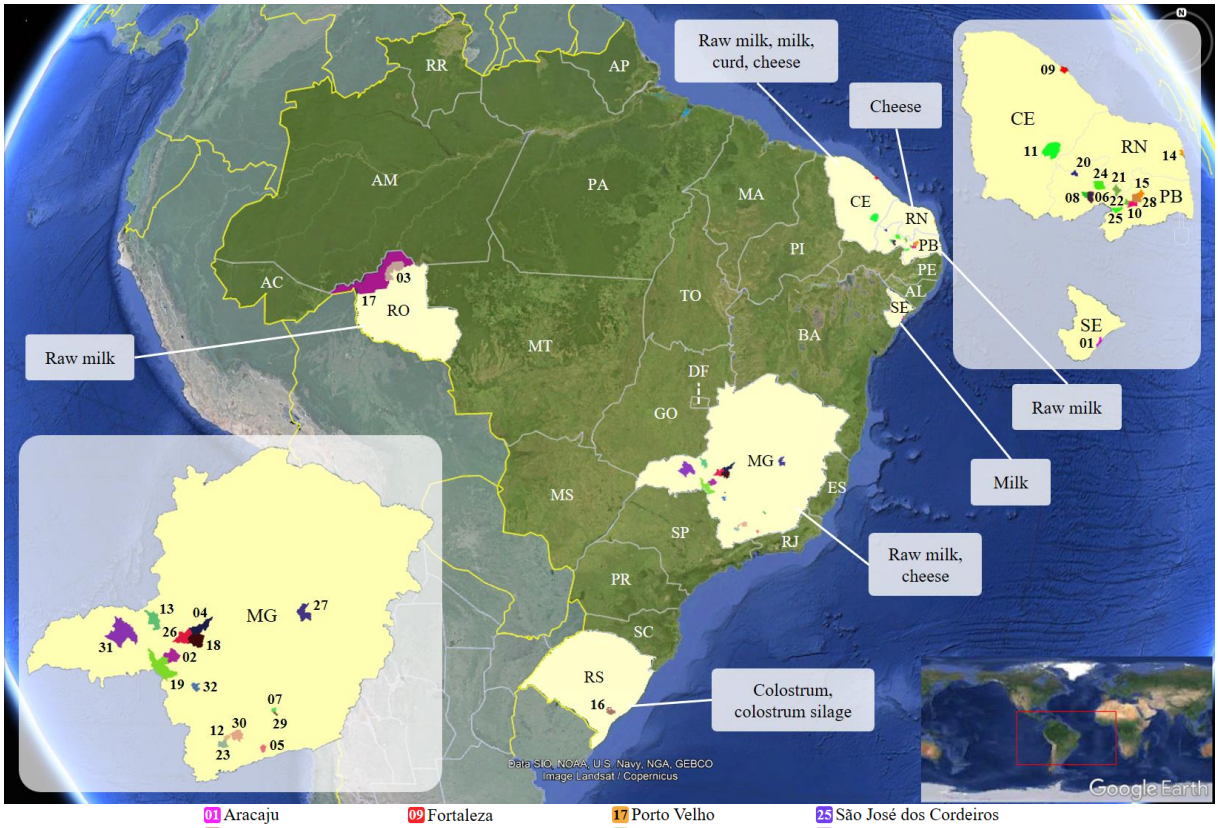
The objective of this study is to select lactic acid bacteria, in the Brazilian territory, that demonstrate potential application for probiotic purposes, starters, NSLAB and/or biotechnological applications, and that can be stored as powder obtained by spray drying.

2 MATERIALS AND METHODS

2.1 LACTIC ACID BACTERIA: ORIGIN AND STORAGE CONDITION

The regions of Brazil from which LAB (n = 100) were collected are highlighted in Figure 1. These microorganisms were isolated, partially characterized at the level of genus and genetic similarity, and are part of the working collection of the Biotech-CPL Phase 1 Project, which are preserved for the long term at Embrapa Gado de Leite, Juiz de Fora - Minas Gerais, Brazil, at -20 °C in a cryoprotectant medium composed of 10% (w/v) skimmed milk and 10% (v/v) glycerol.

Figure 1 – Brazilian map highlighting municipalities, states and types of source samples for LAB



01 Aracaju
02 Araxá
03 Candeias do Jamari
04 Carmo do Paranaíba
05 Carvalhos
06 Catingueira
07 Coronel Xavier Chaves
08 Emas
09 Fortaleza
10 Gurjão
11 Jaguaribe
12 Monsenhor Paulo
13 Monte Carmelo
14 Natal
15 Olivedos
16 Pelotas
17 Porto Velho
18 Rio Paranaíba
19 Sacramento
20 Santa Cruz
21 Santa Luzia
22 Santo André
23 São Gonçalo do Sapucaí
24 São José de Espinharas
25 São José dos Cordeiros
26 Serra do Salitre
27 Serro
28 Soledade
29 Tiradentes
30 Três Corações
31 Uberlândia
32 Vargem Bonita

Source: created by the author (2024).

2.2 OBTAINING THE INOCULUM

What is described as an inoculum in this work refers to the liquid form used for bacterial inoculation.

Interest bacteria maintained in cryoprotective medium was streaked in a Petri dish containing MRS agar in order to obtain isolated colonies. Petri dishes were incubated for 48 h at 35 °C ± 2 °C. An isolated colony was transferred to 1 mL of MRS broth contained in a test tube and incubated at 35 °C ± 2 °C for 24 h. From the previous culture, 100 µL was transferred to 900 µL of MRS broth contained in an Eppendorf microtube and incubated at 35 °C ± 2 °C for 18 h to reach the stationary phase in order to preserve the adapted cells in an active state. After incubation, the bacterial cell mass was collected by centrifugation (6000 x g, 5 min, 25 °C in a Thermo Scientific Megafuge 8R Centrifuge, Germany), washed twice with PBS solution and resuspended in 1 mL of PBS solution.

2.3 OBTAINING BACTERIAL ISOLATES

What is described as an isolate in this work refers to a solid colony to be inoculated or used for making a smear.

Interest bacteria were streaked on MRS agar from the stock at -20 °C to obtain isolated colonies. Incubation was carried out at 35 °C ± 2 °C for 48 h. The use of isolated colonies was then proceeded according to each experiment.

2.4 OBTAINING BACTERIAL ISOLATES USED AS POSITIVE AND NEGATIVE CONTROLS

Interest bacteria from American Type Culture Collection (ATCC) or *Instituto Adolfo Lutz* (IAL) were streaked on BHI agar from the stock at -80 °C to obtain isolated colonies. Incubation was carried out at 37 °C for 24 h. The use of isolated colonies was then proceeded according to each experiment.

2.5 BACTERIAL MAINTENANCE

The Petri dishes with colony growth obtained after each experiment were stored in a refrigerator wrapped in sealing film (Parafilm) and used in the subsequent experiment to select the most resistant strains to temperature variations.

2.6 SCREENING TESTS

2.6.1 Heat stress

A 400 µL aliquot of each inoculum obtained as described in Section 2.2 was transferred to a sterile test tube and kept in a water bath at 60 °C ± 1 °C for 5 min. Then transferred to an

ice bath. The remaining bacterial suspension, not exposed to stress, was used as a control. Methodology described by Paéz et al. (2012) with modifications. The test count was performed in triplicate.

2.6.2 Osmotic stress

An aliquot of 200 µL of each inoculum obtained as described in Section 2.2 was transferred to a sterile Eppendorf microtube and added with 200 µL of sterile 2.0 M NaCl solution, kept in a water bath at 30 °C ± 1 °C for 30 min, and then 10 µL is diluted in 10 mL of PBS solution, contemplating the 10^{-3} dilution. The remaining bacterial suspension, not exposed to stress, was used as a control, where 200 µL of PBS solution was added, and like the test, it was kept in a water bath at 30 °C ± 1 °C for 30 min., and then 10 µL is diluted in 10 mL of PBS solution, contemplating the 10^{-3} dilution. Then transferred to an ice bath. Methodology described by Zotta et al. (2009) with modifications. The test count was performed in triplicate.

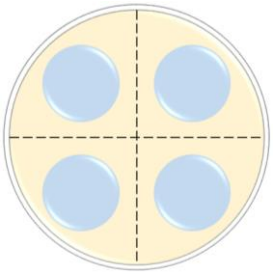
2.6.3 Oxidative stress

An aliquot of 200 µL of each inoculum obtained as described in Section 2.2 was transferred to a sterile Eppendorf microtube and added with 200 µL of 5.0 mM H_2O_2, kept in a water bath at 30 °C ± 1 °C for 3 h, and then 10 µL is diluted in 10 mL of PBS solution, contemplating the 10^{-3} dilution. The remaining bacterial suspension, not exposed to stress, was used as a control, where 200 µL of PBS solution was added, and like the test it was kept in a water bath at 30 °C ± 1 °C for 3 h, and then 10 µL is diluted in 10 mL of PBS solution, contemplating the 10^{-3} dilution. Then transferred to an ice bath. Methodology described by Palud et al. (2018) with modifications. The test count was performed in triplicate.

2.7 ENUMERATION BY MICRODROPLET TECHNIQUE

MRS agar plates that receive the microdroplet are positioned open in a laminar flow hood with the flame on and the UV light on for 15 min so that the medium loses excess moisture and absorbs the inoculum more easily. Afterwards, the plates are covered, but remains ajar long enough time near the flame until the lid is free of condensation. The serial dilution of the bacterial suspensions was carried out in PBS solution until the estimated concentration was reached, in addition to one above and one below this. The dilutions were plated in triplicate and each 20 µL microdroplet was positioned in a quadrant of the Petri dish, as exemplified in Figure 2.

Figure 2 – Scheme of plating the 20 µL microdroplet



Source: created by the author (2022).

A slight circular movement must be made with the Petri dish after each microdroplet has been dripped so that bacteria do not accumulate at the edges.

Incubation was carried out at 35 °C ± 2 °C for 48 h. And the colony count took place preferably in dilutions that included 8 to 80 CFU.

To obtain the result in CFU/mL, equation (1) was used:

$$\mathrm{CFU/mL} = \frac{\bar{x}_{CFU} \times \mathrm{D} \times (-1)}{\mathrm{V_{mL}}} \quad (1)$$

Where:

$\mathrm{CFU/mL}$ = mean concentration of bacteria

$\bar{x}_{CFU}$ = mean triplicate count

D = dilution

$\mathrm{V_{mL}}$ = volume used in mL

To evaluate the reduction in viability resulting from stress, equation (2) was used:

$$\mathrm{RV} = \log \mathrm{N} - \log \mathrm{N_0} \quad (2)$$

Where:

RV = reduction in viability

N = final viable cells

$\mathrm{N_0}$ = initial viable cells

2.8 BACTERIAL SELECTION CRITERIA

The bacteria went through selection filters to meet some requirements, which were: reduction in viability of a maximum of 99% (2 log cycles) after the stress tests (Barbosa et al., 2015; Dijkstra et al., 2014; Ferreira et al., 2017), count greater than 1,0 x 10^7 CFU/mL after

each stress (Barbosa et al., 2015; Lipan et al., 2020) and visibly abundant growth during entire period of handling and storage between experiments (Abbasiliasi et al., 2017).

2.9 IDENTIFICATION BY MATRIX-ASSISTED LASER DESORPTION IONIZATION – TIME OF FLIGHT (MALDI-TOF)

The taxonomic identification of strains (n = 14), selected as described in section 2.8, was also carried out by MALDI-TOF, following the procedure proposed by Nacef et al. (2017). Protein profiles were acquired in triplicate from bacterial cultures, following the extraction protocol using ethanol/formic acid. Two hundred microliters of culture broth were collected and washed twice with sterile Milli-Q water by centrifugation at 16000 x g for 1 min. After removing the supernatant, the cell precipitate was gently resuspended in 300 μL of sterile Milli-Q water. The bacteria were then inactivated with the addition of 900 μL of absolute ethanol. After centrifugation at 16000 x g for 2 min, the supernatant was discarded, and the sediment was dried in room air for 10 min. Extraction of cellular proteins was performed by adding 10 μL of 70% formic acid to the sediment, followed by the addition of 10 μL of pure acetonitrile, after homogenization. After another centrifugation at 16000 x g for 2 min, 1 μL of the supernatant was applied to a well of the MALDI plate (Bruker Daltonics, USA) and dried in room air. Each sample was coated with 1 μL of the matrix for MALDI, α-19 cyano-4-hydroxycinnamic acid, in a solution containing 50% acetonitrile and 2.5% trifluoroacetic acid (v/v), followed by air drying. Mass spectra were acquired using an Autoflex III SmartBeam mass spectrometer (Bruker Daltonics, USA) equipped with a 200 Hz laser, operating in positive linear mode, with a mass range of 2000 to 20000 Daltons. External calibration was performed using Bacterial Test Standard calibrant mix (Bruker Daltonics, USA). Equipment parameters included an IS1 source voltage of 20 kV, IS2 source voltage of 18.55 kV, lens voltage of 8.80 kV, and an ion extraction delay time of 240 ns. Random laser shots were performed with a peak sampling rate of 0.5 GS/s, 513 totaling 2000 spectra, which were summed and processed using the centroid peak detection algorithm of the FlexControl 3.3 program (Bruker Daltonics, USA), resulting in the raw spectrum of each sample, being considered high confidence identification when the score values were between 3.0 and 2.0; medium confidence when scores were between 1.70 and 1.99; and scores lower than 1.69 as non-identifiable. A standard strain of *Escherichia coli* was used as positive control.

2.10 QUALITATIVE ASSESSMENT OF PATHOGENICITY FACTORS: DNASE, GELATINASE AND HEMOLYTIC ACTIVITY

For DNAse test, Kateete et al. (2010) protocol was used with modifications. Bacterial isolates, obtained per Sections 2.3 and 2.4, underwent DNase agar spot smear incubation at 37 ºC for 18 to 24 h. After covering colonies with 2 N HCl, excess was removed, and a 2-min wait preceded result reading, verifying DNase activity through transparent halos. Positive and negative controls were Staphylococcus aureus ATCC 25923 and Streptococcus epidermidis ATCC 12228, respectively.

Gelatinase test was applied according to Pereira et al. (2009) with modifications. Tubes, comprising 1% (w/v) yeast extract, 1.5% (w/v) tryptone, and 12% (w/v) bacteriological gelatin, were prepared. Bacterial isolates were inoculated in 3 mL of the medium and incubated at 30 ºC for 7 days, and kept at 5 ºC ± 2 °C for 30 min before readings. Gelatin hydrolysis positivity was indicated by the medium remaining liquid after refrigeration. Staphylococcus aureus ATCC 25923 and Escherichia coli ATCC 25922 served as positive and negative controls.

Hemolysis was tested according to the protocol with modifications by Ferrari et al. (2016). Blood agar was prepared, bacterial isolates were smeared and then underwent incubation at 37 ºC for 24 to 48 h. Results were classified as partial hemolysis or alpha hemolysis (α) when a greenish halo was observed around the colony; total hemolysis or beta hemolysis (β) when a transparent halo was present around the colony; and the absence of hemolysis or gamma hemolysis (γ) in the absence of a halo. Staphylococcus aureus ATCC 25923, Streptococcus epidermidis ATCC 12228, served as positive and negative controls, respectively.

A guide from ANVISA (2013) was used as source for the ATCC strains. All experiments of pathogenicity factors were made in biological duplicate.

