Susceptibility of foodborne pathogens to lactic acid bacteria isolated from Portuguese ready-to-eat raw fermented meat products

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// RESUMO

A segurança dos alimentos é uma das principais preocupações da indústria alimentar e das entidades reguladoras, devido às graves implicações para a saúde pública que o consumo de alimentos contaminados pode causar. As bactérias patogénicas de origem alimentar (BPAs) possuem elevada morbilidade e mortalidade especialmente em indivíduos susceptíveis. O controlo destes agentes patogénicos é crucial para garantir a segurança dos alimentos e reduzir o risco de surtos de doenças. Com o objetivo de melhorar a segurança dos alimentos há um interesse crescente na utilização de microrganismos ditos funcionais, como as bactérias ácido lácticas (BAL), na produção de alimentos fermentados. As BAL são amplamente utilizadas, em todo o mundo, na fermentação de alimentos devido ao seu potencial para inibir o crescimento dos agentes patogénicos. Os produtos cárneos fermentados prontos a consumir (RTE) são um importante grupo de alimentos em Portugal, sendo muito apreciados pelas suas características sensoriais. Estes produtos são produzidos através de fermentação natural, no entanto é importante avaliar a capacidade destas BAL para inibir agentes patogénicos de origem alimentar em condições reais de produção, para garantir a segurança microbiológica destes produtos. Por esta razão, o objetivo desta tese foi avaliar a capacidade das BAL, naturalmente presentes em enchidos crus fermentados RTE, como chouriças e linguiças, para inibir agentes patogénicos de origem alimentar, de forma a aumentar a segurança destes alimentos. Para tal, várias BAL foram isoladas destes produtos tradicionais portugueses, e caraterizadas nas suas propriedades antimicrobianas e de produção de metabolitos. Para tal, foram adquiridos setenta chouriços de catorze produtores artesanais da região nordeste de Trás-os-Montes. Em primeiro lugar, procedeu-se ao isolamento e à confirmação de bactérias lácticas e agentes patogénicos relevantes (*Salmonella* spp., *Listeria monocytogenes* e *Staphylococcus aureus*) presentes nos choriços. As BAL foram submetidas a testes de antagonismo (propriedades antimicrobianas), de acidificação, de atividade proteolítica e de produção de ácido lático. Os dados foram analisados por análise de componentes principais (PCA) e análise fatorial (AF), com o objetivo de sintetizar a informação fornecida pelos parâmetros tecnológicos, bem como pelas suas inter-relações. Com as metodologias utilizadas, foram isoladas 207 BAL, para testes posteriores foram selecionadas 33 BAL que apresentaram maior diâmetro de inibição contra a *L. monocytogenes*. O maior diâmetro de inibição contra foi de 37,29 mm, contra o *S. aureus* foi

de 16,9 mm e contra a *Salmonella* foi de 24,3 mm. De acordo com os resultados de inibição, as bactérias ML2CA (93), ML4CE (104), ML5CD (108) e AG3CC (18) apresentaram excelentes raios de inibição para os três agentes patogénicos testados. Estas BAL mostraram, também, uma boa capacidade de acidificação, especialmente a ML5CD, a qual reduziu o pH de 7,000 para 5,665 (uma redução de 1,340) após 24 horas de incubação. No entanto, em termos de atividade proteolítica, as BAL acima referidas não apresentaram grandes raios de proteólise, destacando-se a AG3CD, com um raio médio de 8,87 mm. As análises de PCA e AF, mostraram que a inibição da *L. monocytogenes* está intimamente relacionada com a capacidade de acidificação e com a concentração de ácido lático. Observou-se, também, que a inibição da *Salmonella* e do *S. aureus* estava relacionada com a atividade proteolítica e com a concentração de ácido lático. Observou-se, também, que a atividade proteolítica das BAL é independente da capacidade de acidificação, sendo as duas opostas no gráfico de correlação de variáveis. Ao analisar a correlação entre os locais e as propriedades microbiológicas, foi possível concluir que não existe uma tendência ou associação entre os produtores e as propriedades tecnológicas das BAL testadas. O potencial de acidificação parece não estar correlacionado com a rapidez de acidificação das BAL. Este facto sugere que as BAL com crescimento inicialmente lento são ainda capazes de atingir baixos níveis de pH no final do processo. Os resultados deste trabalho mostram que os produtos cárneos fermentados tradicionais são ricos em bactérias BAL, que ocorrem naturalmente e que contribuem para a segurança microbiológica destes alimentos. As BAL isoladas e testadas neste trabalho apresentam grande potencial tecnológico, pelo que importa estudar e compreender o seu comportamento e propriedades de inibição de agentes patogénicos em condições reais de produção.

Palavras-chave: Capacidade antimicrobiana; capacidade acidificante; atividade proteolítica; ensaios in vitro; ácido láctico; linguiça; salpicão; enchidos artesanais.

ABSTRACT

Susceptibility of foodborne pathogens to lactic acid bacteria isolated from Portuguese ready-to-eat fermented meat products

Food safety is one of the main concerns of the food industry and regulatory bodies, due to the serious implications for public health that the consumption of contaminated food can cause. Foodborne pathogenic bacteria (FABBs) have a high morbidity and mortality rate, especially in susceptible individuals. Controlling these pathogens is crucial to ensuring food safety and reducing the risk of disease outbreaks. In order to improve food safety, there is growing interest in the use of so-called functional microorganisms, such as lactic acid bacteria (LAB), in the production of fermented foods. LAB are widely used throughout the world in food fermentation due to their potential to inhibit the growth of pathogens. Ready-to-eat fermented meat products (RTE) are an important food group in Portugal and are highly appreciated for their sensory characteristics. These products are produced through natural fermentation, but it is important to evaluate the ability of these LAB to inhibit food-borne pathogens under real production conditions in order to guarantee the microbiological safety of these products. For this reason, the aim of this thesis was to evaluate the ability of LAB, naturally present in raw fermented (RTE) sausages, such as chorizos and linguiças, to inhibit food-borne pathogens, in order to increase the safety of these foods. To this end, several LAB were isolated from these traditional Portuguese products and characterized in terms of their antimicrobial properties and metabolite production. To this end, seventy chorizos were purchased from fourteen artisan producers in the northeast region of Trás-os-Montes. Firstly, the lactic acid bacteria and relevant pathogens (*Salmonella* spp., *Listeria monocytogenes* and *Staphylococcus aureus*) present in the chorizo were isolated and confirmed. The LAB were tested for antagonism (antimicrobial properties), acidification, proteolytic activity and lactic acid production. The data was analyzed using principal component analysis (PCA) and factor analysis (FA), with the aim of synthesizing the information provided by the technological parameters, as well as their interrelationships. With the methodologies used, 207 LAB were isolated, and for subsequent tests 33 LAB were selected that showed the greatest diameter of inhibition against *L. monocytogenes*. The largest diameter of inhibition against *L. monocytogenes* was 37.29 mm,

against *S. aureus* it was 16.9 mm and against *Salmonella* it was 24.3 mm. According to the inhibition results, the bacteria ML2CA (93), ML4CE (104), ML5CD (108) and AG3CC (18) showed excellent inhibition rays for the three pathogens tested. These LAB also showed a good acidification capacity, especially ML5CD, which reduced the pH from 7.000 to 5.665 (a reduction of 1.340) after 24 hours of incubation. However, in terms of proteolytic activity, the BAL mentioned above did not show large proteolysis radium, with AG3CD standing out with an average radius of 8.87 mm. The PCA and AF analyses showed that the inhibition of *L. monocytogenes* is closely related to the acidification capacity and the concentration of lactic acid. It was also observed that the inhibition of *Salmonella* and *S. aureus* was related to proteolytic activity and the concentration of lactic acid. It was also observed that the proteolytic activity of LAB is independent of the acidification capacity, the two being opposite in the correlation graph of the variables. By analyzing the correlation between the sites and the microbiological properties, it was possible to conclude that there is no trend or association between the producers and the technological properties of the LAB tested. The acidification potential does not seem to correlate with the acidification speed of the LAB. This suggests that initially slow-growing LAB are still capable of reaching low pH levels at the end of the process. The results of this work show that traditional fermented meat products are rich in naturally occurring LAB bacteria, which contribute to the microbiological safety of these foods. The LAB isolated and tested in this work have great technological potential, so it is important to study and understand their behavior and pathogen-inhibiting properties under real production conditions.

Keywords: Antimicrobial capacity; acidifying capacity; proteolytic activity; in-vitro assays; lactic acid production; *linguiça*; *chouriça*; artisanal sausages.

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1. Literature Review

1.1 Portuguese fermented sausages

Animal casing sausages made of seasoned meats, and let to ferment, emerged as a way to preserve food. As in many other countries around the globe, fermented sausages are one of the oldest processed foods representative of a Portuguese cultural heritage.

Sausages are meat products, which can be defined as processed products resulting from the transformation of raw meat in such a way that the cut surface to the eye no longer exhibits the characteristics of fresh meat (Regulation (EC) No. 853/2004). Moreover, the traditional Portuguese sausages have geographical origins, so that the product can be linked to the breed of animal, nature of the soil, vegetation, climate and by the production format.

In general, raw fermented sausages are prepared from seasoned pork and/or lean meat and pork fat that are stuffed in animal casings, let to ferment, and mature for some time (Lucke, 1998). Traditionally, this product can be further defined as made from a mixture of meat, fat in varying proportions and salt, possibly including, sugar, nitrates and/or nitrite (Fontana, Cocconcelli & Vignolo, 2005).

According to Demeyer (2004), fermented products have their specific properties (pH and water activity) due to the effect of bacterial metabolism. Due to the action of bacteria, raw fermented sausages reach a pH of 5.3 or less. In addition, by smoking and drying, between 20 and 50% of the water is removed, forming a product in which the water/protein ratio lies between 1.0 and 2.3 (Hui et al., 2004; Ockerman & Basu, 2007).

In this way, one can consider raw fermented sausages as products in which the color, aroma, and flavour are produced naturally by the meat and fat combination itself. It should be noted that the phenomena of meat coloration, acidification and protein hydrolysis are carried out by the specific microbial flora present; and are based on a series of biochemical, microbiological factors, physical and sensory changes, which develop under specific temperature and humidity conditions (Casaburi et al., 2007).

1.1.1 Types of sausages in Portugal

Portugal's traditional and artisanal sausages are one of the country's main gastronomic attractions, as they are regarded as distinctive culinary treasures guarded in every region of the country. Sausages are prepared in many ways and with different types of meats, seasonings, and spices, thus producing a wide variety of flavors. Taking into consideration the processing technology adopted for production, two major groups of fermented sausages can be differentiated: (1) dry cured, without adding water, in which the curing agents are solubilized only with the natural moisture of the meat; and (2) wet cured, in which the solubilization of the agents is done with the addition of water in order to form a saline solution, the brine (Flores, 1997).

According to Anonymous (2014), the main types of traditional sausages in Portugal include:

1. *Chouriças and linguiças*: are the most popular and well-known types of RTE raw fermented sausages in Portugal. They are made with pork, seasoned with pepper and other spices, and are usually smoked and dried. There are several variations of this type of sausages according to the region, yet all receive the generic term of chorizo.
2. *Alheira*: is a typical non-RTE sausage from Trás-os-Montes, Northern Portugal. It is made with poultry and pork meat, bread, olive oil, garlic, and other spices. Although it was traditionally a poultry/pork sausage, nowadays it is also produced with venison, rabbit and codfish meat.
3. *Morcela*: is a type of RTE sausage made with pig's blood, seasoned with onion, garlic, red wine, and spices. It is one of the most popular sausages in Portugal and is usually served with boiled potatoes.
4. *Farinheira*: is a non-RTE sausage made with wheat flour, pork fat and spices. It is traditionally served sliced, fried, or boiled, and is usually served with potatoes or beans.
5. *Paio*: is a type of non-RTE sausage made with pork, seasoned with garlic, red wine, and sweet bell pepper. It is usually smoked and dried; and is one of the most popular sausages in Portugal.

1.1.2 Composition of raw fermented sausages (chorizo)

Essential and optional ingredients used in the elaboration of Portuguese chorizo are shown in Table 1. According to NP 589 (2006), the traditional meat sausage must have a phosphate/protein ratio lower or equal to 0.03%.

Table 1. Ingredients and optional ingredients of chorizo (*chouriça* and *linguiça*)

	Essential Ingredients	Optional ingredients
Chouriça and linguiça	Pig meat Pig fat	Couratos, water, paprika, garlic, wine, blood in quantity strictly necessary to reinforce the color, salt, sugar (or dextrose), spices, flavorings, and liquid smoke (only for ordinary meat sausage and for extra meat sausage), proteins of animal and/or vegetable origin (only for ordinary meat sausage)

Source: Adapted from Inês Filipa Martins de Almeida (2009).

In Portugal, there are two qualities of chorizo sausages, thus, it is of interest to characterize the traditional Portuguese sausages according to their physical-chemical characteristics. Their differences in composition are shown in Table 2.

Table 2. Composition of extra meat sausage and normal meat.

	Extra meat chorizo	Normal meat chorizo
Free fat	Less than twice the total protein content	Less than three and a half times the total protein content
Total protein	Not less than 19%	Not less than 17%
Moisture content of defatted product	Lower than 65%	Lower than 65%
Collagen	Less than 13% of total protein content	Less than 30% of total protein content

Source: Adapted from Inês Filipa Martins de Almeida (2009).

According to Table 1, the traditional Portuguese chorizo has several components that provide the product flavour and aroma; whereas, according to the physicochemical characteristics presented in Table 2, chorizo sausage is a food rich in protein, yet the proportions of fat, moisture and collagen will define their quality.

1.1.3 Artisanal chorizo sausage

Artisanal Portuguese sausages are part of the national culture and ethnography, as well as being of great socioeconomic importance for the sustainability of rural communities and local economies, providing financial independence, employment, and autonomous livelihoods (Almeida, 2009). Portuguese chorizos are not only appreciated for their taste but also for their cultural significance, as they are strongly associated with rural life, the work in the countryside, and the traditions and feasts of rural communities (Cavignac, Dantas, 2021).

Traditionally, in Portugal, sausage production occurred mainly during the winter season, because climatic conditions, especially temperature, were more favorable for the preservation of meat products.

Sousa and Ribeiro (1983) defined Portuguese chorizo sausages as dry fermented raw sausages similar in composition and seasoning to Spanish chorizo, yet with coarser meat cuts and stuffed in thinner casings of no more than 22 mm caliber.