2.11 ASSESSMENT OF TECHNOLOGICAL POTENTIAL

2.11.1 Diacetyl production

Methodology of King (1948) modified was used. Tubes containing 2 mL MRS broth were prepared. Bacterial inoculum was obtained as described in Section 2.2, and was introduced 1% (v/v) from the volume of MRS broth. Incubation took place at 35 °C ± 2 °C for 24 h. Then, 0.6 mL of 5% (w/v) α-naphthol and 0.2 mL of 40% (w/v) KOH solution were added to 1 mL of the cell suspension. The mixture was incubated at 35 °C ± 2 °C for 10 min. The production

of diacetyl was indicated by the formation of a red ring at the top of the tubes. Experiments were made in biological duplicate.

2.11.2 Proteolytic activity

Plate count agar (PCA) supplemented with 1% (v/v) reconstituted skimmed milk 10% (w/v) were prepared. Bacterial inoculum was obtained as described in Section 2.2, and 5 µL aliquots were spot-dropped. Incubation occurred at 35 °C ± 2 °C for 48 h. Proteolytic activity was identified by the formation of a clear zone around the colonies. Methodology described by Franciosi et al. (2009) with modifications. Experiments were made in biological duplicate.

2.11.3 Lipolytic activity

In the first experiment, described by Hantsis-Zacharov & Halpern (2007) with modifications, plates of PCA supplemented with 1% (v/v) tributyrin were prepared, and 5 µL aliquots of the bacterial inoculum, obtained as described in Section 2.2, were spot-dropped. Incubation took place at 35 °C ± 2 °C for 72 h, with lipolytic activity indicated by a clear zone around the colony growth.

For the second experiment, plates of Luria-Bertani agar supplemented with 2 g/L $CaCl_2$ and 10 g/L Tween 80 were prepared, and 5 µL aliquots of the bacterial isolate were spot-dropped by piercing the agar. Incubation occurred at 35 °C ± 2 °C for 48 h, and lipolytic activity was identified by the formation of opaque halos around the colonies. Methodology described by Barbosa et al. (2010).

In the third experiment, plates of Luria-Bertani agar supplemented with 2 g/L $CaCl_2$ and 10 mL/L butter were prepared. The butter was pasteurized at 62 °C to 65 ºC for 30 to 35 min with agitation for subsequent addition to sterile agar in an aseptic environment. Aliquots of 5 µL of the bacterial isolate were spot-dropped on agar plates. Incubation occurred at 35 °C ± 2 °C for 48 h, and lipolytic activity was identified by the formation of opaque halos around the colonies. Experiments were made in biological duplicate.

2.11.4 Amylolytic activity

Plates of modified MRS agar, without glucose, with 0.2% (w/v) soluble starch (MRS-A), were prepared. Bacterial inoculum was obtained as described in Section 2.2, and 5 µL aliquots were spot-dropped. Incubation occurred at 35 °C ± 2 °C for 48 to 72 h. The plate was flooded with Gram's iodine solution. Clear halos indicated the production of α-amylase.

Methodology described by Adesulu-Dahunsi et al. (2018) with modifications. Experiments were made in biological duplicate.

2.11.5 Exopolysaccharides production

Carbohydrate-modified MRS agar plates with 2% (w/v) glucose, sucrose or lactose were prepared. Bacterial inoculum was obtained as described in Section 2.2, and 5 µL aliquots were spot-dropped. Incubation occurred at 35 °C ± 2 °C for 72 to 120 h. The production of EPS was verified by the formation of viscous colonies. Methodology described by Smitinont et al. (1999) with modifications. Experiments were made in biological duplicate.

2.11.6 Ability to ferment carbohydrates

Plates of purple agar base supplemented with 2% (w/v) glucose, sucrose or lactose were prepared. Bacterial inoculum was obtained as described in Section 2.2, and 5 µL aliquots were spot-dropped. Incubation occurred at 35 °C ± 2 °C for 48 h. Fermentation was verified by the change in culture medium's color from purple to yellow. Adapted methodology from Upham et al. (2023). Experiments were made in biological duplicate.

2.11.7 Gas production

Test tubes containing 3 mL of MRS broth with 5% (w/v) glucose and Durham tubes were prepared. Bacterial inoculum was obtained as described in Section 2.2, and was introduced 1% (v/v) from the final volume of MRS broth. Incubation occurred at 35 °C ± 2 °C for 48 h. Gas production was indicated by bubbles at the top of Durham tube. Modified methodology from Ferrari et al. (2016). Experiments were made in biological duplicate.

2.11.8 Milk coagulation

Ten milliliters of sterilized reconstituted skimmed milk 10% (w/v) was prepared. Bacterial inoculum was obtained as described in Section 2.2, and was introduced 1% (v/v) from the final volume of skimmed milk. Incubation occurred at 35 °C ± 2 °C, and the coagulation of the milk was observed on days 1, 3, 5, and 7. Results were described from 0 to 5. Modified methodology from Tsigkrimani et al. (2022). Experiments were made in biological duplicate.

2.11.9 Acidification activity

Modified methodology of Ayad et al. (2004) was applied. Sterilized reconstituted skimmed milk 10% (w/v) was prepared. Bacterial inoculum was obtained as described in Section 2.2, and was introduced 1% (v/v) from the final volume of skimmed milk. Incubation occurred at 35 °C ± 2 °C and the pH was checked at 0, 2, 4, 6, and 24 h with a benchtop pH meter (Hanna HI 8417, Italy). A sample aliquot was taken from each sample to measure the pH without causing cross-contamination. Acidification rate was calculated using the equation (3). Strains were considered as fast, medium or slow acidifying when a DpH of 0.4U was achieved after 3, 3 to 5, or more than 5 h, respectively. Experiments were made in biological duplicate.

$$DpH = pH_{tx} - pH_{t0} \quad (3)$$

Where:

DpH = pH difference

pH_{tx} = pH at defined time

pH_{t0} = pH at time zero

2.11.10 Ability to survive in different concentrations of NaCl

MRS broth supplemented with 0%, 2%, 6%, and 10% (w/v) NaCl was prepared. Bacterial inoculum was obtained as described in Section 2.2, and was introduced 1% (v/v) from the final volume of MRS broth with different NaCl concentrations. Incubation occurred at 35 °C ± 2 °C for 24 h. Serial dilutions were then performed in PBS solution to achieve an enumerable concentration, considering the increasing stress caused by sodium chloride. Enumeration was carried out as described in Section 2.7, in triplicate. Modified from Dal Bello et al. (2012).

2.11.11 Tolerance to simulated gastric (SGJ) and pancreatic juice (SPJ)

The SGJ was prepared with the following composition: NaCl 9.0 g/L, pepsin 3.0 g/L, distilled water for dilution, and HCl adjusting the pH to 1.5, 2.0, and 3.0. Sterilization was carried out using a 0.45 µm filter. The SPJ was prepared with the following composition: $NaHCO_3$ 150 mM, pancreatin 1.9 g/L, and distilled water for dilution. The pH was maintained between 7.8 and 8.3. Sterilization was performed using a 0.45 µm filter. Bacterial inoculum was obtained as described in Section 2.2, and was inoculated at a ratio of 1:100 in SGJ (G), SPJ (P), and a blank (B90). Incubation took place at 37 °C for 90 min. A sample was taken for serial dilutions in PBS solution to achieve an enumerable concentration. Enumeration of G, P, and

B90 was carried out as described in Section 2.7, in duplicate. Methodologies with modification was applied, Liao et al. (2017) for gastric and Ferrari et al. (2016) for pancreatic test.

2.12 SELECTION OF PROMISING BACTERIAL PROFILES

To reduce the number of samples in subsequent stages, a selection filter was applied to identify bacteria that stood out in the previous analyses. The reduced number of samples or repetitions is indicated when there are difficulties related to cost, quantity, and/or rarity of samples, for example (Girma & Machado, 2013).

2.13 ANTIMICROBIAL SUSCEPTIBILITY

Antimicrobial susceptibility tests were performed using the disc diffusion method proposed by Bauer et al. (1966) with modifications made by Santos et al. (2020). The strains were reactivated in MRS agar at 37 ºC for 24 h with three exhaustions to obtain isolated colonies. After growth and confirmation of genetic purity, 3 isolated colonies were collected for inoculation in MRS broth and incubated for 24 h at 37 °C. After growth, 100 μL of the culture was inoculated into Petri dishes containing 25 mL of MRS agar and spread with the aid of a Drigalski loop throughout the dish. Then, 9 discs containing antimicrobials (Oxoid®, Basingstoke, England) were placed in the Petri dish. The antimicrobials used were: penicillin G (10 μg), ampicillin (10 μg), vancomycin (30 μg), gentamicin (10 μg), streptomycin (10 μg), tetracycline (30 μg), chloramphenicol (30 μg), erythromycin (15 μg), in addition to co-trimoxazole (1.25 μg of trimethoprim and 23.75 μg of sulfamethoxazole). The plates were incubated in anaerobiosis for 24 h, at 35 °C ± 2 °C. To evaluate the results, the inhibition zones (mm) were measured with a millimeter ruler and the strains were classified as sensitive or resistant for each antimicrobial, as proposed by Charteris et al. (2001).

2.14 ANTIBACTERIAL ACTIVITY

The evaluation of the antagonistic activity of the selected strains at the end of the experiment was carried out using the spot-on-the-lawn method, as proposed by Tagg et al. (1976), with modifications. Three indicator pathogens were used: *Listeria monocytogenes* ATCC 5779, *Escherichia coli* IAL1848, *Staphylococcus aureus* ATCC 25923 and *Enterococcus faecalis* ATCC 29212.

Initially, lactic acid and indicator bacteria were inoculated onto MRS and BHI agar, respectively, and incubated at 35 °C ± 2 °C for 24 h. After growth, 3 colonies isolated from the

test bacteria were inoculated into 1000 μL of MRS broth and incubated at 35 °C ± 2 °C for 24 h. Then, 5 μL of the broth containing the test bacteria in different spots (5 per plate) were added to a Petri dish containing MRS agar and incubated at 35 °C ± 2 °C for 24 h, in anaerobiosis. The indicator bacteria were inoculated into 10 mL of BHI broth and incubated at 37 ºC for 4 h. After the incubation period, 100 μL of the broth containing the indicator bacteria was added to 7 mL of semi-solid BHI agar and poured onto the MRS plate, forming a layer over the spots. The Petri dish was re-incubated for 14 to 18 h at 35 °C ± 2 °C in anaerobiosis. The inhibition halos were measured (in mm) with the aid of a millimeter ruler, with the presence of a halo considered favorable evidence of the ability of the test bacteria to inhibit the indicator (Lewus & Montville, 1991). Strains that presented a halo/colony ratio < 1 were considered non-antagonistic (-); strains that presented a halo/colony ratio > 1 were considered to have good antagonistic activity (+); and the strains that presented a halo/colony ratio > 2 were considered to have excellent antagonistic activity (++).

2.15 PREPARATION FROM THE INOCULUM TO THE POWDER

Bacterial isolate was inoculated into 10 mL of MRS broth. Incubation occurred at 35 °C ± 2 °C for 24 h. Then it was transferred to 390 mL of MRS broth, constituting 2/3 (Khem et al., 2016; Lipan et al., 2020; Martins, Cnossen, Silva, Cezarino, et al., 2019) of the total drying medium volume. It was incubated in a shaker (New Brunswick Model G-25, USA) at 150 rpm at 35 °C ± 2 °C for 18 h. The drying medium was prepared following the Section 2.16. The bacterial mass that grew in the shaker was centrifuged in 50 mL Falcon tubes at 3200 × g for 5 min at 25 °C (Thermo Scientific Megafuge 8R Centrifuge, Germany). The pellet was washed once with PBS solution. It was then resuspended in the drying medium, and the Section 2.17 was carried out. The cell concentrate (drying medium with cellular mass added) was kept at temperatures up to 40 °C until drying. The Section 2.18 was followed. The powder was divided into three samples and stored: a) under 5 °C ± 2 °C, b) 35 °C ± 2 °C, and c) room temperature (which in Juiz de Fora – MG, Brazil, from August to September of 2023, meant a range from 12 °C to 33 °C), for 30 days in laminated and sealed packages. The Section 2.19 was carried out.

2.16 DRYING MEDIUM

The drying medium was adapted from Y. Zhang et al. (2016) to a final concentration of 25% skim milk powder and 0.5% trehalose. The powders were weighed for 600 g of drying

medium and the mass was completed with water. The powders were dissolved in a beaker with the aid of a glass rod. The mixture was transferred to a 500 mL Schott flask. Low time low temperature (LTLT) pasteurization was performed at 60 °C to 65 °C for 30 min in a water bath. It was kept in the refrigerator for a minimum of 12 h for greater hydration until bacterial inoculation.

2.17 PRE-ADAPTATION

Cell concentrate remained for 15 min at 50 °C and then had the temperature reduced to proceed with drying (Y. Zhang et al., 2016).

2.18 DRYING PARAMETERS

The drying parameters for each cultivation were applied according to Y. Zhang et al. (2016) and are shown on Table 1.

Table 1 – Drying parameters of powders with different cultivated bacteria

Drying parameters	Bacteria			
	Enterococcus durans CV58	*Enterococcus durans* 1023	*Weissella paramesenteroides* 17(43)G	POOL
Feed pump (L/h)	0.88 – 1.08	0.46 – 0.98	1.03 – 1.08	0.93 – 1.03
Flowmeter (L/min)	33	30 – 33	30	37
Air flow rate (m^3/h)	2.7	2.5	2.9 – 2.7	2.8
Inlet air (°C)	169	155 – 170	170	170
Outlet air (°C)	95 – 90	88 – 96	90 – 92	85 – 88
Inlet product (°C)	47 – 41	42	40 – 46	48.9 – 41.8
Outlet product (°C)	55.2 – 67.6	51.7 – 63.0	52.3 – 60.7	52.0 – 63.5

Source: created by the author (2024).

2.19 ANALYSIS ON CELL CONCENTRATE AND POWDER

2.19.1 pH

The pH of cell concentrate was determined in triplicate using a benchtop pH meter (Hanna HI 8417, Italy) right before spray drying.

2.19.2 Moisture content

Moisture content of the microparticles was determined in an oven at 105 °C until constant weight, according to the methodology proposed by AOAC (2005).

2.19.3 Water activity

Water activity (a_w) of the powders was recorded in Nov-labswift device (TECNAL, Brazil), in triplicate, right after drying (Paula et al., 2023).

2.19.4 Scanning Electron Microscopy (SEM)

The herein investigated powders were morphologically featured based on SEM (Hitachi TM 3000, Japan) at magnifications of 500x, 1000x and 1500x (Paula et al., 2023).