1.1.4 Production of the Portuguese chorizo

According to Incze (1998), the process of sausage production follows the following steps: selection of proper raw materials, seasoning of meat, preparation of batter, maceration, stuffing in casings, and maturation/drying.

The process of producing chorizo starts with selecting the appropriate cuts of meat, which are mainly pork meat with some fat. The meat mixture is usually made with a lean-to-fat ratio of 70/30 or 80/20. The meat and fat used in chorizo production come mainly from the pork belly, loin, and ham (Elias et al, 2006).

The meat is cut into small pieces and marinated with a paste made of garlic, *pimentão* (sweet paprika), and salt for 1 to 2 days. After this period, white wine is added to the marinade. The marinated meat mixture is then stuffed into natural casings, usually made from pig intestines. The hung sausages are then slowly dried over smoke and allow them to mature. The smoking process can take place in different ambient conditions, depending on the artisanal producer's preferences (Elias et al, 2006).

1.2.Microbiological safety of chorizo

In general, raw fermented sausages are considered safe meat products because of the reduction in water activity (a_w) and pH that occurs during processing and storage, which together inhibit the development of pathogenic microflora. However, it has been reported by several authors that raw fermented sausage and other meat products may contain, during processing and in the final product, some of the well-known pathogenic bacteria often associated with meat products such as: *Escherichia coli*, *Listeria monocytogenes*, *Salmonella* spp. *Staphylococcus aureus*, *Clostridium perfringens* (Moore, 2004; Santos et al., 2005; Ferreira et al., 2006, 2007; Siriken et al., 2006).

One of the most frequent pathogens found in chorizo is *Salmonella* spp. According to Martins et al. (2013), *Salmonella* spp. was isolated from 20.8% of the chorizo samples. The ingestion of this bacterium can provoke symptoms like nausea, vomiting, diarrhea, fever, and abdominal pain (Bula-Rudas; Mobeen; Nizar, 2005)

Other potentially hazardous pathogens that may be found in chorizo include *L. monocytogenes* and *S. aureus* (Gonzalez-Fandos et al, 2021). *L. monocytogenes* can cause listeriosis, a serious infection that primarily affects elderly people, pregnant women, and people with compromised immune systems; whereas *S. aureus* can cause a gastrointestinal intoxication that manifests itself with symptoms like nausea, vomiting, and abdominal pain. Therefore, it is crucial that chorizo and related raw fermented sausages are produced, handled, and stored appropriately to prevent proliferation of pathogenic bacteria.

1.2.1 *Salmonella* spp.

Salmonella is a type of bacteria that can cause gastrointestinal infections in humans. The majority of the time, transmission occurs when contaminated foods including meat, eggs, and dairy products are consumed (Abebe et al, 2020). Salmonellosis can have a range of mild to severe gastrointestinal symptoms, such as diarrhoea, abdominal cramps, vomiting, fever, and dehydration. In more severe cases, the infection may spread to other body parts and result in serious complications like septicaemia (OECD/FAO, 2019).

Salmonella is likely to be present in chorizos since they are made of raw meat and do not undergo strong heat treatment during processing. Contaminated casings can also be an entry point of this pathogens into the sausages. If the temperature is not properly controlled during the production process, *Salmonella* growth in chorizos may occur. Additionally, contamination may occur as a result of improper handling of ingredients or equipment (Beauchat et al, 2013)

1.2.2 *Listeria monocytogenes*

L. monocytogenes is a pathogenic bacteria that can lead to serious human illnesses. Listeriosis infection can result in fever, muscle pain, nausea, vomiting, diarrhea, and, in more severe cases, meningitis and septicemia. This bacterium has a high mortality rate and can be especially dangerous for vulnerable groups like pregnant women, new parents, elderly people, and people with compromised immune systems (Osman et al., 2018).

L. monocytogenes can be found in a variety of foods, such as processed and unprocessed meat, cheese, and lactose-free goods. Raw fermented sausages are one of the RTE processed foods that can support the growth of this bacterium, especially if it is not properly prepared and stored. For instance, a study by Khan et al. (2018) that evaluated 120 samples of chorizos isolated *L. monocytogenes* from over 30% of the samples. In addition, Verhegghe and De Reu (2015) revealed that as much as 13% of the chorizos produced in Portugal were positive for *L. monocytogenes*.

L. monocytogenes growth in sausage can occur at several stages of production. Contamination can occur at the start of a process when the raw material is handled (Mataragas et al., 2014). Additionally, storage at an improper temperature may encourage the growth of bacteria.

One of the crucial factors affecting the growth of *L. monocytogenes* is the chorizo's maturation time (Verhegghe and De Reu, 2015).

1.2.3 *Staphylococcus aureus*

According to the study of Anonymous (2014), *S. aureus*-induced food poisoning is typically characterized by the onset of gastrointestinal symptoms as nausea, vomiting, abdominal cramps, and diarrhea within a few hours of ingesting contaminated food. Gram-positive *Staphylococcus aureus* is a bacterium that can be found in fermented foods like chorizo and can cause food intoxication.

Many varieties of fermented sausages have been associated with outbreaks of *Staphylococcus* food poisoning (Lücke, 1985). *Staphylococcus aureus*, a prevalent bacterium present in minced meat blends and processing facilities (equipment, personnel, etc.), poses a risk due to its tolerance to salt and nitrites, as well as its ability to thrive across diverse environmental conditions. Under suitable conditions, this organism is capable of proliferating and producing enterotoxins, particularly during the initial fermentation phase.

Chorizo is a food that can provide nutrients necessary for *S. aureus* to multiply in relation to bacterial growth. Temperature plays a significant role in the development and proliferation of bacteria, with temperatures between 10 and 46 °C being ideal for the development and proliferation of *S. aureus* (Adams and Moss, 2008).

1.3 Lactic acid bacteria

Lactic acid bacteria (LAB) are a diverse group of microorganisms widely found in many natural environments. They have Gram-positive and catalase-negative characteristics and can present themselves as cocci or bacilli. They can be found in various nutrient sources, including the gastrointestinal tract, urogenital tract, fermented foods such as milk and dairy products, meat, grains, as well as in sewage and silage. They are widely used in the food industry, for fermented foodstuffs, such as fermented milks, yogurts, cheeses, fermented sausages and meats, and alcoholic beverages. LAB contribute to the preservation of food shelf life by lowering the pH and producing

peptides that antagonize other bacteria and modify the sensory properties of products (Reis et al, 2012).

With the increased search for safe and healthy foods, consumers have been looking for options that offer health benefits without the excessive use of chemical preservatives. Some LABs can be used as probiotics, conferring health benefits to consumers when added to food. In animal husbandry, LABs act as probiotics and have immunomodulatory effects, and can be used as alternatives to antibiotics and to increase mucosal protection.

According to Chakraborty et al. (2020), LAB can modulate the immune system, improve digestion and nutrient absorption, and prevent infectious and inflammatory diseases. In addition, these bacteria can produce enzymes, peptides, and organic acids with antimicrobial, antioxidant, and anti-inflammatory activity.

Lactic acid bacteria play an important role in the fermentation and maturation of chorizo. According to the literature, different types of lactic acid bacteria can be naturally found in chorizo, depending on the production method and the geographical region. A study by Pintado et al. (2009) analyzed the microbial flora present in Spanish chorizo. The authors identified a diversity of LAB, including *Lactobacillus sakei, Lactobacillus plantarum* and *Lactobacillus curvatus*, among others. The results indicated that the fermentation and maturation of chorizo were influenced by the presence of these bacteria, resulting in characteristic texture and flavour.

Mora et al. (2013) compared different types of chorizos produced in Spain, Portugal, and France. The authors identified a large variability in the bacterial flora present in each type of chorizo, including lactic acid bacteria. The results indicated that processing conditions and the raw materials used are important factors in determining the microbial composition of chorizo.

In Barbosa et al. (2019), *Lactobacillus plantarum* and *L. sakei* were identified in Brazilian chorizo. Furthermore, they indicated that the microbial composition of Brazilian chorizo is similar to that of chorizos produced in Portugal and Spain.

Rattanachomsri et al. (2019), evaluated the probiotic potential of *L. plantarum* strains isolated from traditional Thai foods. The results indicated that these bacteria exhibited inhibitory activity against human pathogens, produced digestive enzymes and antioxidants, and had a positive effect on gut function in an animal model.

Depending on the production method, geographical region and processing conditions, different types of lactic acid bacteria can be found in chorizo. Understanding the microbial composition of

chorizo is important to develop bio-intervention strategies that enhance the safety and quality of these products.

1.3.1 Lactobacillus sakei

L. sakei is a Gram-positive, facultative anaerobic bacterium, which is present in meats and fermented products such as salami and chorizo (Drosinos et al, 2005). This bacterium is responsible for important applications in the food industry, such as the production of probiotics and food preservation. It is also used in the preparation of foods in a natural way, without chemical additives (Yu, 2020).

1.3.2 Lactobacillus plantarum

L. plantarum is a lactic acid bacterium that is widely distributed in the environment. This bacterium is known for its ability to ferment plants and vegetables and plays an important role in the production of fermented foods. In addition, *L. plantarum* is a probiotic bacterium with several beneficial properties for human health, such as improving digestion and preventing diseases of the gastrointestinal tract (Al-Tawaha, Rahman, and Chen Meng, 2018).

1.3.3 Lactobacillus curvatus

L. curvatus is a Gram-positive bacterium that is used in the production of fermented foods, especially in salami manufacturing. *L. curvatus* is known for its ability to inhibit human pathogens, making it an important bacterium for food safety (Chen et al , 2020).

1.4 Antimicrobial Potential of LAB

The antimicrobial potential of LAB is attributed to several substances produced by these bacteria, such as lactic acid, hydrogen peroxide, carbon dioxide, diacetyl, acetaldehyde and bacteriocins. Bacteriocins are compounds produced by LABs with biopreservative action, which have antimicrobial activity, physical stability, and low toxicity, and can be used to control food spoilage and the development of pathogenic bacteria (Passos et al, 2009).

1.4.1 Bacteriocins

Bacteriocins are proteins or peptides produced in the ribosome and released into the extracellular environment. These molecules have bactericidal action on Gram-positive bacteria, such as *L. monocytogenes, Clostridium botulinum, Bacillus cereus* and *S. aureus*, which are pathogens present in food. Inhibition by bacteriocins occurs through the formation of pores in the cell membrane, causing destabilization and change in the permeability of sensitive cells (Lucke, 2000).

The various bacteriocins have variations as to activity, mode of action, genetic origin, molecular weight, and biochemical properties (Deegan et al., 2006). There are two stages for the interaction between the bacteriocin and the cell to occur, the first being the adsorption of the bacteriocin on the cell membrane of a sensitive cell. In this first part, no damage is caused to the cell and the adsorption is reversible. In the second step, the bacteriocin has irreversible lethal damage to the cell. Thus, the inhibitory activity of these metabolites is restricted to gram-positive bacteria, since gram-negative bacteria have an outer membrane impermeable to these molecules (Abee; Krockel & Hill, 1995).

1.4.2 pH and phenolic compounds

A study by Lopez et al. (2018) investigated the change in pH and the concentration of phenolic compounds generated by LAB during chorizo fermentation. Three LAB strains (*L. sakei, L. curvatus,* and *Leuconostoc mesenteroides*) were evaluated under different temperature and humidity conditions. The results showed that fermentation promoted a reduction in the pH of the sausage, indicating the metabolic activity of LABs. In addition, several phenolic compounds were identified, p-hydroxybenzoic acid being the most abundant in all samples.

However, the concentrations of phenolic compounds varied according to the LAB strain and the fermentation conditions. The *L. sakei* strain produced the highest concentrations of phenolic compounds, while *L. curvatus* produced the lowest concentrations. Variation in humidity and temperature also affected the production of phenolic compounds, with the highest levels being achieved at lower temperature and higher humidity conditions.

Due to their antimicrobial activity, the phenolic compounds produced by LABs during chorizo fermentation may help extend the shelf life of the product. However, high levels of phenolic compounds can make chorizo bitter and impair its consumer acceptance. Therefore, it is important to carefully monitor chorizo fermentation and adjust fermentation conditions to ensure product quality and safety.

2. Objective

The objective of this thesis was to evaluate the ability of indigenous LAB bacteria present in Portuguese RTE raw fermented sausages, such as *chouriças* and *linguiças*, to inhibit foodborne pathogens, with views to increasing the microbiological safety of these products through the future development of functional starter cultures. This objective was achieved by isolating a pool of LAB strains from Portuguese raw fermented sausages elaborated across the production zones; and subsequently by screening them for their antimicrobial and metabolite production properties. In parallel, foodborne pathogens, such as *Salmonella spp., L. monocytogenes*, and *S. aureus*, were also investigated in these products.

3. Methodology

To achieve the proposed objectives, the steps shown in Figure 1 were implemented.

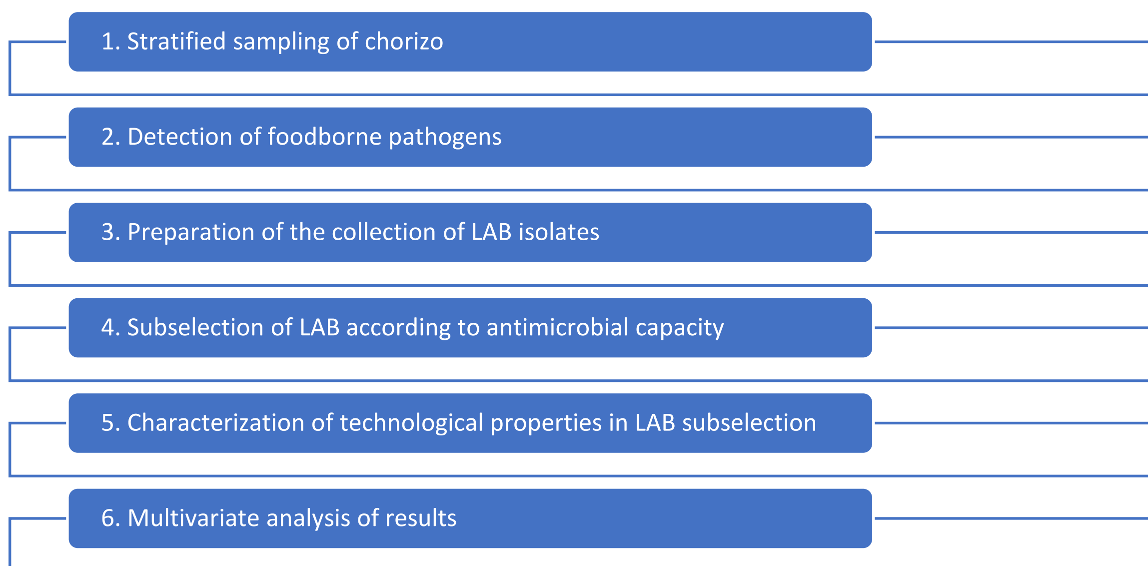

Figure 1. Methodological phases established for the development of the current research work.

As shown in the flow diagram, the methods, tests and experiments were organized in a way that the presence of foodborne pathogens were first investigated, and LAB strains were isolated from the samples. Afterwards, the inhibition diameters produced by each of the LAB isolates against *L. monocytogenes* were measured, and a subselection of the most promising LAB in terms of antimicrobial capacity was selected. To this subset of LABs, the inhibition diameters against *Salmonella* and *S. aureus* were measured as well as the technological properties such as acidification capacity and proteolysis activity. Finally, the full data of the subselected strains were appraised by multivariate analysis.

3.1 Collection of RTE raw fermented sausages from different regions of Portugal

Seventy chorizos (locally referred to as *chouriças* and *linguiças*) from fourteen different artisanal producers establishments located in the towns of Vila Flor, Bragança, Mirandela, Mogadouro, Miranda do Douro, Vimioso, and Vinhais, all of them belonging to the Portuguese northeastern region of Trás-Os-Montes, were purchased. It was known a priori that these establishments are fully artisanal and do not employ starter cultures in the elaboration of their products. To elaborate the location codes, the first two or three initials of the producer name were used, followed by the sample number, which run from 1 to 5; for example AG1, AG2, AG3, AG4 and AG5. For the identification of the samples after the isolation of the LAB colonies, since 5 colonies per sample were isolated, each isolate was identified as colonies A to E; for instance, AG1CA, AG1CB, AG1CC, AG1CD and AG1CE. Table 3 compiles the producers' names, location and code used.

Table 3. Producers' names, location and codes

Producer	Location	Code
Alheiras da Glória	Vila Flor	AG
Deolinda Rodrigues	Vinhais	DR1
Bísaro do Planalto	Mogadouro	BP
AutentiSereia	Vimioso	ATS
PingoD'Orvalho	Miranda do Douro	PDO
Fumeiro da Vareira	Mirandela	FV
Maria Laura	Mirandela	ML
Sofrança	Bragança	SF
Adília Cruz	Vinhais	AC
Isabel Rijo	Vinhais	IR
Alheiras da Judite	Mirandela	AJ
Fumeiro Brízido	Mirandela	FB
Provei e Gostei	Bragança	PG
Salasicharia S.José	Carrazeda de Ansiães	SJ

The location of the Trás-os-Montes region is shown in Figure 2, to illustrate the proximity of the artisanally produced chorizos that were used in the present study.

Figure 2. Map of chorizo producers of Trás-os-Montes region (Taken from Fernandes et al., 2024)

By the Figure 2 and the results obtained it was possible to compare if there was a correlation between the LAB properties of each chorizo and the location of the production.

3.2 Detection of foodborne pathogens: of Salmonella spp., Listeria monocytogenes, and Staphylococcus aureus.

Purchased chorizos were kept under refrigeration (3-4 ºC) and analyzed in batches of 10 units. First, with sterilized material, chorizos were cut into small pieces. Then, twenty-five grams of the sample was mixed with 225 mL of buffered peptone water (BPW, NEOGEN, UK) in a sterile bag.

3.2.1 *Salmonella* spp

The homogenate was incubated at 37°C for 24 h. After incubation, 0.1 ml of BPW were transferred into 10 ml of Rappaport-Vassiliadis Soya Broth (RVS, Liofilchem, Italy) and 1 ml into 10 ml of Muller Kauffmann Tetrathionate Novobiocin Broth (MKTTn, Liofilchem, Italy). RVS tubes were incubated at 42 °C for 24 h and the MKTTn tubes at 37 °C for 24 h .A loopful of RVS

and MKTTn culture were streaked on Xylose Lysine Desoxycholate Modified Agar (XLD, Alliance Bio Expertise, France), Bismuth Sulfite Agar (BSA, Sigma-Aldrich, USA) and Hecktoen Enteric agar (HE, Liofilchem, Italy); and plates will be incubated at 37°C/24 h.

Typical colonies of *Salmonella* from each selective agar were selected and submitted to biochemical and serological confirmation. For purification, colonies were streaked onto the surface of Nutrient Agar (NA, Liofilchem, Italy) plates and incubated at 37 C/24 h.

3.2.2 *Listeria monocytogenes*

For detection of *Listeria*, first 25 g of sample were homogenized in 225 mL Demi Fraser broth (Liofilchem, Italy), and incubated at 30°C for 24 hours. After incubation, 0.1 mL of the homogenate were transferred to 10mL of Fraser broth (Liofilchem, Italy) and placed in a 37°C oven for 48 hours.

After this period, plates containing *Listeria* Oxford agar (Liofilchem, Italy) were streaked in duplicate. These were be incubated at 37ºC for 24 hours.

At the end of the incubation time, plates were observed for the presence of greenish-brown colonies with black halo. In case of presence, colonies werestreaked on Trypticase Soy Agar with 0.6% Yeast Extract agar (TSA-YE, Liofilchem, Italy). These plates were incubated at 37°C for 24 hours.

Finally, any blue colonies that may grow on the plate were confirmed with the Listeria biochemical confirmation kit (Listeria System- 18- R, Liofilchem, Italy).

3.2.3 *Staphylococcus aureus*

Volumes of 0.1 mL of the original homogenate, and ten-fold dilutions were spread onto petri dishes containing Baird Parker (BP) agar (Biolife, Italy). From each plate showing growth, at least one presumptive colony was selected and confirm, as described in the method APHA (2001). If there were colonies with a well-defined double halo, at least 3 colonies were selected and transferred to a tube with 9 mL of buffered peptone water at 37º C/24h with agitation. If the

medium darkened, it was spread on nutrient agar plates. Then the isolated colonies were transferred to cryotubes with glycerol and sterilized distilled water to further identification.

3.3 Preparation of the collection of lactic acid bacteria isolates

In addition, on the same day, physicochemical factors such as pH and water activity (aw) were analyzed. For the determination of pH, ten grams sample were added with 90 ml distilled water and liquefied for 30 seconds. Three pH measurements were taken from the liquid sample, using a pH meter (FiveGo F2, Mettler Toledo, China). The samples for aw determination were analyzed in duplicate using the Aqualab equipment (Dew Point Water Activity Meter 4TE, AQUALAB, USA).

From the original homogenate, one mL of appropriate dilutions were inoculated to petri dishes, and then 15 mL of MRS or M17 agar (Liofilchem, Italy) was added using the incorporation technique. After solidification of the medium, 10 mL of 1.2% agar (Liofilchem, Italy) was poured to generate anaerobiosis to the inoculated bacteria. Finally, these plates were sealed with parafilm and incubated at 30ºC for 48 hours.

After the incubation period, the colonies were counted. Then, five typical MRS colonies and 5 M17 colonies per sample were selected and isolated on plates divided in half by streaking. These streaked colonies were incubated at 30° for 48 hours.

The next step was to confirm these bacteria, which was done by two methods, Gram stain and the catalase test (Brasil, 2018; Silva et al., 2013). To refine LAB isolates selection, catalase (3% hydrogen peroxide) and Gram tests, as well as morphologic observation, were performed.

After confirmation, every isolated and purified colony was transferred into a tube containing 10 mL of MRS broth and placed in an incubator at 37º for 24 hours. After this period, with the tubes turbid and containing the activated colonies, the last step was the transfer to the cryotubes, which had been previously labelled with an in-house code. Then, a volume of 500 µL of culture and 500 µl of 50% glycerol were added to a cryotube. These cryotubes were vortexed and then stored frozen at -80°C. For every isolated strain, two cryotubes for preservation were obtained.

3.4 Characterization of LAB for their antimicrobial properties.

The antimicrobial ability of the strains was evaluated as described by Campagnollo et al. (2018), using the spot-on-lawn assay. For the antimicrobial assays, *Staphylococcus aureus* ATCC 6538, *Salmonella enterica* subsp. *enterica* serovar Typhimurium ATCC 43971 and *Listeria monocytogenes* WDCM 00019 from the Polytechnic Institute of Bragança stock collection were used. First of all, the spot-on-lawn essay was carried out to measure the antimicrobial activity of all the LAB isolates against *L. monocytogenes*. With basis on these results, a subset of at least 30 LAB isolates with the best inhibitory capacity against *L. monocytogenes* was selected. *L. monocytogenes* was chosen because it is of greater interest from the point of view of compliance to the microbiological criterion for chorizo as an RTE food (able to support the growth of this pathogen). It is then on this subset of LAB isolates that the spot-on-lawn assays for *S.* Typhimurium and *S. aureus* were conducted.

Pathogens were kept on Nutrient Agar slants throughout the experiment. To activate them, a loop was taken from a slant, and inoculated in BHI broth (10 mL). Broth tubes were incubated for 16 h at 37 °C, following two consecutive inoculations, to reach approximately 10^8 CFU/mL. *L. monocytogenes* required a pre-activation in 5 mL of BHI at 37 °C for 16 h.

Each LAB isolate was cultured separately from the frozen stock into 10 mL of MRS broth (37°C, 24 h), then spotted (3 uL) onto the surface of MRS or M17 agar plates (into the same media of isolation, respectively) divided in four quadrants (quadruplicate), as shown in Figure 3.

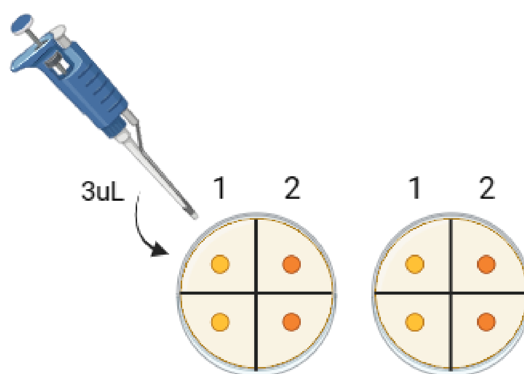

Figure 3. Application of lactic acid bacterium inoculum onto MRS agar plates

Then, the plates were covered with 10 mL of BHI broth with 0.75% (w/v) bacteriological agar seeded with 1 mL of each pathogenic strain (separately) at approximately 8 log CFU/mL. After pre-incubation at 4 °C for 2 h followed by incubation at 37 °C for 16 h , the inhibition zones or diameters were measured with a caliper The inhibition diameter (ID) was compared to LAB colony diameter (Dc), as shown in Figure 4, when looking for zones of inhibition in the pathogen cell lawn on the plates. IDs were then obtained by the difference of the diameter of the halos measured, and they had to be well marked and clear to measure. If halos (diameter) were not conclusive, the assay was repeated. For every strain, four replicates of ID in mm were at least obtained.

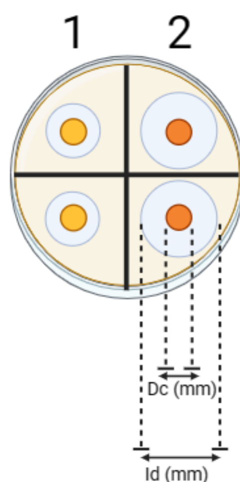

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Figure 4. Measurement of inhibition diameter

3.5 Characterization of technological properties in LAB subselection

In order to characterize the technological properties of the LAB subselection (33 isolates selected), three assays were carried out: acidifying activity, proteolytic activity and production of lactic acid in broth.

3.5.1 Acidifying and proteolytic activity

With a few minor adjustments, acidifying and proteolytic activity were measured as per Durlu-Ozkaya et al. (2001) and Franciosi et al. (2009), respectively. A chosen subset of 33 LAB stock strains was cultured in sterile reconstituted skim milk (10% w/v) supplemented with yeast extract (0.3% w/v) and glucose (0.2% w/v) for two successive subcultures (30°C, 24 h) in tubes of 10 mL, before being used for the acidification profiling. One mL of an active culture was added to 100 ml of sterile reconstituted skim milk and them went for incubation at 30°C for 24 hours, as shown in Figure 5.

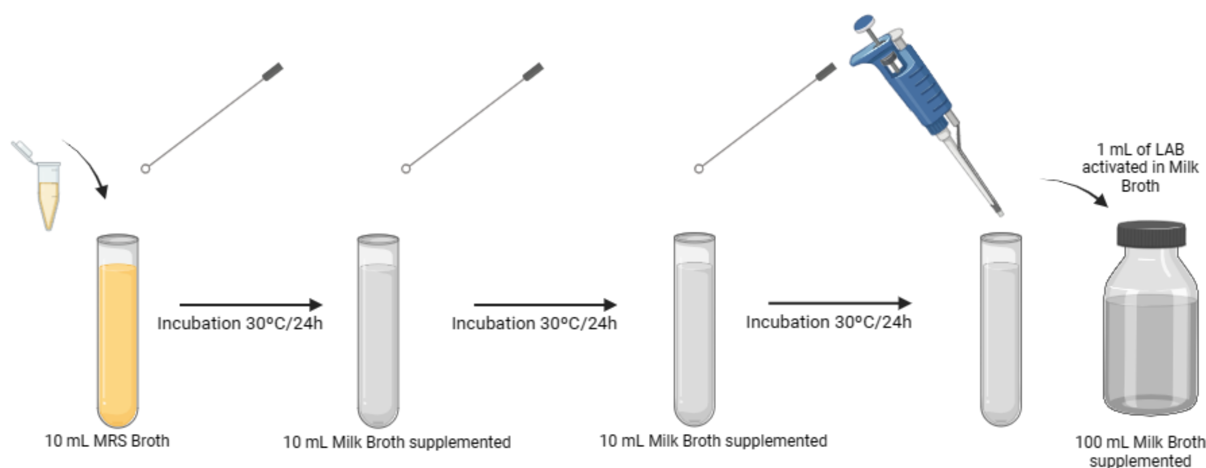

Figure 5. Activation of LAB strains in milk broth for the test of acidification

During the incubation, the pH was assessed every two hours for six hours (t = 0, 2, 4, 6 h) and then again at 24 hours after inoculation, in duplicate. To determine each strain's capability for acidification, pH measurements over time were fitted to a decline curve, to estimate acidification descriptors. Fitting of pH curves are explained in Section 3.6.1.