2.19.5 Viable cell count in cell concentrate or powder

Powder or cell concentrate, 1 g or 1 mL respectively, was diluted in 9 mL of PBS solution until an optimal dilution for counting as described in Section 2.7. Equation 4 was applied to correct the count of powders with respect to dry matter:

$$CFU/mL = CP \div (DM_P \div DM_{CC}) \quad (4)$$

Where:

$CFU/mL =$ powder count corrected in CFU/mL

$CP =$ count in CFU/g obtained at the end of the microdroplet technique

$DM_P =$ powder dry matter

$DM_{CC} =$ cell concentrate dry matter

2.20 STATISTICAL ANALYSIS

Normality was analyzed using the Shapiro-Wilk ($n \leq 30$) or Kolmogorov-Smirnov ($n > 30$) tests. The paired samples T Test was applied to results with normal distribution and two paired groups (before and after heat stress, osmotic stress, gastric and pancreatic simulation). For results with normal distribution and the number of paired groups greater than or equal to 3, the repeated measures ANOVA test was performed (comparison between four bacterial cultures), with Tukey's post-hoc test to compare means ($p < 0.05$). Results with normal distribution from three or more unpaired groups were analyzed using the one-way ANOVA test (before and after pH and a_w between the four bacterial cultures), with Tukey's post-hoc test to compare means ($p < 0.05$). For results with non-normal distribution and two paired groups, the Wilcoxon test was applied (before and after oxidative stress). The Friedman test was applied to results with non-normal distribution with paired groups greater than or equal to 3 (different concentrations of NaCl, analyzes in cellular concentrate and powder over time and at different

temperatures), with a post-hoc Durbin-Conover test to compare means ($p < 0.05$). These statistical analyzes were performed using the Jamovi program (version 2.3.28, Australia).

3 RESULTS AND DISCUSSION

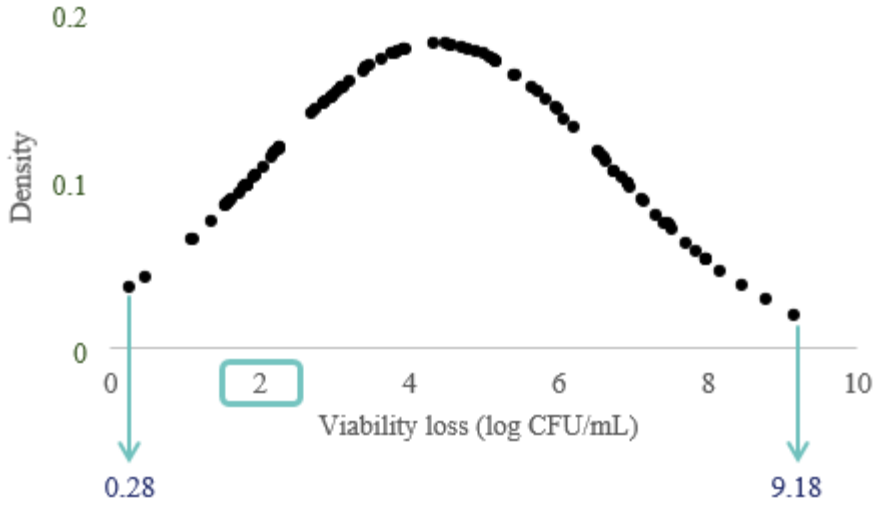
3.1 SCREENING TESTS

3.1.1 Heat stress

Bacteria that exhibit lower robustness, meaning higher viability loss, in heating tests tend to repeat these results during spray drying (Dijkstra et al., 2014).

After the thermal stress test, it was observed that the loss of bacterial viability followed a normal, or Gaussian, distribution (Kolmogorov-Smirnov normality test, p = 0.224), as shown in Figure 3.

Figure 3 – Viability loss after heat stress



Source: created by the author (2024).

In this figure, microorganisms more resistant to the treatment at 60 °C ± 1 °C for 5 min are closer to the left side, while the less resistant ones are on the opposite side. Higher values indicate a greater reduction in bacterial viability, while lower values indicate a smaller reduction.

The paired samples T-test described a significant difference in the count before and after thermal stress at a 5% probability level ($p < 0.001$), wherein the test samples showed a reduction in the count. At the end of the experiment, values ranging from 0.28 to 9.18 were observed, where the more resistant cultures had values similar to those in the literature, and the more susceptible ones showed greater loss than those in the literature (Dijkstra et al., 2014; Gardiner et al., 2000).

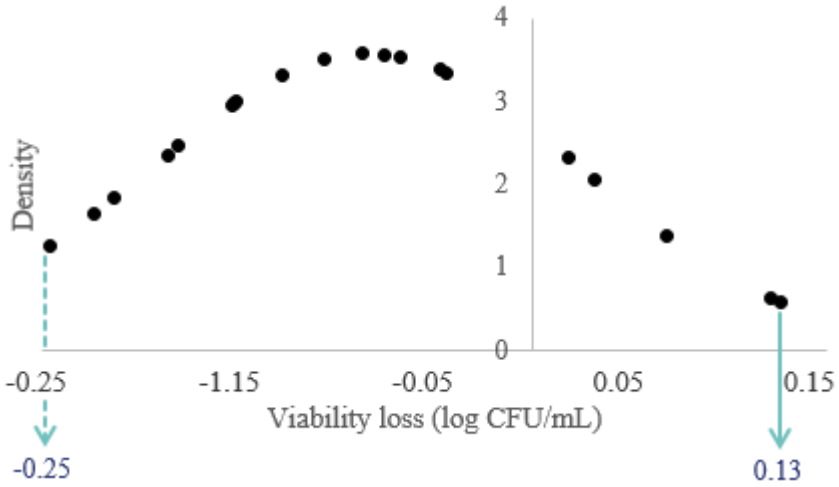
Among the 100 LABs studied in this work, 20 showed a reduction in cell viability of up to 2 logarithmic units, a count greater than 7 log CFU/mL and abundant visible growth after the test. Since they met the selection criteria, they proceeded to the subsequent experiments.

3.1.2 Osmotic stress

Osmotic stress should be tested to predict the bacteria's resistance to dehydration and rehydration, which occur during the water loss in drying and during the rehydration of the powder, respectively.

The results (Figure 4) of the 20 selected and tested LAB showed a normal distribution ($p = 0.321$). Paired samples t-test showed statistical difference at a 5% probability level ($p = 0.003$), where the test sample (viability loss of -0.25 log CFU/mL) exhibited higher growth than the control (viability loss of 0.13 CFU/mL).

Figure 4 – Viability loss after osmotic stress



Source: created by the author (2024).

These results indicate the robustness of the tested bacteria against dehydration and rehydration conditions. Similar results exist in the literature (Sáez et al., 2018; Zotta et al., 2009), but no result where the tested bacteria grow more than the controls, which occurred in our study.

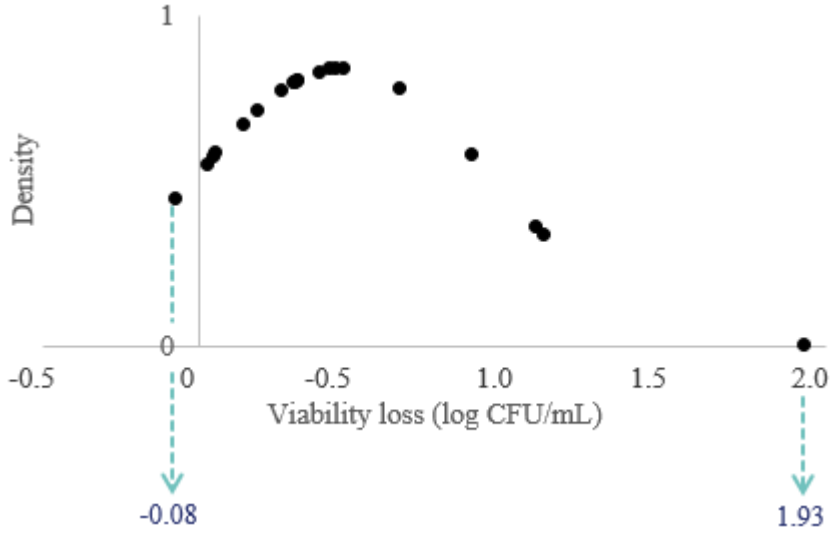
3.1.3 Oxidative stress

Oxidative stress occurs when the environmental oxygen comes into contact with the bacteria in the cell concentrate through the hot air of the spray dryer. It also occurs when the particles are dry, as most of the water surrounding the bacteria, which are catalase-negative, is removed. Catalase is an enzyme that helps protect cells from damage caused by hydrogen peroxide, a toxic byproduct of oxygen metabolism. Therefore, this test indicates the bacteria's resistance and consequent survival when exposed to oxygen.

The same collection of strains presented non-normal distribution of results ($p < 0.001$). Wilcoxon test showed that there was a statistical difference ($p < 0.001$) where the test showed a reduction in counts, unlike González et al. (2020) where there was no significant reduction. Even so, the result indicates that the bacteria tested survive in an aerobic environment, a fact that could be noticed during the culture maintenance stages and after incubation.

The results of reduction in log CFU/mL counts ranged from -0.08 to 1.93, just as other studies showed variability in resistance (Dijkstra et al., 2014; Zotta et al., 2009), also pointing to variability from strain to strain. Bacterial resistance to oxidative stress can increase their survival during and after spray drying (Zotta et al., 2009), a fact that points to the good strategy of cultivating culture in an aerobic environment as a form of selection as well.

Figure 5 – Viability loss after oxidative stress



Source: created by the author (2024).

3.4 BACTERIAL MASS

There was reduced cell mass growth in 5 bacteria. Therefore, they were discontinued in the following experiments, since to prepare an experimental culture, growth should be tested (Abbasiliasi et al., 2017), and this bacterium should preferably be easy to handle and capable of abundant multiplication. Then, 15 strains were selected for subsequent tests.

3.5 MALDI-TOF IDENTIFICATION

Identification at species level of lactic acid bacteria (n = 15) was performed by MALDI-TOF (Table 2). One of the cultures was identified as *Streptococcus pneumoniae* and was discontinued.

Table 2 – Identification by MALDI-TOF at species level, diacetyl production and proteolytic activity

MALDI-TOF identification	Number of strains	Diacetyl production	Proteolytic activity
Enterococcus durans	2	0/2	2/2
Enterococcus faecium	9	8/9	9/9
Lacticaseibacillus rhamnosus	1	1/1	0/1
Lactococcus garvieae	1	0/1	1/1
Weissella paramesenteroides	1	1/1	1/1

Source: created by the author (2024).

Weissella spp. is not a genus generally recognized as safe (GRAS) (Fessard & Remize, 2017), as there have been reported cases of opportunistic infections, but it is a treatable bacterium that exhibits beneficial health activities (Ahmed et al., 2022).

Enterococcus spp. is a genus used in commercial products despite also not having GRAS status (Im et al., 2023).

Therefore, both genera have few scientific studies in the area of spray drying being commercially applied in food for biotechnological, probiotic, starter, or NSLAB purposes (Moumita et al., 2018; Stummer et al., 2012). For such uses, each strain must be thoroughly studied, as each one presents different genotypic and phenotypic characteristics (Gomez-Zavaglia et al., 2015).

The current genus *Lacticaseibacillus* spp. derives from the reclassification of the former genus *Lactobacillus* spp. (Zheng et al., 2020). This genus is well studied, including in the area of spray drying, and most of its strains have GRAS status (Oguntoye & Ezekiel, 2024).

Lactococcus garvieae is not a widely studied species in the field of spray drying. Most the *Lactococcus* spp. members are considered as GRAS (Baig et al., 2022).

Even though the last two genera have GRAS status for most of their components, if intended for use in animals or humans, they must be more carefully studied.

All 14 strains proceeded to the subsequent analyses.

3.6 PATOGENICITY ASSAY

All 14 bacteria showed negative results for DNAse, gelatinase and hemolysis, a suitable result for application in food.

3.7 TECHNOLOGICAL POTENTIAL

3.7.1 Diacetyl production

Diacetyl is an important substance in dairy products for providing a buttery flavor and inhibiting food-borne pathogens (Ferrari et al., 2016; Thierry et al., 2015).

Works in the literature demonstrate the production of such substance through the fermentation of carbohydrates by LAB (Terzić-Vidojević et al., 2020). In our work 71.4% of studied LAB produced diacetyl (Table 2).

Strains that produce diacetyl are preferred for use in the production of starter or adjunct cultures, given that this is a useful technological characteristic.

3.7.2 Proteolytic activity

Proteolytic system of LAB is essential for growth in milk and protein-rich substrate (Hati et al., 2018), in addition it plays an important role in the development of texture and taste of the products (Amiri et al., 2022; Xia et al., 2020). Also, many dairy cultures are proteolytic, so they can generate bioactive peptides that will be present in fermented dairy products (Abbasiliasi et al., 2017). In the study by Terzić-Vidojević et al. (2020), among all bacteria 25.4% showed proteolytic activity, in our work 92.9% showed this activity (Table 2).

3.7.3 Lipolytic activity

All bacteria had negative lipolytic activity. Lipolytic activity is important for the development of flavor and odor of products (Amiri et al., 2022; Xia et al., 2020). However, lactic acid bacteria are considered weakly lipolytic compared with many other groups of bacteria (Medina et al., 2004), a fact that corroborates the findings of this study, since none of the LAB showed such activity, as in other studies (Sharma et al., 2018). In the work of Margalho et al. (2021) 0.5% of studied bacteria (5/1002) had lipolytic activity, and for this reason this is described as an understated characteristic of LAB.

3.7.4 Amylolytic activity

All bacteria had negative amylase activity. Amylolytic activity is important for cereal fermentation and in the additive industry for lactic acid production and the enzyme itself, for example (Adesulu-Dahunsi et al., 2018; Grujović et al., 2020). Some studies (Grujović et al., 2020) indicate strong amylase production by *Enterococcus* spp., but this activity did not occur in our study, it is worth noting that the applied analysis method was different (spectrophotometry). The former genus *Lactobacillus* spp. appeared in studies as an amylase producer (Adesulu-Dahunsi et al., 2018; Grujović et al., 2020), unlike the genus *Weissella* spp. (Sharma et al., 2018).

3.7.5 Exopolysaccharides production

None of the bacteria showed EPS production with 2% (w/v) of carbohydrates. EPS are capable of positively influencing the texture, rheology, and technological properties of the foods produced (Adesulu-Dahunsi et al., 2018), since smooth and creamy products are more appealing to consumers (Meng et al., 2018). The higher the carbon supplementation in the medium, the greater the production of this biopolymer by microorganisms with such ability (Ferrari et al., 2016). In our study, bacteria were cultured in a medium with 2% of each tested carbohydrate, which may have been an insufficient amount for the production of this substance. Studies have shown EPS production by different LAB genera (Ayad et al., 2004; Ferrari et al., 2016), with emphasis on the *Lactococcus* spp. and former *Lactobacillus* spp. genera. The work of Ramos et al. (2023) described 61.8% (76) of the strains studied as EPS producers.

3.7.6 Ability to ferment carbohydrates

Lactic acid bacteria are a kind of microorganisms that use carbohydrates as the only or main carbon source, and from them produces lactic acid, for instance (Yaqi Wang et al., 2021). In this work, all tested bacteria were capable of fermenting all applied carbohydrates.

3.7.7 Gas production

None of the bacteria were able to produce gas. Heterofermentative bacteria produce compounds other than lactic acid, such as carbon dioxide, and can improve the flavor of food by producing some aromatic compounds. There are reports in the literature that gas production is dependent on the composition of the substrate (type and concentration) and the cultivation temperature (Lampien et al., 2023; Ortakci et al., 2015).

3.7.8 Milk coagulation

As in the literature, our study corroborates the results that point to coagulation being more strain-dependent than species-dependent (Tsigkrimani et al., 2022), since similar species exhibit different coagulation times. It is worth highlighting that milk coagulation is promoted by lactic acid production by LAB as well as coagulant enzymes activity.