On the same day of the measurement of pH, the exocellular proteolytic activity was assayed in quadruplicate, as shown in Figure 6.

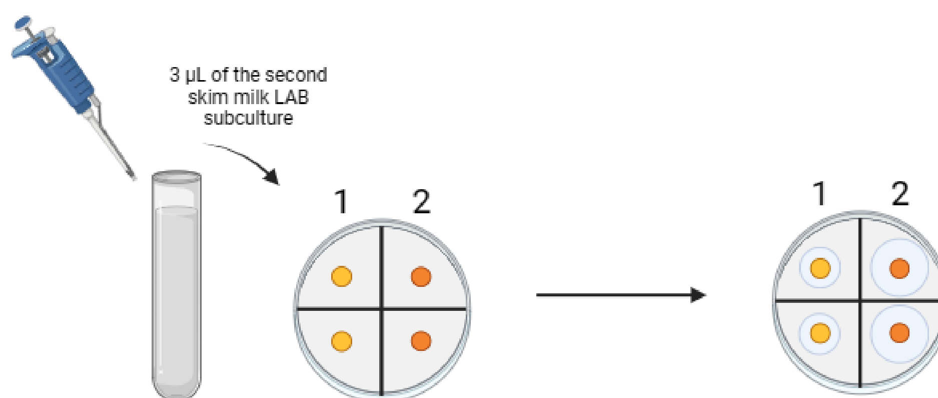

Figure 6. Assay for exocellular proteolytic activity.

A volume of 3 µL of the second skim milk LAB subculture was dotted onto the surface of reconstituted skim milk (3% w/v) agar (1.5% w/v), divided in 4 quadrants (Figure 6); and incubated at 35°C for 4 days. Around the colonies, proteolytic activity was identified as a clear zone, and the diameter of these halos were measured in mm. A value of zero was assigned to LAB isolates that did not produce any clear halo.

3.5.2 Characterization of LAB bacteria to determine the lactic acid concentration

The 33 LAB stock strains selected were initially cultivated in 10 mL of MRS broth (30°C, 24 h) in order to determine the concentration of lactic acid [LAC] produced at the end of a given incubation period. Depending on the source media, a loop of the inoculated broth was streak plated onto MRS or M17 agar, and incubated at 30°C for 48 h. To attain a density of approximately 0.5 McFarland, two or more well-isolated colonies were diluted in 5 mL of sterile saline solution and vortexed to homogenize the suspension. A spectrophotometer was used to confirm the absorbance at 625 nm, ensuring a value of 0.08 to 0.13. If the value was superior to 0.13 (too concentrated) it was necessary to dilute with saline solution, or if it was too diluted (lower than 0.08) one more colony needed to be added, then vortexed again and measured to ensure that the value was in the range required.

One thousand µL of the adjusted solution was added to a tube containing 4 mL of MRS broth, and the tube was then incubated at 30°C for 4 hours. Next, 1 mL was pipetted and transferred to a

microtube, which was centrifuged at 13,000 rpm for 5 minutes. After centrifugation, 10 uL of the supernatant was diluted in 500 µL of sterile distilled water in a microtube and vortexed. Following the directions on the lactic acid determination kit (NZYTech, Portugal), this suspension was used to estimate the L-lactic acid concentration, and, in this manner, quantify the capacity of every selected LAB strain to produce lactic acid.

By obtaining the absorbances at 340 nm of the blank and the samples, it was possible to calculate the according to Equation (1),

$$C(L - latic\ acid) = 0.3204 * \ \Delta A_{L-latic\ acid} \qquad \text{Equation (1)}$$

where $\Delta A_{L-latic\ acid}$ is the difference between the blank and the target sample.

3.6 Statistical analysis

Two statistical analyses were carried out on the data obtained in this work: (1) the fitting of non-linear model (i.e., Weibull decay model) to the acidification capacity data of each strain; and (2) the multivariate modelling of the technological properties of the selected LAB (i.e., principal component analysis, factor analysis and cluster analysis).

3.6.1. Modelling the pH drop in broth medium

The Weibull function finds widespread application in reliability engineering, with numerous parameterizations available (Murthy et al., 2004). In predictive microbiology, Weibull models have gained popularity due to their simplicity, ease of fitting, and ability to model both concave and convex curves (Couvert, Gaillard, Savy, Mafart, & Leguerinel, 2005).

Data on pH drop obtained from the acidifying capacity experiments were modelled using a Weibull function of three parameters,

$$pH(t) = pH_0 \times exp^{\left[-\left(\frac{t}{k}\right)^P\right]} \qquad \text{Equation (2)}$$

where: the scale and shape parameters of the underlying Weibull distribution are k and P, respectively. If shape parameter $P > 1$, convex curves are obtained, and if $P < 1$, concave curves are represented. Although the Weibull model is basically empirical, Van-Boekel (2002) suggested that $P < 1$ presumes that the microorganisms at any point in the curve display the capacity to adapt to the applied stress (a medium every time more acidified), whereas $P > 1$ indicates that the remaining cells become increasingly susceptible to pH. The parameter k is called scale parameter (a characteristic time). pH is defined as above, and the parameter pH0 represents the initial pH, and t represents the time in hours.

For every LAB isolate, the following descriptors were extracted from the fitted curves: ΔpH02: pH decrease between $t = 0$ h and $t = 2$ h; ΔpH06: pH decrease between $t = 0$ h and $t = 6$ h; ΔpH26: pH decrease between $t = 2$ h and $t = 6$ h; and pH6: pH at $t = 6$ h. The non-linear Weibull model was adjusted using the nls function in the R software (version 3.6.2, R Foundation for Statistical Computing, Vienna, Austria).

3.6.2. Multivariate analysis of technological properties of LAB

The data on the technological properties obtained from the subset of 33 strains was subjected to three types of multivariate analysis: principal component analysis, factor analysis and cluster analysis, with the objectives of (1) understanding any interrelationship between variables; (2) reducing the dimensionality of redundant variables; (3) appreciating the diversity of lactic acid bacteria within the produced technological properties maps; and (4) evaluating any effect of the geographical region of production of chorizos on the clusters of lactic acid bacteria thereof isolated.

a. Principal component analysis

Principal Component Analysis (PCA) was conducted to assess the influence of antimicrobial, proteolytic, and acidifying properties on the differentiation of isolates. A solution for three principal components was adopted in order to account for at least 60% of the total variance of the multivariate data. Subsequently, two bidimensional PCA maps representing the antimicrobial,

acidifying, and proteolytic characteristics of chorizo were generated by projecting sample scores onto the principal components' span. The function prcomp from the factoextra package was used in the R software (version 3.6.2, R Foundation for Statistical Computing, Vienna, Austria).

b. Cluster analysis

The cluster analysis was carried out using two methods: k-means clustering and hierarchical clustering. The function kmeans and the function hclust implemented in the cluster package were used. A solution for two clusters or groups were chosen according to the elbow method, which was carried out using the function fviz_nbclust from the factoextra package. The R software was used (version 3.6.2, R Foundation for Statistical Computing, Vienna, Austria).

c. Factor analysis

Factor analysis was used to know whether the covariances between a set of variables could be "explained" in terms of a smaller number of latent (unobservable) variables or common factors. Understanding these results enables the analysis of correlations between factors and properties to identify commonalities and diagnostic insights.

By applying the factor analysis, the following statistics were obtained:

-Factor loadings (R), which are correlation coefficients indicating the relationship between factors and variables. Higher loadings imply that the variable is more representative of the factor.

Commonality (H^2), which measures the proportion of variance in each variable that is accounted for by the factors.

Singularity (U^2), also known as noise, which represents the portion of variance in each variable that remains unexplained by the factors.

Eigenvalues: Eigenvalues indicate the amount of variance explained by each factor.

The factor analysis was carried out using the function fa() from the psych library in the R software (version 3.6.2, R Foundation for Statistical Computing, Vienna, Austria).

4. Results and Discussion

Whereas the presence of the two foodborne pathogens, *Salmonella* spp. and *L. monocytogenes* chorizo samples could not be detected (95% confidence interval: 0.00 – 4.74%), *S. aureus* was below the limit of quantification; this is <1.7 log CFU/g). A high number of LAB isolates were obtained. This collection constituted the LAB bank which became the basis of our research work. In addition, positive results regarding the sought properties and antimicrobial capacity of LAB against foodborne pathogens potentially present in chorizo were found. The next Sections deal with such results.

4.1 Isolation and confirmation of LAB

By culturing, a total of 380 colonies grown on MRS or M17 were isolated. Approximately, 70% of these were gram positive, catalase negative and of typical morphology, therefore totalizing a bank of 267 presumptive LAB. Figure 7 shows an example of a microscope view that was considered as gram positive for presenting the purple coloration. Under the microscope, it was also possible to evaluate the shape of the cells. Samples with morphological shape of cocci or bacilli were considered for inclusion in the bank of LAB. A percentage of 77.5% LABs (207/267) were isolated on MRS whereas 22.5% (60/267) on M17.

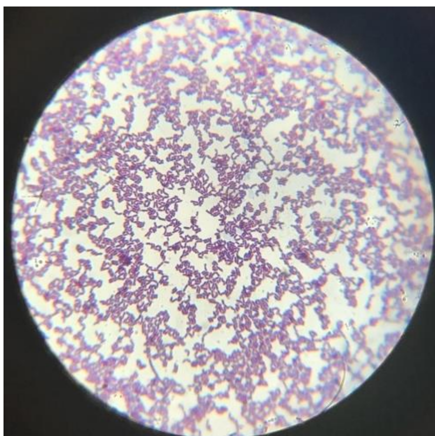

Figure 7. Microscopic view of gram positives bacteria

4.2 LAB Antimicrobial Properties Against *L. monocytogenes, Salmonella* and *S. aureus*

The totality of isolated LABs was tested for their in-vitro antagonism against *L. monocytogenes.* In the majority of cases, the halos formed were well defined and visible, facilitating therefore the procedure of measuring them with the caliper (Figure 8). In Figure 8(A), the bacteria code #190 had a smaller halo of inhibition, while the one with code #191 had a much larger halo of inhibition, thus representing the different inhibition potential of each isolated LAB. In Figure 8(B), the isolate #183 did not have an inhibition halo, which means that it was very likely that this strain was not effective in inhibiting *L. monocytogenes*. While on the right side of the same plate, an example of the outside halo marking is shown for isolate #182.

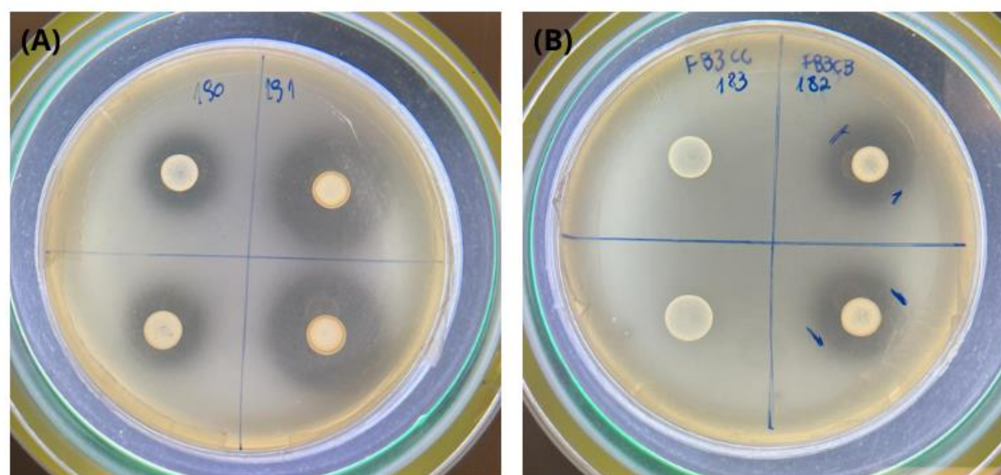

Figure 8. Inhibition halos against *Listeria*

The repeatability reached by the protocol was also very high, with very low standard deviation in inhibition halos between the four replicates. This can be observed in Figure 9, which shows quadruplicate halos for 6 LAB isolates. The halos from the six isolates (#70, 71, 86, 87, 186 and 187) in front of the light had clear and visible margins, so that the colony and inhibition diameters could be measured properly. Also, obtaining very similar results in the quadruplicates confirm that the technique was well executed.

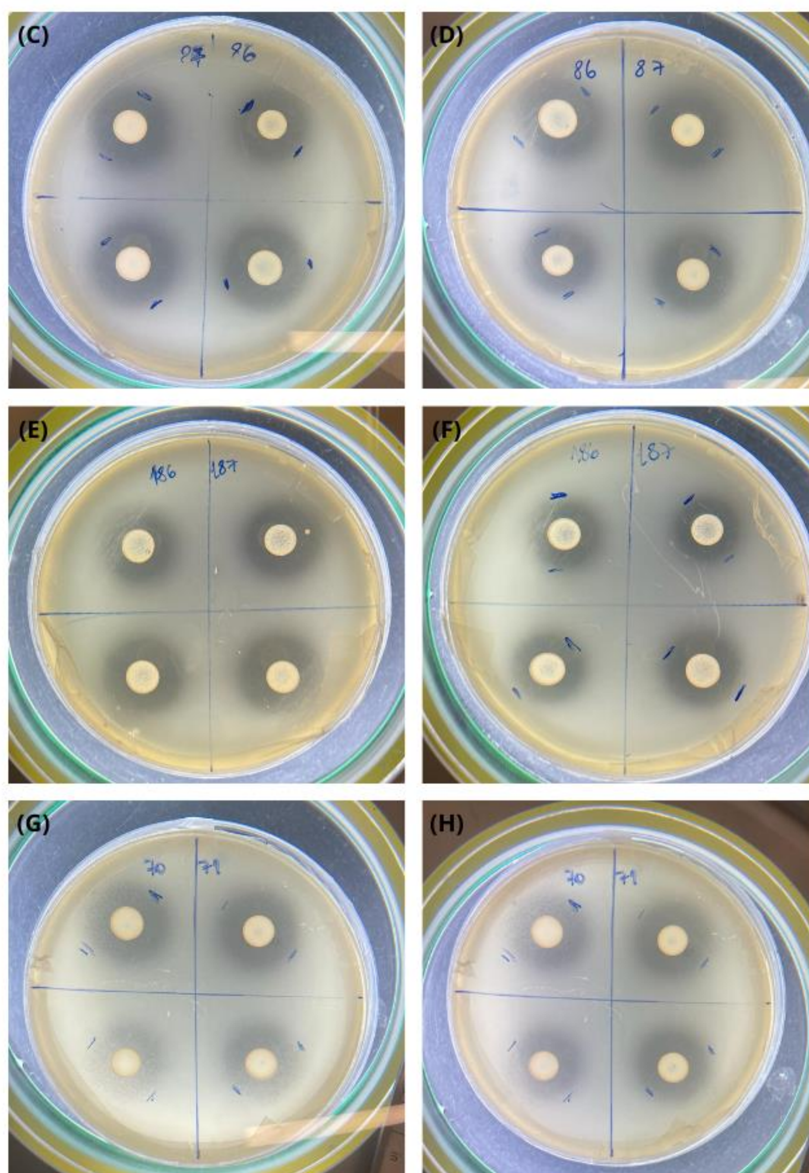

Figure 9. Inhibition halos in quadruplicate for six LAB isolates

Figure 10 shows the inhibition halos in mm in decreasing order. The mean and standard deviation of the halos were 11.48 mm and 2.65 mm, respectively. They ranged from 37 mm to 0 mm of inhibition. The largest halo was 37.29 mm for the LAB code ML2CA (#93), followed by the ML5CD (#104) with 23.34 mm of diameter.