The analysis of milk coagulation, together with the analyses of proteolytic and acidifying activities, can indicate the characteristics and possible uses of a particular bacterial strain. For example, if a bacterium provides rapid coagulation, low proteolytic activity, and

high acidifying activity, it can be inferred that the high production of acid is responsible for the acidic coagulation of milk, and this would be a suitable culture for yogurt.

Another example: if the results show rapid coagulation, high proteolytic activity and low acidifying activity, this enzymatic activity is responsible for the coagulation of the milk since the acidity is slowly reduced. Thus, this microorganism could be a source of genetic or enzymatic bioproducts for industries with such interests.

In our study, the results (Table 3) showed slow coagulation (taking more than a day for a weak gel to appear), presence of proteolytic activity (Section 3.7.2), and slow acidification (next Section). These microorganisms can be used as adjunct cultures for ripened cheeses, as their slow proteolysis aids in the gradual development of texture and flavor.

Table 3 – Milk coagulation assay over seven days

Group of bacteria	Day 1	Day 3	Day 5	Day 7
Enterococcus faecium (8), *Weissella paramesenteroides* (1)	+	++++	++++	+++++
Lacticaseibacillus rhamnosus (1)	+	+++	++++	+++++
Enterococcus durans (1)	-	++	+++	+++++
Enterococcus durans (1), *Lactococcus garvieae* (1)	-	-	+	+++
Enterococcus faecium (1)	-	-	-	+
Control	-	-	-	-

- still liquid; + start to gel; ++ weak gel; +++ coagulum with fines; ++++ coagulum with whey; +++++ cohesive coagulum.
Source: created by the author (2024).

3.7.9 Acidification activity

When we talk about acidification, this is an important technological and functional property in the selection of LAB as a starter culture (Abbasiliasi et al., 2017), but it is not desirable in the adjunct cultures, since it could negatively affect the quality of the final products (Tsigkrimani et al., 2022). Fast acidifying ability is also interesting since rapidly decreasing pH enables reduction of undesirable microbiota (Terzić-Vidojević et al., 2020). Just like coagulation, literature indicates that acidification seems to be more strain-dependent than species-dependent (Tsigkrimani et al., 2022). Our study is in agreement with previous information, since all species of bacteria, without distinction, were slow acidifiers. Because even with 6 h none of them reached a pH decrease of 0.4 U (Ayad et al., 2004), therefore they couldn't be applied as starter bacteria.

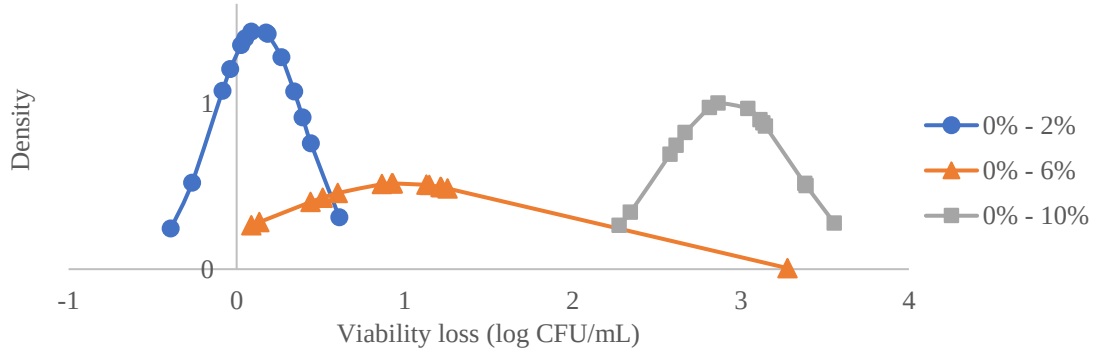
Optimal NSLAB are characterized by slow to moderate acidification activity (Meng et al., 2018). They exhibit unique technological and probiotic features that are significant both as a base for scientific research and for the development of innovative starter cultures for

functional dairy products (Terzić-Vidojević et al., 2020). Bacteria selected in our study have characteristics that resemble NSLAB.

3.7.9 Ability to survive in different concentrations of NaCl

The sodium chloride assay aimed to simulate bacterial resistance in environments containing sodium chloride, such as cheese, for example, in order to predict the behavior of cultures in the food itself. Cheeses can have NaCl concentrations of up to almost 6% (Morandi et al., 2022), and the halotolerance of LAB is important because some cheeses are produced with salt in the curd (Ferrari et al., 2016). Propitious to this fact, studies show LAB survival in sodium chloride concentrations of up to 6% (Margalho, Kamimura, et al., 2021; Ortakci et al., 2015; Prabhurajeshwar & Chandrakanth, 2017). In our study, there was a non-normal distribution when reaching 6% (w/v) NaCl ($p < 0.001$). Friedman test and pairwise comparisons (Durbin-Conover) confirmed that there is a significant difference between all counts, with a reduction in viability as sodium chloride increased, as shown in Figure 4.

Figure 6 – Viability loss after stress with different percentages of NaCl



Source: created by the author (2024).

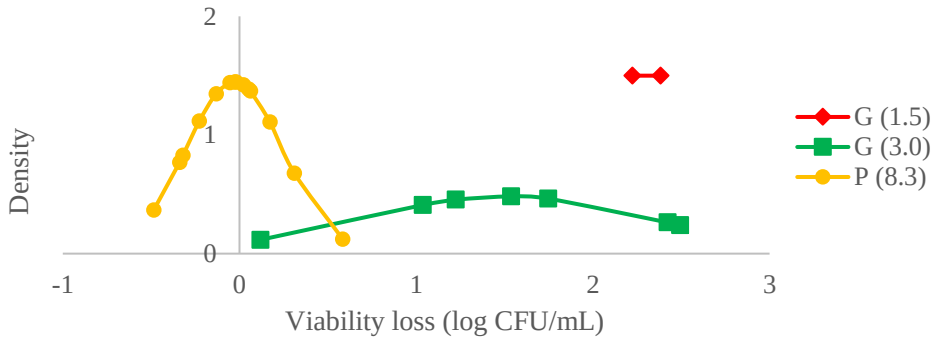
Even with a statistically significant difference after the test, the logarithmic reductions for 2% (w/v) NaCl and 6% (w/v) NaCl were less than 2 units and the counts remained above 7 log CFU/mL. The mean ± standard deviation bacterial count after the test was: 0% NaCl with 9.4 ± 0.2 log CFU/mL, 2% NaCl with 9.2 ± 0.2 log CFU/mL, 6% NaCl with 8.4 ± 0.8 log CFU/mL and 10% NaCl with 6.4 ± 0.3 log CFU/mL.

It is important to redo the tolerance test after spray drying as well, since studies have shown strains becoming sensitive to salt following spray drying (Gardiner et al., 2000).

3.7.10 Tolerance to simulated gastric (SGJ) and pancreatic juice (SPJ)

Tests of tolerance to SGJ and SPJ (Figure 7) aim to predict the survival of bacteria in hostile environments with extreme pH variations and the presence of digestive enzymes when ingested. These tests, along with others, are essential to indicate whether a particular strain can be used as a probiotic, for example.

Figure 7 – Viability loss after simulated gastrointestinal stress with SGJ and SPJ



Source: created by the author (2024).

Paired samples T-Test confirmed that samples submitted to SGJ pH 1.5 showed a significant difference at the 5% probability level, with a reduction after the test ($p = 0.022$). Counts showed a mean value of the post-gastric stress samples of 5.7 log CFU/mL. Only bacteria *W. paramesenteroides* 17(43)G and *E. durans* 1023 presented counts.

For that samples under SGJ pH 3.0, paired samples T-Test also showed a significant difference in counts ($p = 0.003$) with a reduction after the test. Only seven out of fourteen strains showed counts, and all genera were able to survive: *E. faecium* (3 strains), *W. paramesenteroides*, *E. durans* (2 strains), *L. garvieae*. Counts presented a mean value of post-gastric stress samples of 7 log CFU/mL.

In the literature, there are studies where bacteria show a significant reduction in counts, and even complete inviability at pH 1.0. At pH 3.0, some bacteria are capable of surviving. However, in none of the cases there was exposure to the enzyme pepsin as in our work (Banik et al., 2023; Sharma et al., 2018; Srinivash et al., 2023). In studies where enzymes like pepsin are employed along with pH values of 1.5 and 3.0, there is a drastic decrease in cell counts, reaching a reduction of 7 log units (Bove et al., 2013). In our study, the reduction was up to 2.5 log units. In the work of Tsigkrimani et al. (2022), bacteria did not survive at pH 1.0. At pH 3.0, 88.9% of the tested strains survived. In our work, 50% of the strains survived. In contrast, the study by Barbosa et al. (2015) showed that at pH 3, there was no reduction in the count of the strains used.

The test results for SPJ pH 8.3 exhibited normal distribution ($p = 0.873$). Paired samples T-Test showed that the counts of the test samples did not differ significantly from the control samples at a 5% probability level ($p = 0.698$) differently from the literature, which shows a reduction in count, regardless of whether the strains had previously been subjected to simulated gastric stress (Nunes, Etchepare, et al., 2018) or not (Ferrari et al., 2016; Rama et al., 2021). Counts showed a mean value of the post-pancreatic stress samples of 8.4 log CFU/mL.

Literature show that the resistance of LAB to gastrointestinal stress is strain-dependent (Bradford et al., 2019; Sharma et al., 2018). This ability is due to the presence of defense mechanisms which include transporters that regulate decreased pH, chaperone proteins to aid with folding of misfolded proteins, and transport systems which maintain the correct osmolarity (Bradford et al., 2019). And when we think about these bacteria being applied in a dried form, there is a case in the literature that describes increased resistance to gastrointestinal stress after spray drying (Łopusiewicz et al., 2021).

One of the requirements for a bacterium to be classified as probiotic is that it survives passage through the gastrointestinal tract (Frakolaki et al., 2020). In our study of the 14 LAB strains, two demonstrated viability at SGJ pH 1.5, seven at SGJ pH 3.0, and all at SPJ pH 8.3. Consequently, the selection for the subsequent stages of analysis will preferably focus on those strains that survived both the SGJ and SPJ tests.

3.8 SELECTION OF PROMISING BACTERIAL PROFILES

To create a product that attracts attention, it must have unique characteristics. Similarly, when selecting bacterial strains to compose a probiotic product or an adjunct culture, for example, they must have features that distinguish them from others.

For this reason, the bacteria that remained within the limit established in Section 2.8 and stood out from others in the analyses (Table 4) were chosen for the subsequent stages of antimicrobial susceptibility, antibacterial activity and spray drying. Additionally, a pool of these bacteria was spray dried.

Table 4 – Features of the bacteria to be selected for antimicrobial susceptibility, antibacterial activity and spray drying

Features	Bacterium ID				Scores of the features and final sum*			
	17(43)G	1023	Lb219	CV58				
Heat stress reduction (log CFU/mL)	1.72	1.38	0.28	0.49	0	1	3	2
Osmotic stress reduction (log CFU/mL)	-0.04	-0.15	-0.11	-0.19	0	2	1	3
Oxidative stress reduction (log CFU/mL)	0.26	0.05	0.04	0.64	1	2	3	0
2% NaCl stress reduction (log CFU/mL)	0.44	0.03	0.18	0.05	0	3	1	2
6% NaCl stress reduction (log CFU/mL)	1.26	0.93	1.22	1.13	0	3	1	2
Survival under pH 1.5	+	+	-	-	3	3	0	0
Gastric stress reduction (log CFU/mL)	1.54	0.12	1.04	1.75	1	3	2	0
Pancreatic stress reduction (log CFU/mL)	-0.34	-0.02	0.05	-0.03	3	1	0	2
pH after 24 h skimmed milk	5.57	5.73	6.14	5.87	3	2	0	1
Coagulation after 1 day in skimmed milk	1	0	0	0	3	0	0	0
Coagulation after 3 days in skimmed milk	4	2	0	0	3	2	0	0
Coagulation after 5 days in skimmed milk	4	3	0	1	3	2	0	1
Coagulation after 7 days in skimmed milk	5	5	1	3	3	2	0	1
Diacetyl production	+	-	-	-	3	0	0	0
Final sum of features values	26	26	11	14	**26**	**26**	**11**	**14**
Species by MALDI-TOF	*W. paramesenteroides*	*E. durans*	*E. faecium*	*E. durans*				
Score value of MALDI-TOF	1.84	2.17	2.02	1.89				
Order of analysis among the 100	14	56	74	92				
Municipality – State of sample	Catingueira - PB	Fortaleza - CE	Fortaleza - CE	Cel. Xavier Chaves - MG				
Sample source	Raw milk	Cheese	Cheese	Raw milk				
Type of profile / Potential application	Adjunct culture	Probiotic	Discarded	Heat resistance				

*On a scale from 0 to 3, a value was assigned to the characteristics of the bacteria, with higher values given for the best results, which were greener in color. The bacterium with the lowest sum of the four was not tested in the subsequent stages of antimicrobial susceptibility, antibacterial activity, and spray drying.
Source: created by the author (2024).

3.9 ANTIMICROBIAL SUSCEPTIBILITY

Antimicrobial resistance has been an inherent characteristic of bacteria since the discovery of Penicillin in 1941, the World Health Organization (WHO) estimates that by 2050 there will be no bacteria sensitive to currently available antimicrobials (ANVISA, 2022). This makes the sensitivity analysis of LAB to the most commonly used antibiotics indispensable.

Based on the results obtained by the disk diffusion (Table 5), the three evaluated strains were susceptible to most tested antibiotics (resistance to 3 and 4/9 antibiotics). All strains were susceptible to ampicillin and chloramphenicol and resistant to erythromycin and streptomycin. Different to the others, *E. durans* 1023 was resistant to cotrimoxazole and gentamicin, *E. durans* CV58 was resistant to penicillin and tetracycline and only *W. paramesenteroides* 17(43)G was resistant to vancomycin.

Table 5 – Results of bacterial susceptibility to antibiotics

Antibiotic	*Enterococcus durans* CV58	*Enterococcus durans* 1023	*Weissella paramesenteroides* 17(43)G
Ampicillin[1]	S	S	S
Penicillin[1]	R	S	S
Streptomycin[2]	R	R	R
Gentamycin[2]	S	R	S
Erythromycin[3]	R	R	R
Tetracycline[4]	R	S	S
Vancomycin[5]	S	S	R
Chloramphenicol[6]	S	S	S
Cotrixazole[7]	S	R	S
Multi resistance	4	4	3

S means susceptible; R means resistant.
Superscript numbers means antibiotic class: [1]β-lactams; [2]Aminoglycoside; [3]Macrolide; [4]Tetracycline; [5]Glycopeptide; [6]Amphenicois; [7]Combination of sulfonamides + diaminopyrimidine.
Source: created by the author (2024).

E. durans strains 1023 and CV58 showed different susceptible profile, but both were resistant to erythromycin and streptomycin. Although the enterococci strains have an intrinsic resistance to cephalosporin, cotrimoxazole, lincomycin, and low levels of penicillin and aminoglycosides (Pandova et al., 2023) resistance of enterococci isolated from foods of animal origin to other classes of antibiotics, including erythromycin and tetracycline, has previously been described as a common feature (Hammad et al., 2015; Nieto-Arribas et al., 2011).