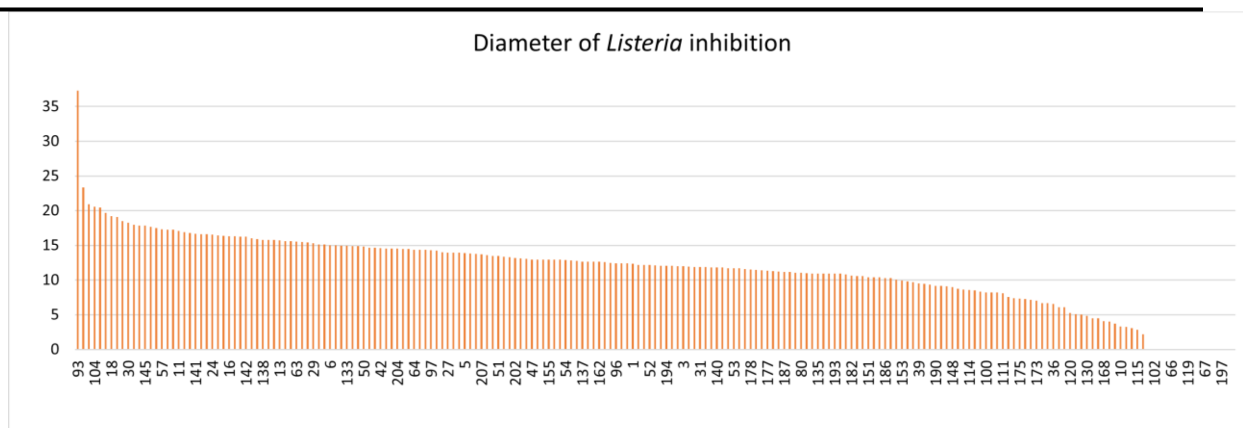

Figure 10. Susceptibility of *L. monocytogenes* (diameter in mm versus LAB number).

In Benito et al. (2007), culture filtrates of 3 LAB isolates from industrial salchichón and chorizo samples showed weak antimicrobial activity against *L. moncytogenes* with clearing zones between 5 and 7 mm. If we were to assume that diameters until 7 mm are weak in inhibition, then 19% of the LAB tested in our study could be considered as having negligible inhibition properties.

In comparison with the study carried out by Perin (2011), in which the same spot-on-the-lawn methodology was used, the largest diameter obtained by *Lactococcus* spp. was 30 mm, and the smallest value was 7 mm of inhibition against *L. monocytogenes*. This shows that the LAB tested in the present study, have similar inhibition halos to *Lactococcus* spp, which is a LAB species known for having some strains with capability of pathogen inhibition. These results were encouraging as the LAB isolated from artisanal Portuguese chorizo may have good potential for inhibiting pathogens, thereby paving the way for further studies as biopreservatives in food.

Andrade et al. (2014) evaluated the antagonistic activity of *Lactobacillus* spp. isolated from Minas cheese against *L. monocytogenes*, and obtained inhibition halos between 19.25 and 30.53 mm, while in De Marco (2018), 26.66% of the LAB isolated in sausage showed antagonistic activity against the pathogenic microorganisms tested, presenting halos in the range of 19.00 and 21.75 mm for *L. monocytogenes*. Of the results obtained in this study, eleven LAB isolates presented inhibition values greater than 19 mm; such big difference can be explained by the environment of the food in which it is isolated, since Portuguese chorizos present very high salt content, low pH and very low water activity.

Compared to Tönz (2024), of the 274 LAB strains tested against *L. monocytogenes*, the author considered that 51 (18.6%) showed the most promising anti-*Listeria* activity in agar stain

assays because they had halos greater than 14.9 mm. In our study, based on a threshold of 16 mm, thirty-three LAB isolates were selected as promising, which represents a fraction of 16%, comparable to that of Tonz (2024) (18.6%). To this subselection of LAB, the in vitro antimicrobial capacities against *S. aureus* (Figure 11) and *Salmonella* (Figure 12) were measured.

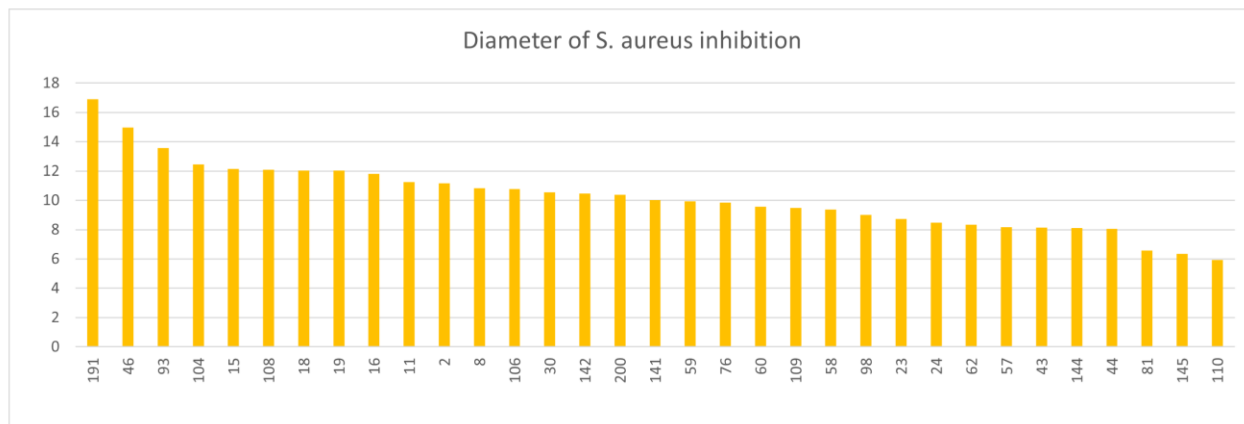

Figure 11. Susceptibility of *S. aureus* (diameter in mm versus LAB number).

Among the 33 LAB tested against *S. aureus*, the average diameter was 10.23 mm and the standard deviation was 1.18mm. The best inhibition was obtained with isolate PG3CC (#191) with a diameter of 16.9 mm. As with inhibition against *L. monocytogenes*, isolates ML3CA (#93) and ML4CE (#104) presented large diameters of inhibition, 13.56 and 12.44 mm, respectively.

According to Guessas et al (2005), in raw goat and ewes' milk, the LAB tested (*Lactoccocus lactis subsp lactis*) produced an average inhibition zone of 10 mm against *S. aureus*. In comparison with our study, and using such a threshold, 54% of the LAB isolated from Portuguese chorizo obtained halos larger than 10 mm.

In a study conducted in alheira sausage, another Portuguese fermented sausage (Fernandes et al., 2023), the largest and smallest halos against *S. aureus* were 13.88 and 4.49 mm. In our study, the range for halos from LAB obtained from chorizo was very comparable to that of alheira, since we obtained halos in the range of 16.9 and 5.9 mm; both of them slightly superior to those obtained in Fernandes et al. (2023). In this way, the LAB isolated obtained from chorizo had also the potential to case inhibition of *S. aureus*, in addition to *L. monocytogenes*.

The largest diameter obtained was 24.3 mm for isolate ML2CA (#93), which also showed large inhibition halos against *L. monocytogenes* and *S. aureus*. Isolate ML4CE (#104) also produced large halos against the three pathogens, with a diameter of 20.3 mm against *Salmonella*. These two LAB isolates (ML2CA and ML4CE) had great inhibition potential, since they obtained promising results for the three pathogens tested against and will be subsequently identified by molecular techniques.

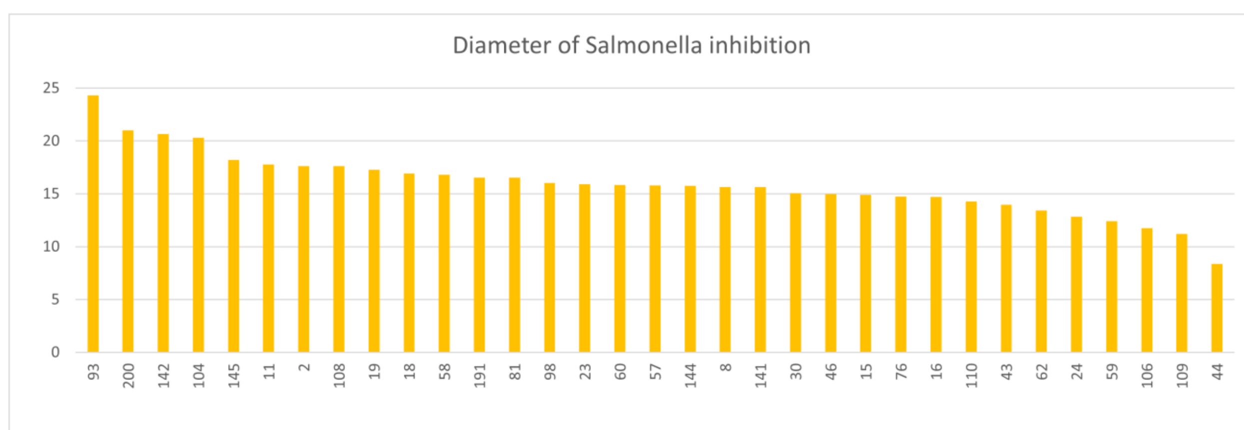

Figure 12. Susceptibility of *Salmonella* (diameter in mm versus LAB number).

Among the 33 LAB tested against *Salmonella*, the average susceptibility was 15.90mm mm and the standard deviation was 1.51mm. In the study of Samar, Doaa and Naeima (2024), the minimum and maximum inhibition zones for *L. monocytogenes* were 6.67 mm and 26.67 mm, and in case of *Salmonella* Typhimurium were 0.00 mm and 9.33 mm, respectively. Comparing to the results shown in Figures 10 and 12, it is possible to observe that the susceptibility of *L. monocytogenes* was greater, for some LAB strains, than the susceptibility of *Salmonella*. However in term of means, both susceptibilities were very close, suggesting that the LAB tested share similar inhibition properties. On average, the capacity of LABs to inhibit *S. aureus* was lower.

Capita et al. (2006) explained that chemical compounds (antibiotic-like substances) excreted by LAB and the decrease in pH as a result of their high acidifying capability may partially explain the reduction and disappearance of some microbial groups (*Enterobacteriaceae*, enterococci, and pseudomonads) in fermented meat products. Such microbial groups were not investigated in the present study.

The results suggest that some LAB strains may be producing bacteriocins of broad antibacterial spectrum considering 9 mm as the cut-off inhibition diameter that reveals high level of antimicrobial activity (Pan et al., 2009).

4.3 Acidifying Capacity of Selected LAB

The acidification tests were carried out with the 33 LAB selected as the best inhibitors against *L. monocytogenes*, in order to characterize their ability to acidify broth. As described in the Methodology, the acidification profile was obtained by measuring the pH of broth inoculated with a LAB strain.

The pH descriptors obtained from the Weibull function fitted to each data set were variable from isolate to isolate; reinforcing therefore that we were in the presence of distinct lactic acid producing strains. Such variability in the pH descriptors originated from the variability in pH profiles, with LABs that had a faster acidification profile, thus having a high pH drop in the first few hours, and, on the other end, LABs that took longer to acidify. Examples of acidification profiles for three LAB isolates are shown in Figures 13, 14 and 15.

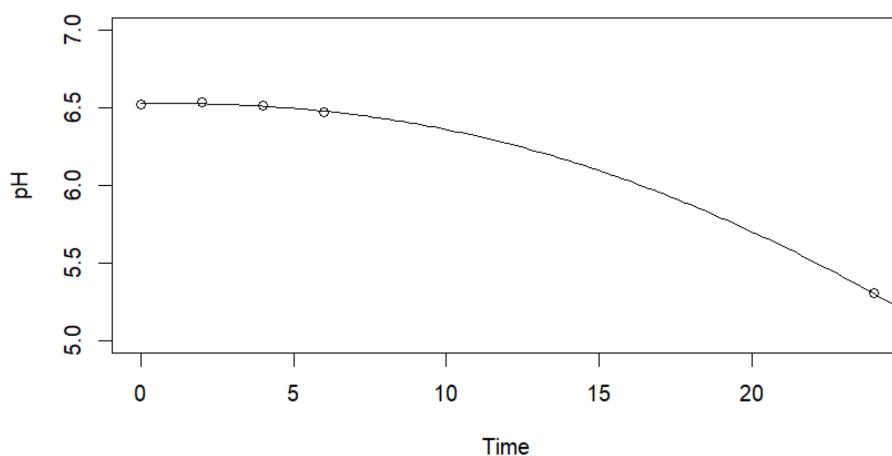

Figure 13. Acidification profile of LAB isolate FV4CD (pH versus Time in h). Fitted Weibull decay model is shown.

In Figure 13, it was possible to observe that pH gradually but slowly decreases, so that until the first 6 hours of acidification, it only changed from approximately 6.5 to 6.4. However, after 24 hours, a final pH of 5.3 was obtained, which was lower compared to other LABs that had faster initial acidification. This means that there were LAB isolates that acidified very well yet needing a longer incubation period to perform their best.

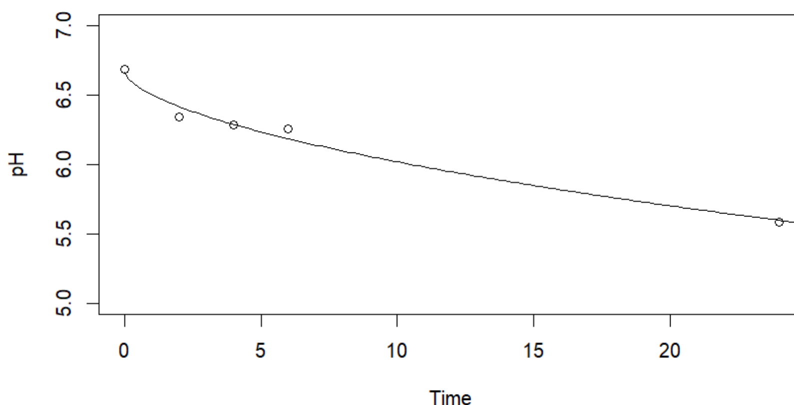

Figure 14. Acidification profile of LAB isolate ML2CA (pH versus Time in hours)). Fitted Weibull decay model is shown.

In contract, the LAB isolate presented in Figure 14 showed higher initial acidification, with a pH variation of 0.42 within the first 6 hours, demonstrating that this LAB is rapid in starting and performing the fermentation. However, after 24 hours of incubation, the pH obtained is 5.58, which was higher than that of the LAB isolate FV4CD, shown in Figure 13. Thus, it is expected that isolates from the Portuguese chorizo's LAB bank are very different in physiology and therefore genetically. It is worth mentioning that these acidification essays were performed in duplicate, to filter out the variability due to LAB activation.

In a study by LV et al (2023), during the fermentation of sour meat by *Saccharomyces cerevisiae*, the pH decreased rapidly within 7 days (from approximately 6.1 until 4.8), after which the rate of decrease slowed down and gradually leveled off during the rest of the fermentation (28 days). So, by comparing the results obtained in one day of fermentation, pH decrease of 6.7 until 5.58, it is possible to affirm that the LAB tested have performed a good fermentation and acidification.

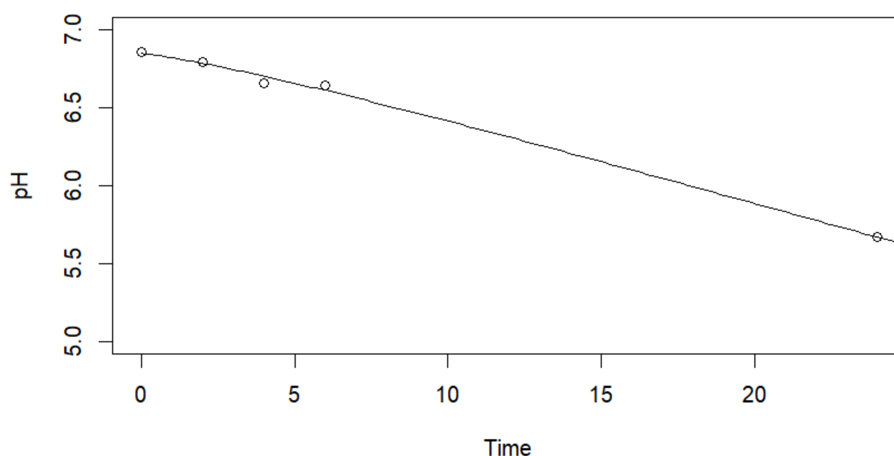

Figure 15. Acidification profile of LAB isolate ML5CE (pH versus Time in hours). Fitted Weibull decay model is shown.

In Figure 15, an initial behavior similar to the behavior in Figure 13, but as time goes by the LAB maintained its acidification rate over the 24 hours, practically obtaining a straight line of acidification. In this way, among the LAB isolates studied, these were the three most frequent profiles; namely faster acidification at the beginning; better final acidification with initially slow LAB; and LAB with rather constant acidification throughout the incubation period.

The decrease in pH is due to the organic acids produced by LAB during carbohydrate metabolism, which is in line with the gradual increase in lactic acid content during fermentation according to the behavior of each LAB.

The acidification process varied among the different LAB of fermented sausages, but this variability served to validate the efficacy of the LAB strains that would eventually be present in a starter cultures for acidifying the sausages. Dellaglio, Casiraghi, and Pompei (1996) stated that poor acidification may stem from the presence of LAB with limited acidifying capabilities or from a potential decline in acidifying and deaminase activities.

4.4 Proteolytic Activity of Selected LAB

Using the 33 LAB selected, the assay was carried out to test the proteolytic activity, as shown in Figure 6. After 4 days of incubation, the results obtained showed that 20 LAB isolates (60%) produced in-vitro proteolytic activity; and within these, there was a high variation in the size of the halos measured (Figure 16).

A clear zone around a colony is an indicator that soluble nitrogenous compounds are formed, and in the case of bacteria also producing acid from fermentable carbohydrates present in the medium, the clearer/more transparent the zone will be. This may be the explanation for the two types of zones observed in this assay.

The LAB isolate with the largest average radius of proteolysis obtained was AG3CD, with an average radius of 8.87 mm. Followed by strain code AG3CECB with an average radius of 8.62 mm and strain AG2CA with average radius halos of 8.46mm, these three LAB were the ones with values greater than 8 mm, thus being the ones with the greatest proteolytic activity.

Compared to Fernandes et al. (2023), which obtained the best diameter of proteolytic action of 8.26 mm in LAB isolated from alheira sausage, it is possible to observe that the LABs isolated from chorizo presented a proteolytic action similar to those tested in alheira, once the halos obtained were almost the same size.

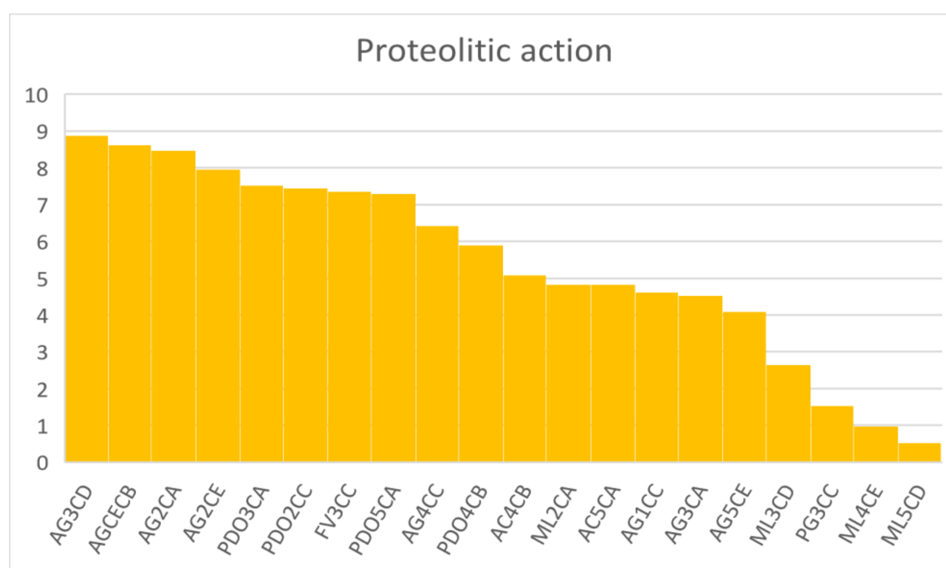

Figure 16. Proteolytic activity of LAB (diameter in mm versus LAB code).

The difference between these and the LAB with weaker proteolytic activity was clearly visible on the plate (Figure 17), Figure 17(A) shows the halos obtained in quadruplicate for strain AC4CB; whereas Figure 17(B) shows the halos obtained for isolate AG3CA and isolate AG2CA, which produced one of the greatest proteolytic activity.

The halos in Figure 17(A) correspond all to the same LAB isolate, and show the consistency in the results obtained in this work.. Figure 17 (B) shows the big difference between the proteolysis capacity of AG3CA compared to AG2CA. Therefore, as the halos obtained were clearly visible and demarcated, it was possible to carry out the measurements, and find the LAB strains with the highest proteolytic activity.

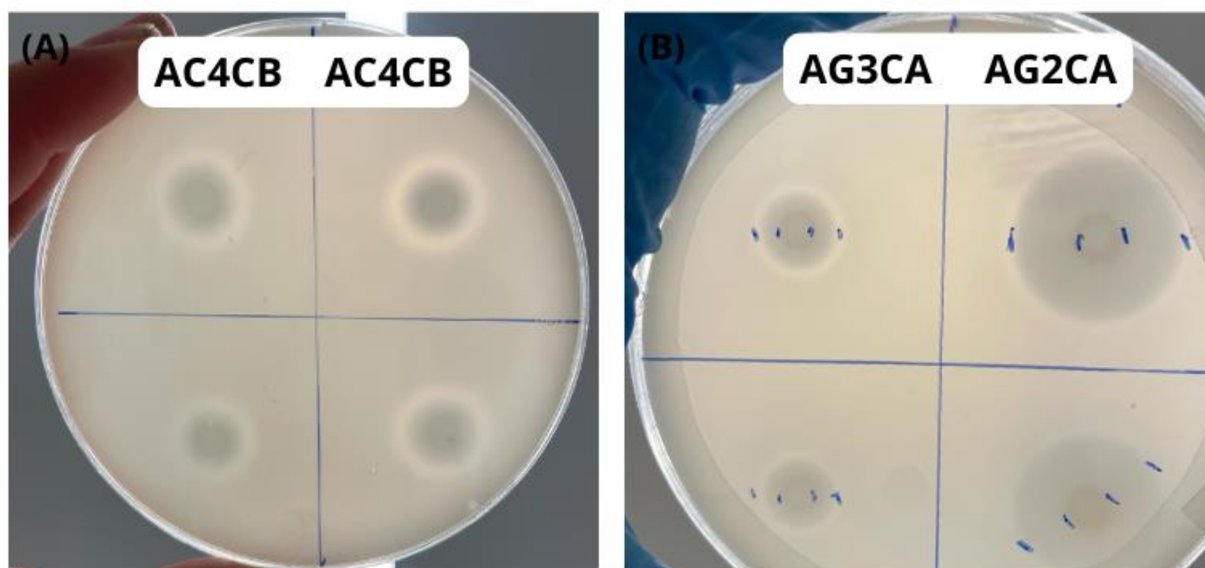

Figure 17. Plates with halos of proteolytic activity on agar.

4.5 Production of Lactic Acid by Selected LAB

The potential of starter cultures to produce antimicrobial compounds to prevent food spoilage and to inhibit growth of food pathogens has attracted much attention. The primary antimicrobial effect exerted by LAB is the production of lactic acid (LAC) and reduction of pH (Daeschel, 1989).

To measure the production of LAC, after applying the L-Lactic acid Kit for UV method, the cuvettes were measured in the spectrophotometer. In order to confirm the effectiveness of the measurements, a positive control present in the kit was used and also to confirm that there was no contamination in the results, a negative control was measured. With the absorbances obtained, the concentration of LAC [LAC] was obtained for each of the selected LABs. Table 4 presents the mean results for every isolate and in descending order. The highest concentration was obtained by isolate ML2CA at 0. 929 g/L and the lowest by isolate ML4CE at 0.016 g/L. This range was comparable with the results for the LAB isolated from Portuguese alheira sausage (Fernandes et al., 2023), which were in the range between 0.663 and 0.019 g/L.~

In the study realized by Benito et al (2007) in traditional Iberian dry-fermented salchichón and chorizo sausages, the authors considered that the LAB isolated from industrial chorizo had a good capacity to generate lactic acid, once these produced 0.0178–0.0355 g/L LAC in 34.4% of the strains; and 0.0356-0.0533 g/L LAC in 34.9% of the strains; whereas only 17.7% generated less than 0.0178 g/L LAC after 12 h of incubation. Comparing to the results obtained in the present work, in 24 hours of incubation, it was possible to obtain concentrations higher than 0.0533g/L in 91% of the LAB. From the 33 LAB tested, 42% had concentrations greater than concentration of 0.25 g/L. The results, however, are not directly comparable because the incubation period in our study doubled the incubation period of the protocol used by Benito et al. (2007).

LAB isolate ML2CA produced more LAC (0.9292 g/L) among all the selected LAB, and was the best isolate inhibiting the three pathogens. It is like that this isolate has the abilities to quicky ferment and to produce bacteriocins. It is important to consider its capacity to produce lactic acid rapidly, in selecting it for the development of a starter culture (Buckenhuskes, 1993).

Table 4. L-latic acid concentration in descending order for each LAB isolate

LAB CODE	Lactic Acid Concentration (g/L)
ML2CA	0.9292
AG3CA	0.7529
FV3CC	0.6088
PDO4CB	0.5927
FV4CD	0.5767
ML4CE	0.4806
AC4CB	0.4486
AGCECB	0.3524
PDO3CA	0.3364
AG3CC	0.3204
PG3CC	0.3204
PDO4CA	0.3044
AG4CD	0.2563
SJ3CA	0.2563
AG3CD	0.1922
AG4CC	0.1602
ML3CD	0.1602
AC5CA	0.1602
AG5CE	0.1282
AG1CC	0.1121
AG2CA	0.0961
ATS1CA	0.0961
ML5CD	0.0961
IR1CA	0.0961
IR1CB	0.0961
ATS2CA	0.0801
ATS3CB	0.0801
PDO5CA	0.0801
PDO2CC	0.0641
SF1CA	0.0320
ML5CB	0.0160
ML5CE	0.0160

In the production of raw fermented sausages, the acidic character of medium produced by the metabolism of LAB becomes unsuitable for the growth of the pathogen (Massaguer, 2006). The acidic nature of LAB, and their remarkable ability to adapt to often extreme conditions, together with the high concentrations of lactic acid and molecules with antimicrobial activity (bacteriocins)

produced, allows to inhibit the growth of other microorganisms, and to use them in the manufacture of fermented products, such as chorizo.