Unlike our results, Pieniz et al. (2015) did not find resistance to antibiotics commonly used in animal production, such as erythromycin, tetracycline, vancomycin, gentamicin and penicillin in a strain of *E. durans* previously isolated from Minas Frescal cheese.

W. paramesenteroides 17(43)G was the only vancomycin resistant strain. Yadav et al. (2022) found the same resistance to vancomycin in a strain of *W. paramesenteroides* from dairy products. In addition, the authors also observed resistance to most tested antibiotics (resistance to kanamycin, tetracycline, ceftazidime, nalidixic acid and penicillin – G; and susceptibility to ampicillin and cefotaxime), different from our results.

Vancomycin is considered a key indicator in evaluating the safety of LAB (Chen et al., 2019). However, several reports demonstrate that the resistance of lactic acid bacteria to vancomycin may be intrinsic, requiring more specific studies for better conclusions on safety related to sensitivity to this antibiotic (Colombo et al., 2020). In other studies, with lactic acid bacteria, no resistance to vancomycin was determined in enterococci strains (Mohammed & Çon, 2021; Pieniz et al., 2015; Yerlikaya & Akbulut, 2020), as was observed in this current study.

To be used in products for human consumption, bacterial strains can be recognized as safe (GRAS) if they have only a minimal possibility of transferring antibiotic resistance genes (Colombo et al., 2020). The antimicrobial susceptibility results of this current study allow us to infer that, based on this parameter, all three species evaluated can be considered for future studies and potentially be used as probiotics, as they meet one of the requirements of RDC n° 241/2018, dated July 26, 2018, from ANVISA, as they are sensitive to at least two antibiotics, even if used in combination (pool).

3.10 ANTIBACTERIAL ACTIVITY

The application of antimicrobial LAB or yeasts that contain a bioprotective effect in dairy products implies an additional processing advantage, because it improves the safety and increases the quality, constituting an additional obstacle to reduce the risk of transmitted diseases by food (Arqués et al., 2015). The antimicrobial properties of LAB strains are mediated by the antimicrobial molecules produced by them. These antimicrobials can be divided into three main groups: peptide or protein bacteriocins; organic acids (butyric acetic acid and lactic acid); other small molecules, e.g. diacetyl, hydrogen peroxide, acetaldehyde, acetoin, reuterin and reutericycline (Ibrahim et al., 2021).

L. monocytogenes, E. coli, S. aureus and *E. faecalis* are among the most important foodborne pathogens. Yadav et al. (2022) studied the probiotic potential of *W. paramesenteroides* strains isolated from dairy products and observed their antimicrobial activity against *Bacillus cereus, E. coli, S. aureus,* and *Pseudomonas aeruginosa.* In another study, that characterized the probiotic characteristics of enterococci strains isolated from raw milk and traditional dairy products, Yerlikaya & Akbulut (2020) reported that some strains did not show activity against tested bacteria including *S. aureus, B. cereus, L. monocytogenes, P. aeruginosa* and *Aeromonas hydrophila*. Furthermore, the most strains showed an effect on *E. coli*, followed by *L. monocytogenes* and *S. aureus.*

As for antimicrobial capacity of the strains studied here (Table 6), all of them presented a good antagonistic activity against *E. faecalis* and an excellent antagonistic activity against *E. coli.* To *L. monocytogenes, W. paramesenteroides* 17(43)G and *E. durans* 1023 presented an excellent antagonistic activity, and to *S. aureus*, only *E. durans* 1023 didn't present an excellent antagonistic activity.

Table 6 – Antimicrobial capacity determined by the halo/colony ratio (mm) against pathogens

Indicator pathogens	Tested strains		
	E. durans CV58	*E. durans* 1023	*W. paramesenteroides* 17(43)G
L. monocytogenes ATCC 5779	1.91 (+)	2.39 (++)	2.32 (++)
E. coli IAL1848	2.03 (++)	2.73 (++)	2.81 (++)
S. aureus ATCC 25923	2.51 (++)	1.77 (+)	2.14 (++)
E. faecalis ATCC 29212	1.78 (+)	1.82 (+)	1.95 (+)

Halo/colony ratio < 1 = non-antagonistic (-); Halo/colony ratio > 1 = good antagonistic activity (+); > 2 = excellent antagonistic activity (++).
Source: created by the author (2024).

Both strains, *E. durans* and *W. paramesenteroides*, evaluated in the present study showed antagonistic activity against foodborne indicator pathogens. Therefore, these results are very promising and indicate a great potential for the application of these bacteria in the food industry because they have the ability to extend shelf life by inhibiting spoilage organisms; in addition to inhibiting pathogens and their byproducts, which are harmful to human health.

3.11 ANALYZES OF CELL CONCENTRATE AND POWDER

3.11.1 Drying matrix

Studies are conducted for each microorganism to be dried, adapting the drying medium to the characteristics of the microorganism (Agudelo et al., 2017; Archacka et al., 2019; Burca-

Busaga et al., 2020; Martins, Cnossen, Silva, Vakarelova, et al., 2019; Nunes, Etchepare, et al., 2018; Simpson et al., 2005; Y. Zhang et al., 2016). Different compositions are tested to select the best one. However, in this work, the focus was not on testing different compositions. This variation, in fact, should be tested in the future to uncover whether there is a possibility of greater bacterial survival. Therefore, in our work, an adapted composition of Y. Zhang et al. (2016) methodology was used for the drying matrix.

Some macro and micronutrients had their mass calculated (Table 7) for comparison with the literature. The variation in the concentration of these macro and micronutrients to evaluate viability was studied in the works of Huang & Chen (2013), Martins, Cnossen, Silva, Vakarelova, et al. (2019) and Y. Zhang et al. (2016) for lactose variation; Huang & Chen (2013) and Wang et al. (2020) for calcium variations; and Huang (2020) for magnesium variations. The best composition results in these authors works are linked to each strain studied.

Table 7 – Reconstituted skimmed milk at 25% (w/v) solids + 0.5% (w/v) trehalose

Component	Average centesimal composition of skimmed milk	Composition after reconstitution of 25% (w/w)
Lactose	53.0 g	13.2 g
Protein	34.7 g	8.7 g
Calcium	1257 mg	78.3 mM
Magnesium	110 mg	11.3 mM

Source: created by the author (2024).

Viscosity should be studied in the future, as decreased viscosity of the drying matrix results in smaller droplets, which leads to a lower heat requirement to dry the droplet and, consequently, less thermal stress on the encapsulated bacteria, less time in the drying chamber, and higher bacterial viability.

3.11.2 pH

The pH of the cell concentrate was monitored (Table 8) before entering the spray dryer to ensure a standard average value suitable for bacterial survival. There are studies indicating pH values close to neutrality as ideal for this (Khem et al., 2016). And given the positive results of the counts obtained post-drying, indeed the pH of the encapsulating material was adequate.

3.11.3 Water activity and moisture content

Water activity ranges from 0.161 to 0.259 in the powders (Table 8), and these values did not influence bacterial survival statistically, as well as moisture content, that ranged from 2.88% to 3.82%. Literature indicates that to maximize the physical chemical stability of the

powder and, consequently, the survival of bacteria, the ideal range for water activity is 0.180 to 0.220 (Martins, Cnossen, Silva, Cezarino, et al., 2019).

Table 8 – Results of analyzes on cell concentrate and powder

Analyses	*Enterococcus durans* CV58	*Enterococcus durans* 1023	*Weissella paramesenteroides* 17(43)G	POOL
pH	6.41 ± 0.01a	6.32 ± 0.01b	6.27 ± 0.01c	6.28 ± 0.01b
% moisture $_{CC}$	71.70	71.90	72.03	71.90
% moisture $_{P0}$	3.82	3.06	2.88	3.27
a_w	0.259 ± 0.001a	0.185 ± 0.001b	0.161 ± 0.001c	0.174 ± 0.001d

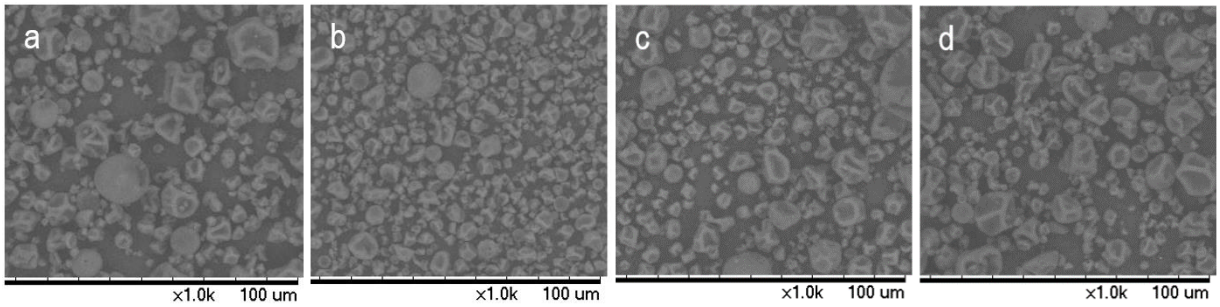
Mean ± standard deviation followed by the same letter, lowercase in the line, do not differ statistically by the Tukey test at 5% significance. Means found in triplicate. CC subscript means cell concentrate; P0 subscript means powder at time zero right after drying.
Source: created by the author (2024).

In experiments that varied a_w and humidity to evaluate bacterial survival over storage time, it was noticed that lower values of a_w (0.11) and humidity (1.7%) allowed viability to be maintained for long periods; on the other hand, higher a_w (0.33) and humidity (5.4%) negatively affected bacterial viability. When the a_w reached 0.52, there was a total loss of microorganisms in less than 1 month (Poddar et al., 2014). Therefore, a_w and moisture content values for the powders obtained in our study were adequate, as bacterial viability was not negatively affected.

3.11.4 Scanning electron microscopy

Scanning electron microscopy (Figure 8) revealed spherical-shaped microparticles, of different sizes, with a deflated or concave structure of surface and others not, without ruptures or cracks.

Figure 8 – Scanning Electron Microscopy images of three encapsulated LAB isolates and one POOL



a) *E. durans* CV58; b) *E. durans* 1023; c) *W. paramesenteroides* 17(43)G; d) POOL.
Source: created by the author (2024).

The concave structures are probably formed due to the shrinkage caused during the early stage of the drying process (Bhagwat et al., 2020), as the wrinkle appearance of spray dried

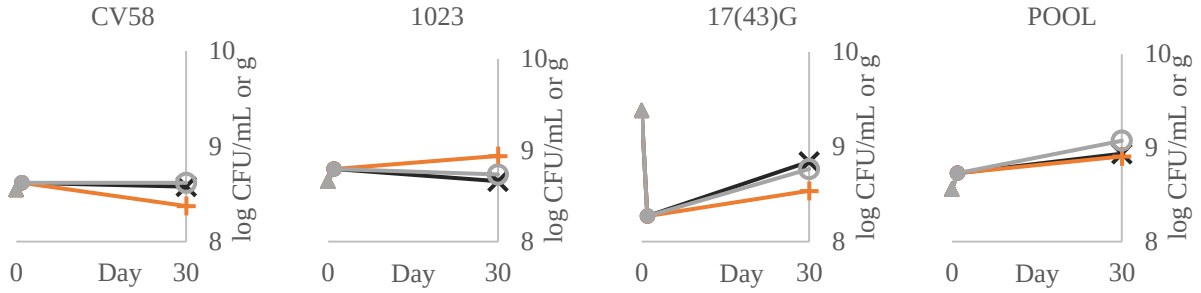
microcapsule is probably a result of a rapid moisture loss (Kamil et al., 2020), and a low dry matter.

Too high and too low drying temperatures, as well as high-viscosity encapsulants and inappropriate solidification conditions, can cause cracks, blow-holes, and hollow structures in microencapsulates, resulting in the lowest encapsulation efficiencies and poorest protection capabilities (Yun et al., 2021). However, the produced microparticles in this study exhibited no visible cracks or fractures on their surfaces, indicating minimal or non-existent air permeability, thus ensuring greater protection of the probiotic microorganisms (Nunes, Etchepare, et al., 2018).

3.11.5 Microbiological analyzes of cell concentrate and powder

Comparing the counts between the moments before drying (cell concentrate), immediately after drying, and after 30 days of storage, no significant difference was found by the Friedman test (p = 0.254). That is, at all moments, the bacteria were viable (Figure 9 and Table 9).

Figure 9 – Counts over time at different storage temperatures



▲ means cell concentrate; ● means powder right after drying; × means powder stored for 30 days at 5 °C ± 2 °C; + means powder stored for 30 days at 35 °C ± 2 °C; ○ means powder stored for 30 days at room temperature (12 °C to 34 °C).
Source: created by the author (2024).

When comparing the counts at different temperatures of 5 °C ± 2 °C, 35 °C ± 2 °C, and room (12 °C to 33 °C), the Friedman test did not indicate a significant difference either (p = 0.726). This means that the studied bacteria can be stored at any of the temperatures employed and remain viable (Figure 9 and Table 9). Unlike the work of Bhagwat et al. (2020) where the microcapsules showed better viability when stored at refrigerated temperatures than room temperature. According to the authors mentioned above, the storage temperature conditions might have an impact on the stability of the probiotics via two mechanisms: 1) increased temperatures lead to the increased metabolic activity of the cells during storage involving

chemical or enzymatic reactions, e.g., lipid oxidation; 2) a_w (molecular mobility), which causes the rubbery state of the matrix.

However, for the comparison between strains *E. durans* CV58, *E. durans* 1023, *W. paramesenteroides* 17(43)G and the POOL, repeated measures ANOVA showed that there is significant difference. Post-hoc Tukey test pointed out the difference between *E. durans* CV58 and POOL counts, where POOL presents bigger counts. All other comparisons showed no significant difference (Figure 9 and Table 9).

Table 9 – Values of LAB counts expressed as mean ± standard deviation of triplicates from *E. durans* CV58, *E. durans* 1023, *W. paramesenteroides* 17(43)G and a POOL. Counts of liquid cell concentrate (L), powder after drying (P), powder after 30 days of storage under 5 °C ± 2 °C (P30 5), powder after 30 days under 35 °C ± 2 °C (P30 35) and powder after 30 days at room temperature (P30 R).

ID	Liquid or powder analyzed				
	L	P	P30 5	P30 35	P30 R
CV58	8.54 ± 0.05^{a}	8.62 ± 0.04^{a}	8.82 ± 0.04^{a}	8.62 ± 0.04^{a}	8.86 ± 0.03^{a}
1023	8.66 ± 0.03^{ab}	9.34 ± 0.02^{ab}	8.88 ± 0.17^{ab}	9.17 ± 0.10^{ab}	8.97 ± 0.10^{ab}
17(43)G	9.26 ± 0.49^{ab}	8.80 ± 0.14^{ab}	9.08 ± 0.06^{ab}	8.77 ± 0.07^{ab}	9.00 ± 0.07^{ab}
POOL	8.70 ± 0.04^{b}	9.26 ± 0.16^{b}	9.18 ± 0.04^{b}	9.15 ± 0.04^{b}	9.31 ± 0.08^{b}

The different lowercase superscript letters within the same column represent statistically significant difference ($p < 0.05$)
Source: created by the author (2024).

These results align with some works in the literature, where resistance to stresses is linked to the strain used, since the only difference between the counts was not due to storage time or temperature, but rather to the bacteria used (Ferreira et al., 2017; Lipan et al., 2020; Nunes, Motta, et al., 2018; Paéz et al., 2012; Rama et al., 2020, 2021).