4.6 Multivariate analysis

The principal component analysis pointed towards a three-dimension solution that jointly accounts for 72.7% of the variability in the multivariate data. Dimension 1 explains 40.4% of the variability, and is positively correlated with the Weibull scale parameter k (the fastest the pH drop, the lower the value of k), with most of the pH descriptors, namely Delta 0_3, Delta 0_6, and Delta 3_6, and with the susceptibility of *L. monocytogenes*. Dimension 2 explains 18.2% of the variability, and is positively correlated with the higher concentration of lactic acid, the susceptibility of *S. aureus* and *Salmonella* spp. and the proteolytic activity. Dimension 3 explains only 14.1% of the data variability, and is positively correlated with Delta 0_24 (i.e., the total change in pH) and inversely with the Weibull scale parameter k. The lower the value of k, the higher the pH drop caused by LAB.

4.6.1 Correlation between technological properties and dimensions

A graph of correlations between variables and dimensions was built to assess the variables that contribute the most to each of the dimensions (Figure 18). The correlation coefficient, ranging between -1.0 to 1.0, is represented by different tonalities between deep red color to deep blue color. Furthermore, the size of the circle marker is proportional to the absolute value (strength) of the correlation coefficient.

In Dimension 1 the inhibition of *L. monocytogenes* was very related to the acidification, as characterized by pH0, Delta 0_3, Delta 0_6, Delta 3_6 and Delta 0_24, meaning that the control of the *L. monocytogenes* growth was being exercised by the rapid lowering of the pH.

According to Costa & Fernandes (2018), when determining the proteolytic activity of meats, they found that some of the isolates that presented high antimicrobial activity also presented high proteolytic activity. This has been found in our study, and made evident in Dimension 2, which correlated with both proteolytic activity and antimicrobial activity against *Salmonella* and *S. aureus*.

For Dimension 3, the pH drop after 24 hours (Delta0_24) was the most important variable, and was moderately correlated with the Weibull parameter k and, more interestingly, with the L-lactic acid concentration, to some extent.

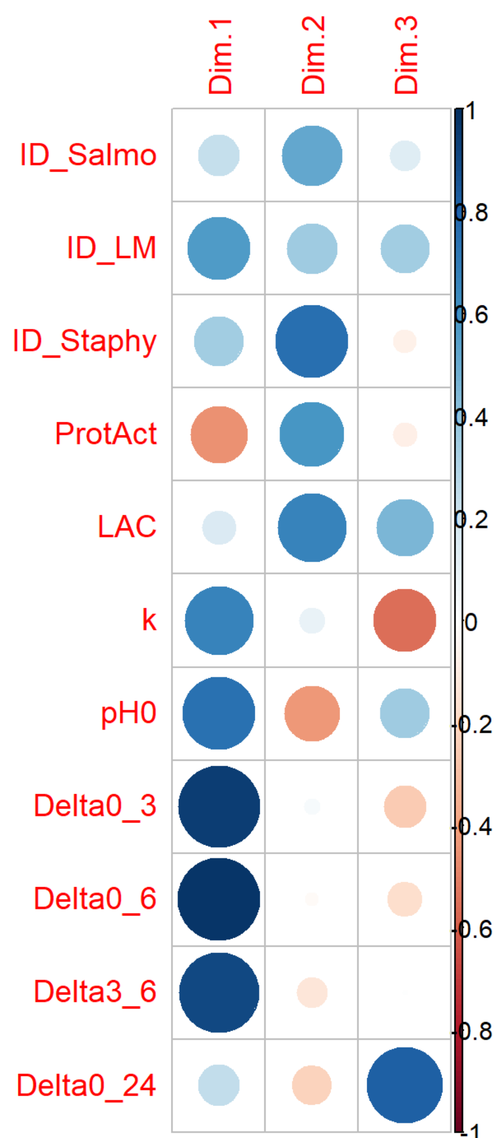

Figure 18 - Correlations of the Factors vs Dimensions. Dim.1: Dimension 1. Dim.2: Dimension 2. Dim.3: Dimension 3. ProtAct: Proteolytic activity. LAC: Latic acid concentration. ID_Salmo: Inhibition diameter for *Salmonella*. ID_Staphy: Inhibition diameter for *S. aureus*. ID_LM: Inhibition diameter for *L. monocytogenes*. k: Weibull scale parameter. pH0: initial pH. Delta (0_3, 0_6, 3_6, 0_24,): pH drops in the time intervals.

4.6.2 Correlation of technological properties

Figures 19 and 20 show the variables correlation bi-dimensional graphs for the three-component PCA solution. These graphs allow a better understanding of the potential association between technological properties.

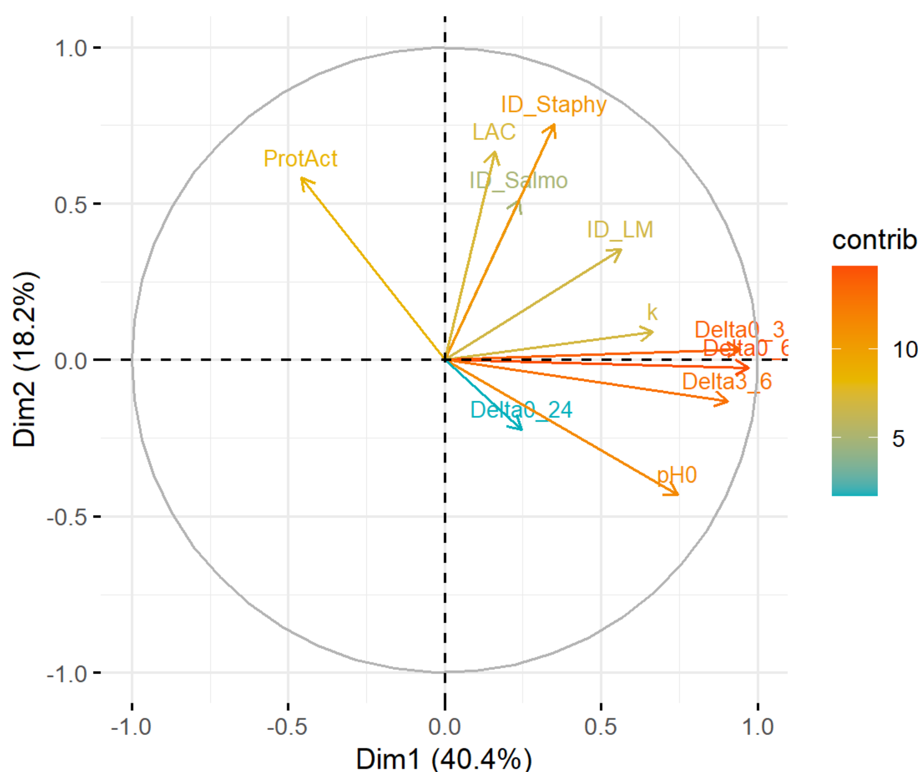

Figure 19. Variable correlations of Dimension 1 (Dim1) vs Dimension 2 (Dim2). ProtAct: Proteolytic activity. LAC: Latic acid concentration. ID_Salmo: Inhibition diameter for *Salmonella*. ID_Staphy: Inhibition diameter for *S. aureus*. ID_LM: Inhibition diameter for *L. monocytogenes*. k: Weibull scale parameter. pH0: initial pH. Delta (0_3, 0_6, 3_6, 0_24,): pH drops in the time intervals.

Figure 19 made clear the close association between L-lactic acid concentration and the inhibition of *S. aureus* and *Salmonella*. The inhibition of *L. monocytogenes* appears less correlated with the L-lactic acid concentration, and uncorrelated with the proteolytic activity. Inhibition of *L. monocytogenes* was greater association with the set of pH drop descriptors than did the inhibition of *S. aureus* and *Salmonella*. It was also observed that the proteolytic activity was independent of

such pH drop. Similarly, the descriptors of pH drop were not associated with the L-lactic acid concentration.

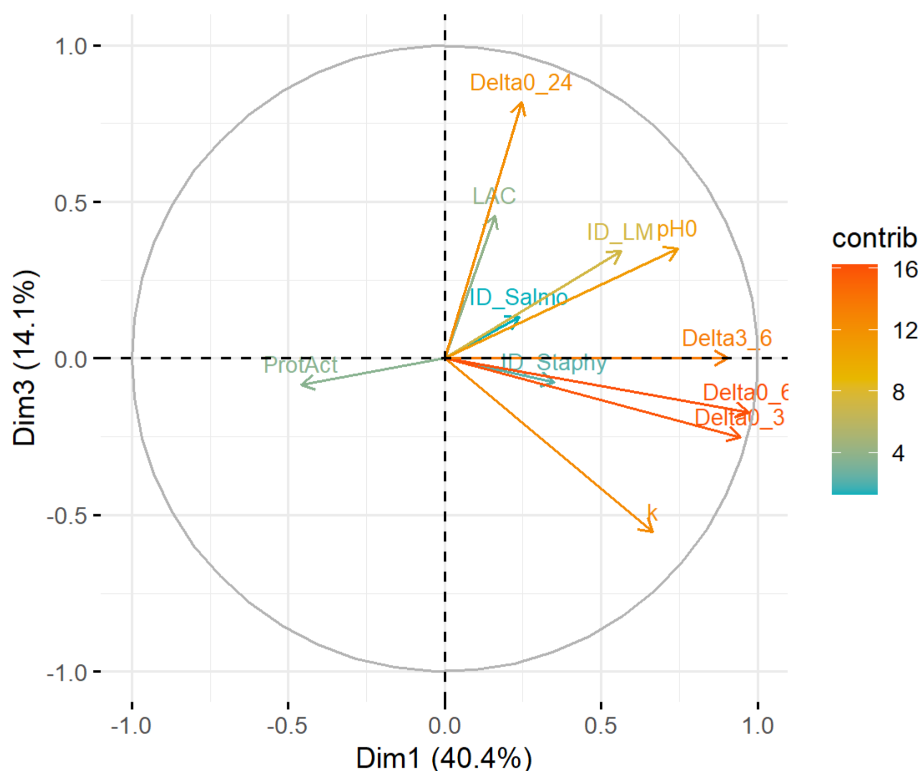

Figure 20.Variable correlations of Dimension 1 (Dim1) vs Dimension 3 (Dim1). ProtAct: Proteolytic activity. LAC : Latic acid concentration. ID_Salmo: Inhibition diameter for *Salmonella*. ID_Staphy: Inhibition diameter for *S. aureus*. ID_LM: Inhibition diameter for *L. monocytogenes*. k: Weibull scale parameter. pH0: initial pH. Delta (0_3, 0_6, 3_6, 0_24,): pH drops in the time intervals.

Interestingly, the descriptor Delta 0_24 did not correlate well with the other pH drop descriptors (Figure 19), meaning that, even if some LAB strains presented a delay time for pH drop, the final pH they reach after 24 hours incubation could still be low. The shoulder in the pH drop profile therefore does not determine the final pH.

The importance of the pH descriptor Delta 0_24 can be appreciated in Figure 20, where this variable is highly correlated with Dimension 3. Another trend can be identified: higher pH drops between 0 and 24 hours are associated with higher L-lactic acid concentrations.

4.6.3 Biplot of technological properties with cluster analysis

Biplots for the three-dimension solution are shown in Figures 21 and 22; where the numbers within the graph indicate the LAB strain. The cluster by the k-means algorithm led to a solution of 2 clusters (k=2). These clusters or groups are indicated in the biplot graphs.

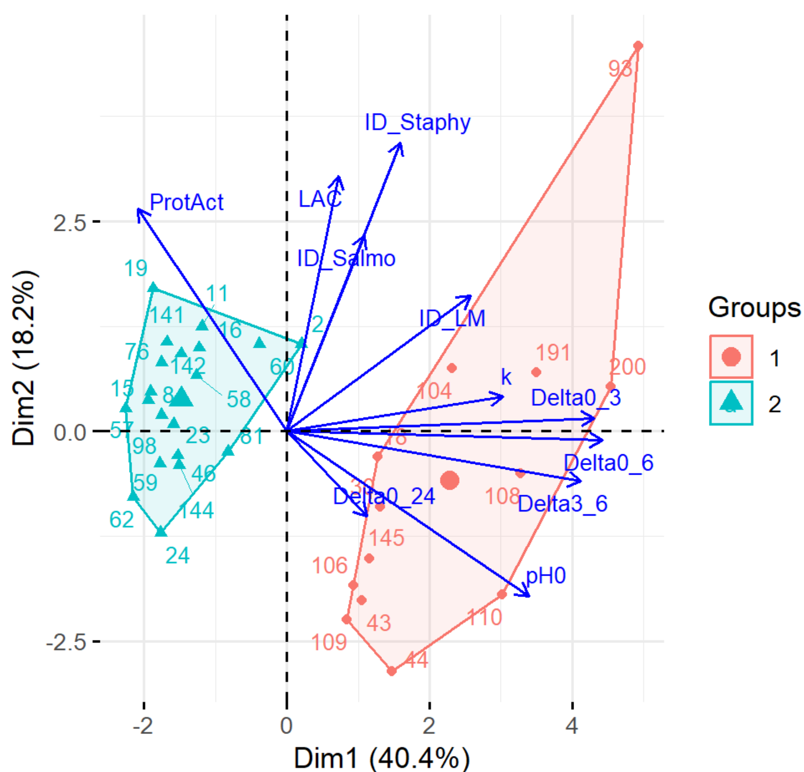

Figure 21. Biplot of Dimensions 1 and 2. ProtAct: Proteolytic activity. LAC : Latic acid concentration. ID_Salmo: Inhibition diameter for *Salmonella*. ID_Staphy: Inhibition diameter for *S. aureus*. ID_LM: Inhibition diameter for *L. monocytogenes*. k: Weibull scale parameter. pH0: initial pH. Delta (0_3, 0_6, 3_6, 0_24,): pH drops in the time intervals.

It can be noticed that Group 1 was deeply related with those LAB isolates that presented a rapid pH drop as well as those LABs that attained lower pH after 24 hours. Those strains with higher antimicrobial activity against L. monocytogenes also presented a higher chance to be accommodated int Group 1 than into Group 2 (Figure 21). Figure 22 shows that the extent of Salmonella susceptibility was not determinant in a LAB belonging to one group or the other. Group

2 clusters those LAB strains that are slower in acidification and with high proteolytic activity. According to Figure 22, the L-lactic acid concentration is another property that does not determine the correspondence to any of the groups.