All of the three strains can be further studied for application in food in its isolated form or in conjunction with other bacteria that have different technological properties, which is the case of POOL. The interesting aspect of using a pool of bacteria is the combination of characteristics from each bacterium in a single "product", whether these characteristics are good (technological or functional properties and higher counts) or bad (antibiotic resistance).

At the time of publication of this work, the completed steps of probiotic evaluation for use in food recommended by the ANVISA (2021) are depicted in Table 10. There are still time-consuming steps to be taken to meet all requirements.

Table 10 – ANVISA recommendation of bacterial assessment for use in food as probiotic

Phase 1	***Confirmation of identity***	***Nomenclature***		
		Culture collection deposit		
		Lineage origin		
		Identification	***Fenotipic tests***	
			Genotipic tests	
		Manufacturing process		
Phase 2	Confirmation of safety	***Identification of the microorganism's risk group or class***		
		History of use		
		Literature review		
		In vitro assays	***Minimum testing***	***Profile of clinically relevant antimicrobial resistance***
				Research on virulence factors
			Additional tests	***Production of antimicrobial substances***
				Production of mucinase
				Production of D-lactate
		Animal trials		
		Human trials		
		Post-market surveillance		
Phase 3	Evidence of benefit	Functional or health claims		
		Studies for the characterization of the probiotic strain		
		Studies to substantiate the benefit of a claim	Types of studies	
			Criteria for study selection	Target population
				Outcome of clinical studies
				Food matrix and dose
		Search for the totality of evidence		
		Evaluation of study quality		
		Assessment of the totality of evidence	Primary studies	
			Systematic review	
		Mechanism of action		
		Substantiation of a claim in probiotic mixtures		

In bold italics are the steps already completed at EMBRAPA Gado de Leite.
Source: Source: created by the author (2024) based on (ANVISA, 2021).

4 CONCLUSIONS

Brazil is a big and diverse country from which we can prospect bacteria with technological potential for application in the food industry, capable of enriching the quality and safety of dairy products.

The viability results after spray drying and storage are high and satisfactory comparing to literature. They encourage research on bacterial cultures spray dried for commercialization without the need for the cold chain.

The bacteria selected in our study have characteristics that resemble NSLAB (adjunct culture), and the results inspire carrying out additional tests to evaluate the probiotic features by using in vitro and in vivo tests before applying these strains to a food system.

REFERENCES

Aakko, J., Sánchez, B., Gueimonde, M., & Salminen, S. (2014). Assessment of stress tolerance acquisition in the heat-tolerant derivative strains of Bifidobacterium animalis subsp. lactis BB-12 and Lactobacillus rhamnosus GG. *Journal of Applied Microbiology*, *117*(1), 239–248. https://doi.org/10.1111/JAM.12520

Abbasiliasi, S., Tan, J. S., Bashokouh, F., Ibrahim, T. A. T., Mustafa, S., Vakhshiteh, F., Sivasamboo, S., & Ariff, A. B. (2017). In vitro assessment of Pediococcus acidilactici Kp10 for its potential use in the food industry. *BMC Microbiology*, *17*(1), 121. https://doi.org/10.1186/s12866-017-1000-z

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

Adu, K. T., Wilson, R., Nichols, D. S., Baker, A. L., Bowman, J. P., & Britz, M. L. (2018). Proteomic analysis of Lactobacillus casei GCRL163 cell-free extracts reveals a SecB homolog and other biomarkers of prolonged heat stress. *PLoS ONE*, *13*(10). https://doi.org/10.1371/JOURNAL.PONE.0206317

Agudelo, J., Cano, A., González-Martínez, C., & Chiralt, A. (2017). Disaccharide incorporation to improve survival during storage of spray dried Lactobacillus rhamnosus in whey protein-maltodextrin carriers. *Journal of Functional Foods*, *37*, 416–423. https://doi.org/10.1016/j.jff.2017.08.014

Ahmed, S., Singh, S., Singh, V., Roberts, K. D., Zaidi, A., & Rodriguez-Palacios, A. (2022). The Weissella Genus: Clinically Treatable Bacteria with Antimicrobial/Probiotic Effects on Inflammation and Cancer. *Microorganisms*, *10*(12), 2427. https://doi.org/10.3390/MICROORGANISMS10122427

Amiri, S., Abotalebi Kohneshahri, S. R., & Nabizadeh, F. (2022). The effect of unit operation and adjunct probiotic culture on physicochemical, biochemical, and textural properties of Dutch Edam cheese. *LWT*, *155*, 112859. https://doi.org/10.1016/J.LWT.2021.112859

ANVISA. (2013). Módulo 5: Tecnologias em Serviços de Saúde: Descrição dos Meios de Cultura Empregados nos Exames Microbiológicos. In *Microbiologia Clínica Para O Controle De Infecção Relacionada À Assistência À Saúde* (3rd ed.).

ANVISA. (2021). Guia para instrução processual de petição de avaliação de probióticos para uso em alimentos. In *Brasília (DF): Ministério da Saúde* (2nd ed., Vol. 1). https://antigo.anvisa.gov.br/documents/10181/5280930/guia+21+v2.pdf/dac5bf5f-ae56-4444-b53c-2cf0f7c15301

ANVISA. (2022). *Resistência antimicrobiana é ameaça global, diz OMS*.

AOAC. (2005). *Official Methods of Analysis of AOAC INTERNATIONAL* (W. Horwitz & G. W. Latimer (eds.); 18th ed.). AOAC INTERNATIONAL. https://www.researchgate.net/publication/292783651_AOAC_2005

Archacka, M., Białas, W., Dembczyński, R., Olejnik, A., Sip, A., Szymanowska, D., Celińska, E., Jankowski, T., Olejnik, A., & Rogodzińska, M. (2019). Method of preservation and type of protective agent strongly influence probiotic properties of Lactococcus lactis: A complete process of probiotic preparation manufacture and use. *Food Chemistry*, *274*, 733–742. https://doi.org/10.1016/j.foodchem.2018.09.033

Arqués, J. L., Rodríguez, E., Langa, S., Landete, J. M., & Medina, M. (2015). Antimicrobial Activity of Lactic Acid Bacteria in Dairy Products and Gut: Effect on Pathogens. *BioMed Research International*, *2015*, 1–9. https://doi.org/10.1155/2015/584183

Ayad, E. H. ., Nashat, S., El-Sadek, N., Metwaly, H., & El-Soda, M. (2004). Selection of wild

lactic acid bacteria isolated from traditional Egyptian dairy products according to production and technological criteria. *Food Microbiology*, *21*(6), 715–725. https://doi.org/10.1016/j.fm.2004.02.009

Baig, M. A., Turner, M. S., Liu, S.-Q., Shah, N. N., & Ayyash, M. M. (2022). Heat, cold, acid, and bile salt induced differential proteomic responses of a novel potential probiotic Lactococcus garvieae C47 isolated from camel milk. *Food Chemistry*, *397*, 133774. https://doi.org/10.1016/j.foodchem.2022.133774

Banik, A., Anjum, H., Habib, H., Abony, M., Begum, A., & Ahmed, Z. (2023). Characterization of lactic acid bacteria isolated from street pickles of Dhaka, Bangladesh. *Heliyon*, *9*(6), e17508. https://doi.org/10.1016/J.HELIYON.2023.E17508

Barbosa, J., Borges, S., & Teixeira, P. (2015). Pediococcus acidilactici as a potential probiotic to be used in food industry. *International Journal of Food Science and Technology*, *50*(5), 1151–1157. https://doi.org/10.1111/ijfs.12768

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

Barbosa, J. I. B., Borges, S., & Teixeira, P. (2015). Influence of sub-lethal stresses on the survival of lactic acid bacteria after spray-drying in orange juice. *Food Microbiology*, *52*, 77–83. https://doi.org/10.1016/j.fm.2015.06.010

Barbosa, J., & Teixeira, P. (2017). Development of probiotic fruit juice powders by spray-drying: A review. *Food Reviews International*, *33*(4), 335–358. https://doi.org/10.1080/87559129.2016.1175016

Bauer, A. W., Kirby, W. M. M., Sherris, J. C., & Turck, M. (1966). Antibiotic Susceptibility Testing by a Standardized Single Disk Method. *American Journal of Clinical Pathology*, *45*(4_ts), 493–496. https://doi.org/10.1093/ajcp/45.4_ts.493

Bhagwat, A., Bhushette, P., & Annapure, U. S. (2020). Spray drying studies of probiotic Enterococcus strains encapsulated with whey protein and maltodextrin. *Beni-Suef University Journal of Basic and Applied Sciences*, *9*(1), 1–8. https://doi.org/10.1186/S43088-020-00061-Z/FIGURES/2

Blajman, J. E., Vinderola, G., Cuatrin, A., Lingua, M. S., & Páez, R. B. (2020). Technological variables influencing the growth and stability of a silage inoculant based on spray-dried lactic acid bacteria. *Journal of Applied Microbiology*, *129*(6), 1486–1496. https://doi.org/10.1111/jam.14750

Bove, P., Russo, P., Capozzi, V., Gallone, A., Spano, G., & Fiocco, D. (2013). Lactobacillus plantarum passage through an oro-gastro-intestinal tract simulator: Carrier matrix effect and transcriptional analysis of genes associated to stress and probiosis. *Microbiological Research*, *168*(6), 351–359. https://doi.org/10.1016/j.micres.2013.01.004

Bradford, R., Reyes, V., Bonilla, F., Bueno, F., Dzandu, B., Liu, C., Chouljenko, A., & Sathivel, S. (2019). Development of milk powder containing Lactobacillus plantarum NCIMB 8826 immobilized with prebiotic hi-maize starch and survival under simulated gastric and intestinal conditions. *Food Production, Processing and Nutrition 2019 1:1*, *1*(1), 1–8. https://doi.org/10.1186/S43014-019-0011-6

Burca-Busaga, C. G., Betoret, N., Seguí, L., Betoret, E., & Barrera, C. (2020). Survival of Lactobacillus salivarius CECT 4063 and Stability of Antioxidant Compounds in Dried Apple Snacks as Affected by the Water Activity, the Addition of Trehalose and High Pressure Homogenization. *Microorganisms*, *8*(8), 1095. https://doi.org/10.3390/microorganisms8081095

Çabuk, B., & Harsa, Ş. (2015). Whey Protein-Pullulan (WP/Pullulan) Polymer Blend for Preservation of Viability of Lactobacillus acidophilus. *Drying Technology*, *33*(10), 1223–1233. https://doi.org/10.1080/07373937.2015.1021008

Campagnollo, F. B., Margalho, L. P., Kamimura, B. A., Feliciano, M. D., Freire, L., Lopes, L. S., Alvarenga, V. O., Cadavez, V. A. P., Gonzales-Barron, U., Schaffner, D. W., & Sant'Ana, A. S. (2018). Selection of indigenous lactic acid bacteria presenting anti-listerial activity, and their role in reducing the maturation period and assuring the safety of traditional Brazilian cheeses. *Food Microbiology*, *73*, 288–297. https://doi.org/10.1016/J.FM.2018.02.006

Charteris, W. P., Kelly, P. M., Morelli, L., & Collins, A. J. K. (2001). Gradient Diffusion Antibiotic Susceptibility Testing of Potentially Probiotic Lactobacilli. *Journal of Food Protection*, *64*(12).

Chen, W., Yu, L., & Shi, Y. (2019). Safety Evaluation of Lactic Acid Bacteria. In *Lactic Acid Bacteria* (pp. 371–409). Springer Nature Singapore. https://doi.org/10.1007/978-981-13-7832-4_11

Coghetto, C. C., Flores, S. H., Brusch Brinques, G., & Ant ^ Onio Z Achia Ayub, M. (2016). *Viability and alternative uses of a dried powder, microencapsulated Lactobacillus plantarum without the use of cold chain or dairy products*. https://doi.org/10.1016/j.lwt.2016.03.020

Colombo, M., Nero, L. A., & Todorov, S. D. (2020). Safety profiles of beneficial lactic acid bacteria isolated from dairy systems. *Brazilian Journal of Microbiology*, *51*(2), 787–795. https://doi.org/10.1007/s42770-020-00227-y

Cosentino, S., Viale, S., Deplano, M., Fadda, M. E., & Pisano, M. B. (2018). *Application of Autochthonous Lactobacillus Strains as Biopreservatives to Control Fungal Spoilage in Caciotta Cheese*. https://doi.org/10.1155/2018/3915615

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

Desmond, C., Stanton, C., Fitzgerald, G. F., Collins, K., & Paul Ross, R. (2002). Environmental adaptation of probiotic lactobacilli towards improvement of performance during spray drying. *International Dairy Journal*, *12*(2–3), 183–190. https://doi.org/10.1016/S0958-6946(02)00040-7

Dijkstra, A. R., Setyawati, M. C., Bayjanov, J. R., Alkema, W., Van Hijum, S. A. F. T., Bron, P. A., & Hugenholtz, J. (2014). Diversity in robustness of Lactococcus lactis strains during heat stress, oxidative stress, and spray drying stress. *Applied and Environmental Microbiology*, *80*(2), 603–611. https://doi.org/10.1128/aem.03434-13

El-Salam, M. H. A., & El-Shibiny, S. (2015). Preparation and properties of milk proteins-based encapsulated probiotics: a review. *Dairy Science & Technology*, *95*(4), 393–412. https://doi.org/10.1007/s13594-015-0223-8

Eratte, D., Gengenbach, T. R., Dowling, K., Barrow, C. J., & Adhikari, B. (2016). Survival, oxidative stability, and surface characteristics of spray dried co-microcapsules containing omega-3 fatty acids and probiotic bacteria. *Drying Technology*, *34*(16), 1926–1935. https://doi.org/10.1080/07373937.2016.1141782

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

Ferreira, A. A., Huang, S., Perrone, Í. T., Schuck, P., Jan, G., & Carvalho, A. F. (2017). Tracking Amazonian cheese microbial diversity: Development of an original, sustainable, and robust starter by freeze drying/spray drying. *Journal of Dairy Science*, *100*(9), 6997–7006. https://doi.org/10.3168/jds.2016-12418

Fessard, A., & Remize, F. (2017). Why Are Weissella spp. Not Used as Commercial Starter Cultures for Food Fermentation? *Fermentation 2017, Vol. 3, Page 38*, *3*(3), 38. https://doi.org/10.3390/FERMENTATION3030038

Frakolaki, G., Giannou, V., Kekos, D., & Tzia, C. (2020). A review of the microencapsulation techniques for the incorporation of probiotic bacteria in functional foods. *Https://Doi.Org/10.1080/10408398.2020.1761773*, *61*(9), 1515–1536. https://doi.org/10.1080/10408398.2020.1761773

Franciosi, E., Settanni, L., Cavazza, A., & Poznanski, E. (2009). Biodiversity and technological potential of wild lactic acid bacteria from raw cows' milk. *International Dairy Journal*, *19*(1), 3–11. https://doi.org/10.1016/j.idairyj.2008.07.008

Gardiner, G. E., O'sullivan, E., Kelly, J., Auty, M. A. E., Fitzgerald, G. F., Collins, J. K., Ross, R. P., & Stanton, C. (2000). Comparative Survival Rates of Human-Derived Probiotic Lactobacillus paracasei and L. salivarius Strains during Heat Treatment and Spray Drying. *APPLIED AND ENVIRONMENTAL MICROBIOLOGY*, *66*(6), 2605–2612. https://journals.asm.org/journal/aem

Girma, K., & Machado, S. (2013). Use of non-replicated observations and farm trials for guiding nutrient management decisions. *Proceedings of the Western Nutrient Management Conference*, *10*, 72–80.

Gomez-Zavaglia, A., Chen, Z., Carasi, P., Abriouel, H., Lavilla Lerma, L., del Carmen Casado Muñoz, M., Pérez Montoro, B., Kabisch, J., Pichner, R., Cho, G.-S., Neve, H., Fusco, V., A P Franz, C. M., Gálvez, A., & Benomar, N. (2015). *The controversial nature of the Weissella genus: technological and functional aspects versus whole genome analysis-based pathogenic potential for their application in food and health*. https://doi.org/10.3389/fmicb.2015.01197

González, E., Herencias, C., & Prieto, M. A. (2020). A polyhydroxyalkanoate-based encapsulating strategy for 'bioplasticizing' microorganisms. *Microbial Biotechnology*, *13*(1), 185–198. https://doi.org/10.1111/1751-7915.13492

Grujović, M., Mladenović, K., Jakovljević, V., & Čomić, L. (2020). Detection of enzymes produced by lactic acid bacteria isolated from traditionally made Serbian cheese and their role in the formation of its specific flavor. *Acta Agriculturae Serbica*, *25*(50), 165–169. https://doi.org/10.5937/AASER2050165G

Hammad, A. M., Hassan, H. A., & Shimamoto, T. (2015). Prevalence, antibiotic resistance and virulence of Enterococcus spp. in Egyptian fresh raw milk cheese. *Food Control*, *50*, 815–820. https://doi.org/10.1016/j.foodcont.2014.10.020

Hantsis-Zacharov, E., & Halpern, M. (2007). Culturable Psychrotrophic Bacterial Communities in Raw Milk and Their Proteolytic and Lipolytic Traits. *Applied and Environmental Microbiology*, *73*(22), 7162. https://doi.org/10.1128/AEM.00866-07

Hao, F., Fu, N., Ndiaye, H., Woo, W., Jeantet, R., Chen, X. D., Thermotolerance, S., & Meng, &. (2021). *Lactic Acid Bacteria After Spray Drying as Affected by the Increase of Growth Temperature*. *Food and Bioprocess Technology*. *10*(1), 6. https://doi.org/10.1007/s11947

Hati, S., Patel, N., & Mandal, S. (2018). Comparative Growth Behaviour and Biofunctionality of Lactic Acid Bacteria During Fermentation of Soy Milk and Bovine Milk. *Probiotics and Antimicrobial Proteins*, *10*(2), 277–283. https://doi.org/10.1007/s12602-017-9279-5

Huang, S. (2020). *Spray drying of probiotic bacteria : From molecular mechanism to pilot-scale productio*. universite bretagne loire.

Huang, S., & Chen, X. D. (2013). Significant effect of Ca2+ on improving the heat resistance of lactic acid bacteria. *FEMS Microbiology Letters*, *344*(1), 31–38. https://doi.org/10.1111/1574-6968.12151

Ibrahim, S. A., Ayivi, R. D., Zimmerman, T., Siddiqui, S. A., Altemimi, A. B., Fidan, H.,

Esatbeyoglu, T., & Bakhshayesh, R. V. (2021). Lactic Acid Bacteria as Antimicrobial Agents: Food Safety and Microbial Food Spoilage Prevention. *Foods*, *10*(12), 3131. https://doi.org/10.3390/foods10123131

Im, E. J., Lee, H. H. Y., Kim, M., & Kim, M. K. (2023). Evaluation of Enterococcal Probiotic Usage and Review of Potential Health Benefits, Safety, and Risk of Antibiotic-Resistant Strain Emergence. *Antibiotics*, *12*(8). https://doi.org/10.3390/ANTIBIOTICS12081327

Kamil, R. Z., Yanti, R., Murdiati, A., Juffrie, M., & Rahayu, E. S. (2020). Microencapsulation of indigenous probiotic Lactobacillus plantarum Dad-13 by spray and freeze-drying: strain-dependent effect and its antibacterial property. *Food Research*, *4*(6), 2181–2189. https://doi.org/10.26656/fr.2017.4(6).280

Kateete, D. P., Kimani, C. N., Katabazi, F. A., Okeng, A., Okee, M. S., Nanteza, A., Joloba, M. L., & Najjuka, F. C. (2010). Identification of Staphylococcus aureus: DNase and Mannitol salt agar improve the efficiency of the tube coagulase test. *Annals of Clinical Microbiology and Antimicrobials*, *9*, 1–7. https://doi.org/10.1186/1476-0711-9-23

Kavitake, D., Kandasamy, S., Devi, P. B., & Shetty, P. H. (2018). Recent developments on encapsulation of lactic acid bacteria as potential starter culture in fermented foods – A review. *Food Bioscience*, *21*(November 2017), 34–44. https://doi.org/10.1016/j.fbio.2017.11.003

Khem, S., Bansal, V., Small, D. M., & May, B. K. (2016). Comparative influence of pH and heat on whey protein isolate in protecting Lactobacillus plantarum A17 during spray drying. *Food Hydrocolloids*, *54*, 162–169. https://doi.org/10.1016/j.foodhyd.2015.09.029

King, N. (1948). A modification of the Voges-Proskauer test for rapid colorimetric determination of acetylmethylcarbinol plus diacetyl in the butter cultures. *Dairy Indust*, *13*(9), 860–861, 878. https://eurekamag.com/research/029/681/029681170.php

Lampien, A., Sekhon, A. S., Unger, P., Sharma, S., Galland, A. T., & Michael, M. (2023). Thermal inactivation kinetics of Paucilactobacillus wasatchensis SH05 in whole milk. *International Dairy Journal*, *145*, 105715. https://doi.org/10.1016/j.idairyj.2023.105715

Lewus, C. B., & Montville, T. J. (1991). Detection of bacteriocins produced by lactic acid bacteria. *Journal of Microbiological Methods*, *13*(2), 145–150. https://doi.org/10.1016/0167-7012(91)90014-H

Liao, L. K., Wei, X. Y., Gong, X., Li, J. H., Huang, T., & Xiong, T. (2017). Microencapsulation of Lactobacillus casei LK-1 by spray drying related to its stability and in vitro digestion. *LWT - Food Science and Technology*, *82*, 82–89. https://doi.org/10.1016/j.lwt.2017.03.065

Lipan, L., Rusu, B., Sendra, E., Hernández, F., Vázquez-Araújo, L., Vodnar, D. C., & Carbonell-Barrachina, Á. A. (2020). Spray drying and storage of probiotic-enriched almond milk: probiotic survival and physicochemical properties. *Journal of the Science of Food and Agriculture*, *100*(9), 3697–3708. https://doi.org/10.1002/jsfa.10409

Łopusiewicz, Ł., Bogusławska-Wąs, E., Drozłowska, E., Trocer, P., Dłubała, A., Mazurkiewicz-Zapałowicz, K., & Bartkowiak, A. (2021). The Application of Spray-Dried and Reconstituted Flaxseed Oil Cake Extract as Encapsulating Material and Carrier for Probiotic Lacticaseibacillus rhamnosus GG. *Materials*, *14*(18), 5324. https://doi.org/10.3390/ma14185324

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

Margalho, L. P., Jorge, G. P., Noleto, D. A. P., Silva, C. E., Abreu, J. S., Piran, M. V. F., Brocchi, M., & Sant'Ana, A. S. (2021). Biopreservation and probiotic potential of a large

set of lactic acid bacteria isolated from Brazilian artisanal cheeses: From screening to in product approach. *Microbiological Research*, *242*, 126622. https://doi.org/10.1016/J.MICRES.2020.126622

Margalho, L. P., Kamimura, B. A., Brexó, R. P., Alvarenga, V. O., Sabri Cebeci, A., Janssen, P. W. M., Dijkstra, A., Starrenburg, M. J. C., Sheombarsing, R. S., Cruz, A. G., Alkema, W., Bachmann, H., & Sant'ana, A. S. (2021). High throughput screening of technological and biopreservation traits of a large set of wild lactic acid bacteria from Brazilian artisanal cheeses. *Food Microbiology*, *100*, 103872. https://doi.org/10.1016/j.fm.2021.103872

Martins, E., Cnossen, D. C., Silva, C. R. J., Cezarino, J. C., Nero, L. A., Perrone, I. T., & Carvalho, A. F. (2019). Determination of ideal water activity and powder temperature after spray drying to reduce Lactococcus lactis cell viability loss. *Journal of Dairy Science*, *102*(7), 6013–6022. https://doi.org/10.3168/jds.2019-16297

Martins, E., Cnossen, D. C., Silva, C. R. J., Vakarelova, M., & Carvalho, A. F. (2019). Short communication: Effect of lactose on the spray drying of Lactococcus lactis in dairy matrices. *Journal of Dairy Science*, *102*(11), 9763–9766. https://doi.org/10.3168/jds.2019-16939

Martley, F. G., & Crow, V. L. (1993). Interactions between non-starter microorganisms during cheese manufacture and repening. *International Dairy Journal*, *3*(4–6), 461–483. https://doi.org/10.1016/0958-6946(93)90027-W

Medina, R. B., Katz, M. B., González, S., & Oliver, G. (2004). Determination of esterolytic and lipolytic activities of lactic acid bacteria. *Methods in Molecular Biology (Clifton, N.J.)*, *268*, 465–470. https://doi.org/10.1385/1-59259-766-1:465

Meng, Z., Zhang, L., Xin, L., Lin, K., Yi, H., & Han, X. (2018). Technological characterization of Lactobacillus in semihard artisanal goat cheeses from different Mediterranean areas for potential use as nonstarter lactic acid bacteria. *Journal of Dairy Science*, *101*(4), 2887–2896. https://doi.org/10.3168/jds.2017-14003

Moayyedi, M., Eskandari, M. H., Rad, A. H. E., Ziaee, E., Khodaparast, M. H. H., & Golmakani, M.-T. (2018). Effect of drying methods (electrospraying, freeze drying and spray drying) on survival and viability of microencapsulated Lactobacillus rhamnosus ATCC 7469. *Journal of Functional Foods*, *40*(July 2017), 391–399. https://doi.org/10.1016/j.jff.2017.11.016

Mohammed, S., & Çon, A. H. (2021). Isolation and characterization of potential probiotic lactic acid bacteria from traditional cheese. *LWT*, *152*, 112319. https://doi.org/10.1016/j.lwt.2021.112319

Morandi, S., Silvetti, T., & Brasca, M. (2022). Content and spatial distribution of dairy-related Clostridium spores in Grana Padano cheese during the ripening period. *LWT*, *167*, 113850. https://doi.org/10.1016/j.lwt.2022.113850

Moreira, M. T. C., Martins, E., Perrone, Í. T., de Freitas, R., Queiroz, L. S., & de Carvalho, A. F. (2021). Challenges associated with spray drying of lactic acid bacteria: Understanding cell viability loss. In *Comprehensive Reviews in Food Science and Food Safety* (Vol. 20, Issue 4, pp. 3267–3283). Blackwell Publishing Inc. https://doi.org/10.1111/1541-4337.12774

Moreira, M. T. C., Martins, E., Perrone, Í. T., Freitas, R., Queiroz, L. S., & Carvalho, A. F. (2021). Challenges associated with spray drying of lactic acid bacteria: Understanding cell viability loss. *Comprehensive Reviews in Food Science and Food Safety*, *20*(4), 3267–3283. https://doi.org/10.1111/1541-4337.12774

Moumita, S., Das, B., Hasan, U., & Jayabalan, R. (2018). Effect of long-term storage on viability and acceptability of lyophilized and spray-dried synbiotic microcapsules in dry functional food formulations. *Lwt*, *96*, 127–132.

https://doi.org/10.1016/j.lwt.2018.05.030
Nacef, M., Chevalier, M., Chollet, S., Drider, D., & Flahaut, C. (2017). MALDI-TOF mass spectrometry for the identification of lactic acid bacteria isolated from a French cheese: The Maroilles. *International Journal of Food Microbiology*, *247*, 2–8. https://doi.org/10.1016/j.ijfoodmicro.2016.07.005
Nieto-Arribas, P., Seseña, S., Poveda, J. M., Chicón, R., Cabezas, L., & Palop, L. (2011). Enterococcus populations in artisanal Manchego cheese: Biodiversity, technological and safety aspects. *Food Microbiology*, *28*(5), 891–899. https://doi.org/10.1016/j.fm.2010.12.005
Nunes, G. L., Etchepare, M. de A., Cichoski, A. J., Zepka, L. Q., Jacob Lopes, E., Barin, J. S., Flores, É. M. de M., da Silva, C. de B., & de Menezes, C. R. (2018). Inulin, hi-maize, and trehalose as thermal protectants for increasing viability of Lactobacillus acidophilus encapsulated by spray drying. *LWT - Food Science and Technology*, *89*, 128–133. https://doi.org/10.1016/J.LWT.2017.10.032
Nunes, G. L., Motta, M. H., Cichoski, A. J., Wagner, R., Muller, É. I., Codevilla, C. F., da Silva, C. D. B., & de Menezes, C. R. (2018). Encapsulation of Lactobacillus acidophilus La-5 and Bifidobacterium Bb-12 by spray drying and evaluation of its resistance in simulated gastrointestinal conditions, thermal treatments and storage conditions. *Ciência Rural*, *48*(6). https://doi.org/10.1590/0103-8478CR20180035
Oguntoye, M. A., & Ezekiel, O. O. (2024). Toxicological safety assessment of provitamin A cassava hydrolysate with free and encapsulated Lacticaseibacillus rhamnosus GG in the Wistar rat model. *Food Chemistry Advances*, *4*, 100641. https://doi.org/10.1016/j.focha.2024.100641
Ortakci, F., Broadbent, J. R., Oberg, C. J., & McMahon, D. J. (2015). Growth and gas production of a novel obligatory heterofermentative Cheddar cheese nonstarter lactobacilli species on ribose and galactose. *Journal of Dairy Science*, *98*, 3645–3654. https://doi.org/10.3168/jds.2014-9293
Paéz, R., Lavari, L., Vinderola, G., Audero, G., Cuatrin, A., Zaritzky, N., & Reinheimer, J. (2012). Effect of heat treatment and spray drying on lactobacilli viability and resistance to simulated gastrointestinal digestion. *Food Research International*, *48*(2), 748–754. https://doi.org/10.1016/j.foodres.2012.06.018
Palud, A., Scornec, H., Cavin, J.-F., & Licandro, H. (2018). New Genes Involved in Mild Stress Response Identified by Transposon Mutagenesis in Lactobacillus paracasei. *Frontiers in Microbiology*, *9*(MAR). https://doi.org/10.3389/fmicb.2018.00535
Pan, Q., Shen, X., Yu, L., Tian, F., Zhao, J., Zhang, H., Chen, W., & Zhai, Q. (2021). Comparative genomic analysis determines the functional genes related to bile salt resistance in lactobacillus salivarius. *Microorganisms*, *9*(10), 2038. https://doi.org/10.3390/MICROORGANISMS9102038/S1
Pandova, M., Kizheva, Y., Tsenova, M., Rusinova, M., Borisova, T., & Hristova, P. (2023). Pathogenic Potential and Antibiotic Susceptibility: A Comprehensive Study of Enterococci from Different Ecological Settings. *Pathogens*, *13*(1), 36. https://doi.org/10.3390/pathogens13010036
Paula, I. L. de, Melquíades, L. L., Perrone, Í. T., Mauricio, E. F., Dias, B. A. de C., Carvalho, A. F. de, Oliveira, L. F. C. de, & Stephani, R. (2023). Reduced and Low-lactose Powdered Milk Production and Determination of its Physicochemical and Microstructural Properties During Storage. *Revista Virtual de Química*, *15*(5), 887–895. https://doi.org/10.21577/1984-6835.20230031
Pereira, V., Lopes, C., Castro, A., Silva, J., Gibbs, P., & Teixeira, P. (2009). Characterization for enterotoxin production, virulence factors, and antibiotic susceptibility of Staphylococcus aureus isolates from various foods in Portugal. *Food Microbiology*,

26(3), 278–282. https://doi.org/10.1016/j.fm.2008.12.008

Pieniz, S., de Moura, T. M., Cassenego, A. P. V., Andreazza, R., Frazzon, A. P. G., Camargo, F. A. de O., & Brandelli, A. (2015). Evaluation of resistance genes and virulence factors in a food isolated Enterococcus durans with potential probiotic effect. *Food Control*, *51*, 49–54. https://doi.org/10.1016/J.FOODCONT.2014.11.012

Poddar, D., Das, S., Jones, G., Palmer, J., Jameson, G. B., Haverkamp, R. G., & Singh, H. (2014). *Stability of probiotic Lactobacillus paracasei during storage as affected by the drying method*. https://doi.org/10.1016/j.idairyj.2014.04.007

Prabhurajeshwar, C., & Chandrakanth, R. K. (2017). Probiotic potential of Lactobacilli with antagonistic activity against pathogenic strains: An in vitro validation for the production of inhibitory substances. *Biomedical Journal*, *40*(5), 270–283. https://doi.org/10.1016/j.bj.2017.06.008

Rama, G. R., Dullius, D., Agnol, W. D., Esquerdo, V. M., Lehn, D. N., & Souza, C. F. V. de. (2021). Ricotta whey supplemented with gelatin and collagen for the encapsulation of probiotic lactic acid bacteria. *Food Science and Technology*, *41*(3), 576–586. https://doi.org/10.1590/fst.19720

Rama, G. R., Kuhn, D., Beux, S., Maciel, M. J., & Souza, C. F. V. de. (2020). Cheese Whey and Ricotta Whey for the Growth and Encapsulation of Endogenous Lactic Acid Bacteria. *Food and Bioprocess Technology*, *13*(2), 308–322. https://doi.org/10.1007/s11947-019-02395-8

Ramos, I. M., Seseña, S., Poveda, J. M., & Palop, M. L. (2023). Screening of Lactic Acid Bacteria Strains to Improve the Properties of Non-fat Set Yogurt by in situ EPS Production. *Food and Bioprocess Technology*, *16*(11), 2541–2558. https://doi.org/10.1007/S11947-023-03080-7/FIGURES/7

RESEARCH AND MARKETS. (2023). *Probiotics Global Market Report 2024* (p. 200). https://www.researchandmarkets.com/reports/5744225/probiotics-global-market-report

Reyes, V., Chotiko, A., Chouljenko, A., Campbell, V., Liu, C., Theegala, C., & Sathivel, S. (2018). Influence of wall material on production of spray dried Lactobacillus plantarum NRRL B-4496 and its viability at different storage conditions. *Drying Technology*, *36*(14), 1738–1748. https://doi.org/10.1080/07373937.2017.1423324

Rudolph, A. S., & Crowe, J. H. (1985). Membrane stabilization during freezing: The role of two natural cryoprotectants, trehalose and proline. *Cryobiology*, *22*(4), 367–377. https://doi.org/10.1016/0011-2240(85)90184-1

Sáez, G. D., Flomenbaum, L., & Zárate, G. (2018). Lactic acid bacteria from argentinean fermented foods: Isolation and characterization for their potential use as starters for fermentation of vegetables. *Food Technology and Biotechnology*, *56*(3), 398–410. https://doi.org/10.17113/ftb.56.03.18.5631

Santos, G. Dos, Nogueira, R. I., & Rosenthal, A. (2018). Powdered yoghurt produced by spray drying and freeze drying: a review. *Brazilian Journal of Food Technology*, *21*. https://doi.org/10.1590/1981-6723.12716

Santos, R. C. S. dos, Finkler, L., & Finkler, C. L. L. (2014). Microencapsulation of Lactobacillus casei by spray drying. *Journal of Microencapsulation*, *31*(8), 759–767. https://doi.org/10.3109/02652048.2014.932026

Santos, K. M. O., Matos, C. R., Salles, H. O., Franco, B. D. G. de M., Arellano, K., Holzapfel, W. H., & Todorov, S. D. (2020). Exploring Beneficial/Virulence Properties of Two Dairy-Related Strains of Streptococcus infantarius subsp. infantarius. *Probiotics and Antimicrobial Proteins*, *12*(4), 1524–1541. https://doi.org/10.1007/S12602-020-09637-8/METRICS

Schuck, P., Dolivet, A., & Jeantet, R. (2012). Analytical Methods for Food and Dairy Powders. In *Analytical Methods for Food and Dairy Powders*. Wiley.

https://doi.org/10.1002/9781118307397
Sharma, S., Kandasamy, S., Kavitake, D., & Shetty, P. H. (2018). Probiotic characterization and antioxidant properties of Weissella confusa KR780676, isolated from an Indian fermented food. *Lwt*, *97*(June), 53–60. https://doi.org/10.1016/j.lwt.2018.06.033
Shu, G., Zheng, Q., Chen, L., Kou, J., Guo, Y., & Zhang, M. (2020). Preparation of goat milk powder with Lactobacillus casei L61 and antioxidant peptides: optimization of the composite thermal protectants and evaluation of storage stability. *Drying Technology*, *38*(14), 1943–1954. https://doi.org/10.1080/07373937.2019.1681447
Simpson, P. J., Stanton, C., Fitzgerald, G. F., & Ross, R. P. (2005). Intrinsic tolerance of Bifidobacterium species to heat and oxygen and survival following spray drying and storage. *Journal of Applied Microbiology*, *99*(3), 493–501. https://doi.org/10.1111/J.1365-2672.2005.02648.X
Smitinont, T., Tansakul, C., Tanasupawat, S., Keeratipibul, S., Navarini, L., Bosco, M., & Cescutti, P. (1999). Exopolysaccharide-producing lactic acid bacteria strains from traditional Thai fermented foods: isolation, identification and exopolysaccharide characterization. *International Journal of Food Microbiology*, *51*(2–3), 105–111. https://doi.org/10.1016/S0168-1605(99)00094-X
Souza, M., Mesquita, A., Veríssimo, C., Grosso, C., Converti, A., & Maciel, M. I. (2020). Microencapsulation by spray drying of a functional product with mixed juice of acerola and ciriguela fruits containing three probiotic lactobacilli. *Drying Technology*, 1–11. https://doi.org/10.1080/07373937.2020.1862182
Srinivash, M., Krishnamoorthi, R., Mahalingam, P. U., Malaikozhundan, B., & Keerthivasan, M. (2023). Probiotic potential of exopolysaccharide producing lactic acid bacteria isolated from homemade fermented food products. *Journal of Agriculture and Food Research*, *11*, 100517. https://doi.org/10.1016/J.JAFR.2023.100517
Stummer, S., Toegel, S., Rabenreither, M. C., Unger, F. M., Wirth, M., Viernstein, H., & Salar-Behzadi, S. (2012). Fluidized-bed drying as a feasible method for dehydration of Enterococcus faecium M74. *Journal of Food Engineering*, *111*(1), 156–165. https://doi.org/10.1016/j.jfoodeng.2012.01.005
Sugimoto, S., Abdullah-Al-Mahin, & Sonomoto, K. (2008). Molecular Chaperones in Lactic Acid Bacteria: Physiological Consequences and Biochemical Properties. *Journal of Bioscience and Bioengineering*, *106*(4), 324–336. https://doi.org/10.1263/jbb.106.324
Tagg, J. R., Dajani, A. S., & Wannamaker, L. W. (1976). Bacteriocins of gram-positive bacteria. *Bacteriological Reviews*, *40*(3), 722–756. https://doi.org/10.1128/BR.40.3.722-756.1976
Tang, H. W., Abbasiliasi, S., Murugan, P., Tam, Y. J., Ng, H. S., & Tan, J. S. (2020). Influence of freeze-drying and spray-drying preservation methods on survivability rate of different types of protectants encapsulated Lactobacillus acidophilus FTDC 3081. *OUP*, *84*(9), 1913–1920. https://doi.org/10.1080/09168451.2020.1770572
Tantratian, S., Wattanaprasert, S., & Suknaisilp, S. (2018). Effect of partial substitution of milk-non-fat with xanthan gum on encapsulation of a probiotic Lactobacillus. *Journal of Food Processing and Preservation*, *42*(7), e13673. https://doi.org/10.1111/jfpp.13673
Terzić-Vidojević, A., Veljović, K., Tolinački, M., Živković, M., Lukić, J., Lozo, J., Fira, Đ., Jovčić, B., Strahinić, I., Begović, J., Popović, N., Miljković, M., Kojić, M., Topisirović, L., & Golić, N. (2020). Diversity of non-starter lactic acid bacteria in autochthonous dairy products from Western Balkan Countries - Technological and probiotic properties. *Food Research International*, *136*. https://doi.org/10.1016/J.FOODRES.2020.109494
Thierry, A., Valence, F., Deutsch, S.-M., Even, S., Falentin, H., Le Loir, Y., Jan, G., & Gagnaire, V. (2015). Strain-to-strain differences within lactic and propionic acid bacteria species strongly impact the properties of cheese–A review. *Dairy Science & Technology*,

95(6), 895–918. https://doi.org/10.1007/s13594-015-0267-9

Tsigkrimani, M., Panagiotarea, K., Paramithiotis, S., Bosnea, L., Pappa, E., Drosinos, E. H., Skandamis, P. N., & Mataragas, M. (2022). Microbial Ecology of Sheep Milk, Artisanal Feta, and Kefalograviera Cheeses. Part II: Technological, Safety, and Probiotic Attributes of Lactic Acid Bacteria Isolates. *Foods*, *11*(3). https://doi.org/10.3390/foods11030459

Umashankar, K., Chandralekha, A., Dandavate, T., Tavanandi, H. A., & Raghavarao, K. S. M. S. (2019). A nonconventional method for drying of Pseudomonas aeruginosa and its comparison with conventional methods. *Drying Technology*, *37*(7), 839–853. https://doi.org/10.1080/07373937.2018.1469140

Upham, J. P., Eisebraun, M., Fortuna, A., & Mallo, G. V. (2023). Substituting Allose as the Primary Carbon Source During Enrichment Helps Improve Detection and Isolation of Lineage II Listeria monocytogenes From Food. *Journal of Food Protection*, *86*(7), 100104. https://doi.org/10.1016/J.JFP.2023.100104

Vera-Peña, M. Y., Rodríguez, M. C., Francia, Y., & Valencia-García, E. (2019). Spray Drying of Lactic Acid Bacteria. *Ingeniería y Ciencia*, *15*(29), 179–213. https://doi.org/10.17230/INGCIENCIA.15.29.7

Vieco-Saiz, N., Belguesmia, Y., Raspoet, R., Auclair, E., Gancel, F., Kempf, I., & Drider, D. (2019). Benefits and inputs from lactic acid bacteria and their bacteriocins as alternatives to antibiotic growth promoters during food-animal production. *Frontiers in Microbiology*, *10*, 57. https://doi.org/10.3389/FMICB.2019.00057/BIBTEX

Vivek, K., Mishra, S., Rama, &, & Pradhan, C. (2021). Optimization of Spray Drying Conditions for Developing Nondairy Based Probiotic Sohiong Fruit Powder. *International Journal of Fruit Science*, *21*(1), 193–204. https://doi.org/10.1080/15538362.2020.1864567

Wang, N., Fu, N., & Chen, X. D. (2022). The extent and mechanism of the effect of protectant material in the production of active lactic acid bacteria powder using spray drying: a review. *Current Opinion in Food Science*, *44*, 100807. https://doi.org/10.1016/J.COFS.2022.01.003

Wang, Yan, Hao, F., Lu, W., Suo, X., Bellenger, E., Fu, N., Jeantet, R., & Chen, X. D. (2020). Enhanced thermal stability of lactic acid bacteria during spray drying by intracellular accumulation of calcium. *Journal of Food Engineering*, *279*, 109975. https://doi.org/10.1016/j.jfoodeng.2020.109975

Wang, Yaqi, Wu, J., Lv, M., Shao, Z., Hungwe, M., Wang, J., Bai, X., Xie, J., Wang, Y., & Geng, W. (2021). Metabolism Characteristics of Lactic Acid Bacteria and the Expanding Applications in Food Industry. *Frontiers in Bioengineering and Biotechnology*, *9*. https://doi.org/10.3389/fbioe.2021.612285

Guidelines for the Evaluation of Probiotics in Food, 11 (2002). http://www.fao.org/es/ESN/Probio/probio.htm

Xia, Y., Yuan, R., Weng, S., Wang, G., Xiong, Z., Zhang, H., Song, X., Liu, W., & Ai, L. (2020). Proteolysis, lipolysis, texture and sensory properties of cheese ripened by Monascus fumeus. *Food Research International*, *137*, 109657. https://doi.org/10.1016/J.FOODRES.2020.109657

Yadav, M., Sunita, & Shukla, P. (2022). Probiotic potential of Weissella paramesenteroides MYPS5.1 isolated from customary dairy products and its therapeutic application. *3 Biotech*, *12*(1), 9. https://doi.org/10.1007/s13205-021-03074-2

Yerlikaya, O., & Akbulut, N. (2020). In vitro characterisation of probiotic properties of Enterococcus faecium and Enterococcus durans strains isolated from raw milk and traditional dairy products. *International Journal of Dairy Technology*, *73*(1), 98–107. https://doi.org/10.1111/1471-0307.12645

Yun, P., Devahastin, S., & Chiewchan, N. (2021). Microstructures of encapsulates and their relations with encapsulation efficiency and controlled release of bioactive constituents: A review. *Comprehensive Reviews in Food Science and Food Safety*, *20*(2), 1768–1799. https://doi.org/10.1111/1541-4337.12701

Zhang, Y., Lin, J., & Zhong, Q. (2016). Effects of media, heat adaptation, and outlet temperature on the survival of Lactobacillus salivarius NRRL B-30514 after spray drying and subsequent storage. *LWT*, *74*, 441–447. https://doi.org/10.1016/j.lwt.2016.08.008

Zhang, Z., Peng, S., Sun, X., Jie, Y., Zhao, H., Zhu, B., Dziugan, P., & Zhang, B. (2020). A novel insight to screen the optimal spray-drying protectants and parameters for manufacturing lactic acid bacteria preparations. *Drying Technology*, *38*(14), 1843–1856. https://doi.org/10.1080/07373937.2019.1674323

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

Zotta, T., Parente, E., & Ricciardi, A. (2009). Viability staining and detection of metabolic activity of sourdough lactic acid bacteria under stress conditions. *World Journal of Microbiology and Biotechnology*, *25*(6), 1119–1124. https://doi.org/10.1007/s11274-009-9972-y