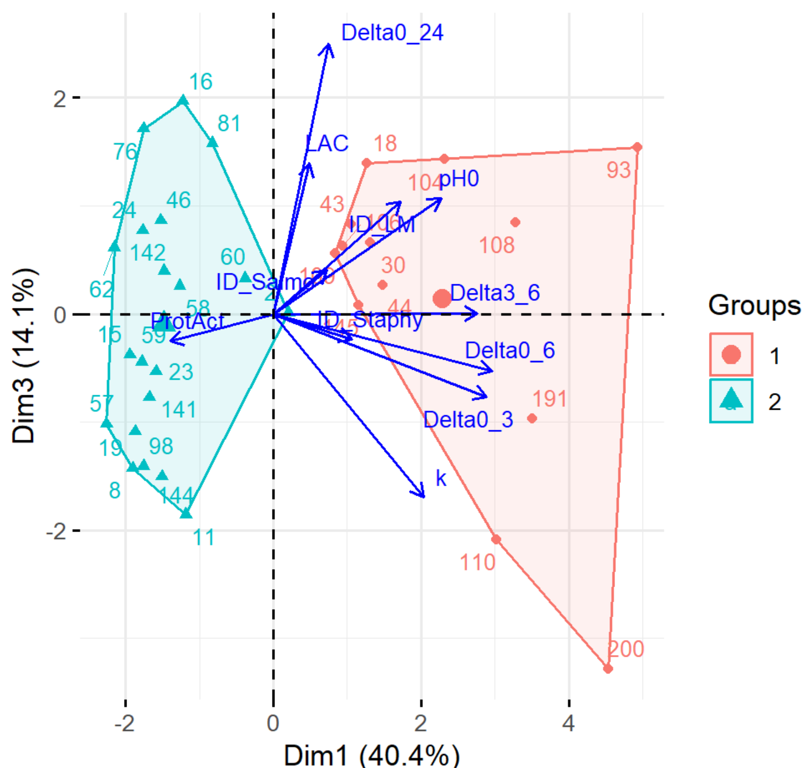

Figure 22. Biplot of Dimensions 1 and 3. ProtAct: Proteolytic activity. LAC: Latic acid concentration. ID_Salmo: Inhibition diameter for *Salmonella*. ID_Staphy: Inhibition diameter for *S. aureus*. ID_LM: Inhibition diameter for *L. monocytogenes*. k: Weibull scale parameter. pH0: initial pH. Delta (0_3, 0_6, 3_6, 0_24,): pH drops in the time intervals.

From Figure 22, it can also be established that LAB with greater acidification potential (Delta 0_24) or higher lactic acid production could be either classified as group 1 or group 2.

4.6.4 Effect of production region on LAB groups

In order to appraise any association between the production region and the LAB technological properties or groups, another cluster representation on the Dimension 1 and 2 was

made by the origin of the chorizo samples (Figure 23). Acronyms of the regions are used and indicated in the figure.

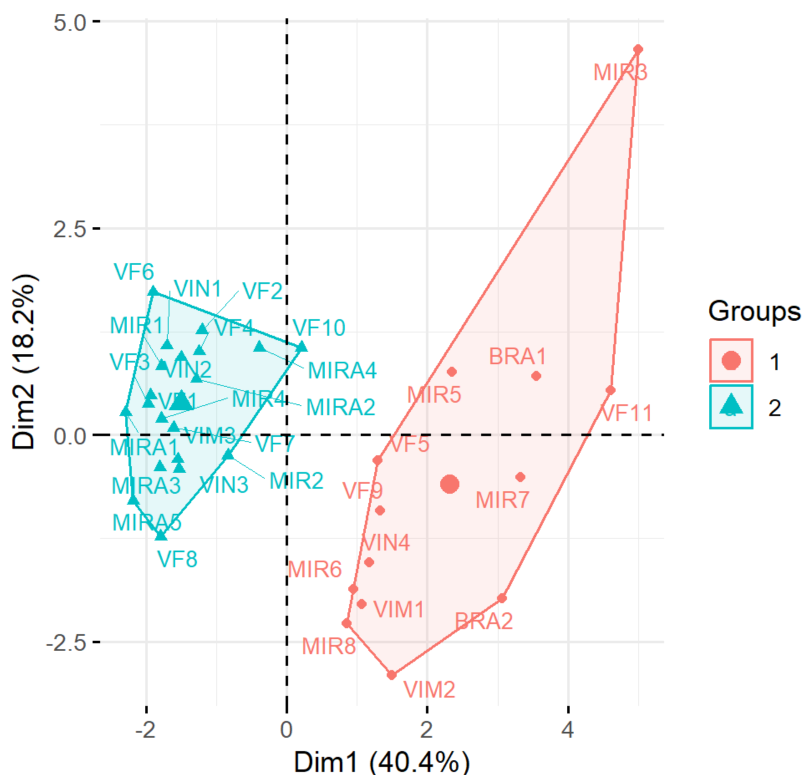

Figure 23. k-means (k=2) cluster representation on principal components 1 and 2, showing the origin of the chorizo samples. BRA: Bragança; MIRA: Miranda do Douro; MIR: Mirandela; VF: Vila Flor; VIM: Vimioso; VIN: Vinhais

Figure 23 implies that there is no regional effect on the properties or specificities of the LAB isolates. Notice that that in both groups there are LAB isolates from the same producer, which demonstrates that the location of the producer did not affect the pool of results obtained. Since LAB from the same producer occupied places in different groups, the factors affecting the properties and potential of LAB were related to metabolic, and physiological factors and not locational.

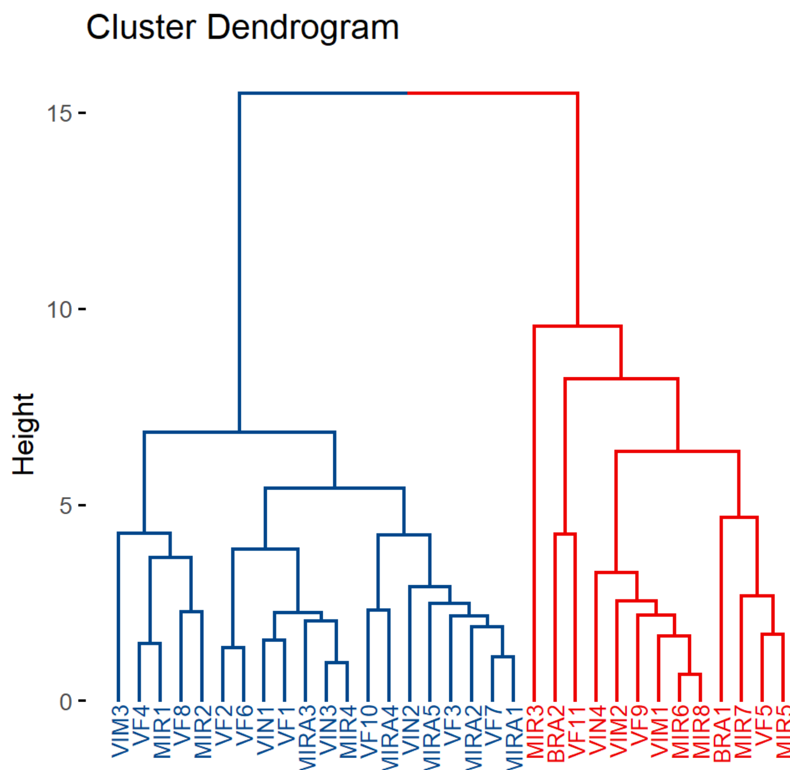

Figure 24. Hierarchical clustering (dendrogram) solution for two groups, showing the origin of the chorizo samples. BRA: Bragança; MIRA: Miranda do Douro; MIR: Mirandela; VF: Vila Flor; VIM: Vimioso; VIN: Vinhais

A hierarchical dendrogram was also produced to corroborate, by using another technique, the absence of a geographical location effect. Figure 24 shows that cluster 1 (blue) was formed of LAB isolates from Mirandela, Vimioso and Vila Flor, and equally for cluster 2 (red). It was possible therefore to conclude that there was no trend or association between producers and the grouping of technological properties.

4.6.4 Factor analysis of technological properties of LAB

Since the principal component analysis pointed out to a solution for three components, when conducting the factor analysis, three factors were also extracted. The factor analysis produced similar results, despite being based on a different multivariate algorithm. Factor 1 was positively

and highly correlated with the pH descriptors Delta 0_3, pH0 and Delta 2_6 high loadings; where it was moderately and inversely correlated with proteolytic activity. Therefore, Factor 1 can be labelled as "*Rapid acidification capacity*". It could be also appreciated that the rapid acidification capacity has a moderate correlation (R=0.39) with the antimicrobial capacity against *L. monocytogenes*. This demonstrates that the acidification of the medium enhances the inhibition of *L. monocytogenes* growth.

Table 5. Coefficients of correlation of the microbiological properties of "chorizo" with three factors with varimax rotation (Factor 1, Factor 2 and Factor 3)

	Factor 1	**Factor 2**	**Factor 3**	**H2**	**U2**	**COM**
ID_Salmo	0.06	**0.41**	0.00	0.17	0.8297	1.0
ID_LM	0.39	**0.52**	0.08	0.43	0.5697	1.9
ID_Staphy	0.06	**0.72**	-0.15	0.55	0.4485	1.1
ProtAct	**-0.50**	0.25	-0.19	0.35	0.6508	1.8
LAC	-0.13	**0.69**	0.22	0.54	0.4603	1.3
Delta0_3	**0.82**	0.32	-0.11	0.78	0.2208	1.3
pH0	**0.81**	-0.06	**0.44**	0.86	0.1441	1.6
Delta2_6	**0.93**	0.21	0.00	0.90	0.0995	1.1
Delta0_24	0.16	0.08	**0.98**	1.00	0.0036	1.1

Factor 2 was correlated with the susceptibilities of the three pathogens and the lactic acid concentration. Thus, Factor 2 can be labelled as "*Antimicrobial capacity*". Factor 3 was heavily correlated with the pH drop after 24 hours, and moderately correlated with the initial pH. This means that when the initial pH is low, there is also a lower potential for pH drop. Factor 3 therefore can be labelled as "*Acidification potential*". As explained before, acidification potential is not correlated at all with the rapid acidification capacity, since LAB isolates that are initially slow in growing and fermenting are able to produce low levels of pH in the end.

Table 6 compiles the analysis of variabilities obtained in the factor analysis. Unlike the principal component analysis, which accounted for 72.7% of the total variability, the factor analysis could explain 62% of the variability. It is worth mentioning that the SS loadings line (Table 6) provides the sum of the squared loads, and in all factors the value obtained was greater than 1.0, which was another reason to maintain them according to Kaiser's rule.

Table 6. Analysis of variabilities obtained in factor analysis with three factors.

	Factor 1: Rapid acidification capacity	**Factor 2: Antimicrobial capacity**	**Factor 3: Acidification potential**
SS loadings	2.63	1.66	1.28
Proportion Var	**0.29**	**0.18**	**0.14**
Cumulative Var	0.29	0.48	**0.62**
Proportion Explained	0.47	0.30	0.23
Cumulative Proportion	0.47	0.77	**1.00**

5. Conclusion

In this study, seventy sausages from 14 different producers in the Trás-os-Montes region were used to investigate the ability of the lactic acid bacteria present in this product to inhibit the most common pathogens, *L. monocytogenes, Salmonella* and *S. aureus.* A total of 380 colonies were isolated, 70% of these were gram positive, catalase negative and of typical LAB morphology. Using microbiological techniques, it was possible to isolate and characterize 207 lactic acid bacteria. Of these 207 isolated bacteria, 33 were chosen for their best inhibition results against *L. monocytogenes*.

According to the inhibition results, five LAB strains ML2CA (#93), ML4CE (#104), ML5CD (#108) and AG3CC #(18) obtained very high inhibition properties against the three pathogens tested. On average, the capacity of LABs to inhibit *S. aureus* was lower than their capacity to inhibit *L. monocytogenes* and *Salmonella*.

The LAB isolates highlighted in the antagonism test also obtained good acidification results with initial and pH values after 24 hours of 6.975 and 5.685 for ML4CE ; 6.68 to 5.58 for ML2CA; 6.83 to 5.515 for AG3CC, and especially 7 to 5.665 for ML5CD. It is worth noting that in terms of lactic acid production, LAB ML2CA (092916 g/L) and ML4CE (0.4806 g/L) were among the LAB to produce the highest concentration of lactic acid in broth, reinforcing the potential of these LAB isolates as strong biopreservatives for ensuring the safety of Portuguese chorizos.

However, in terms of proteolytic activity, the LAB mentioned above did not have large inhibition halos, with LAB AG3CD standing out, with the highest average radius of 8.87 mm,

followed by isolate code AG3CECB with an average radius of 8.62 mm, and isolate AG2CA with average radius of 8.46 mm. These were the only three LAB isolates displaying radius greater than 8 mm, thus being the ones with the greatest proteolytic activity.

The multivariate analysis showed that *L. monocytogenes* susceptibility was closely related to the acidification by the correlation with the pH drop descriptors (pH0, Delta 0_3, Delta 0_6, Delta 3_6); whereas the production of lactic acid was more associated with the inhibition of *S. aureus* and *Salmonella*. The inhibition of *L. monocytogenes* appears less correlated with the lactic acid concentration, and uncorrelated with the proteolytic activity. It was also observed that the proteolytic activity was independent of the aforementioned pH descriptors. Similarly, such pH descriptors were not associated with the lactic acid concentration. Nonetheless, a greater acidification potential (represented by a higher pH drop between 0 and 24 hours, Delta 0_24) was found to be associated with higher lactic acid concentration. Furthermore, isolates that obtained a high proteolytic activity had also a high antimicrobial activity against *Salmonella* and *S. aureus*.

Hierarchical and k-means clustering pointed out to two clusters of LAB: one represented by greater proteolytic activity, and the other having a faster pH drop. In the analysis of the impact of geographical location, since LAB from the same producer occupied places in the two clusters, it was deduced that the technological properties were related to metabolic, and physiological factors and not locational. It was possible therefore to conclude that there was no trend or association between producers and LAB having certain technological properties and not others.

Additionally, through principal component analysis, it became evident that a lower initial pH corresponds to a diminished potential for pH reduction. The acidification potential shows no correlation with the rapid acidification capacity. This suggests that LAB isolates initially slow in kicking off the fermentation are still capable of achieving low pH levels by the end of the process. Furthermore, factor analysis allowed the identification of three latent variables accounting for 62% of the data variability; namely: rapid acidification capacity, antimicrobial capacity and acidification potential.

Through this in-vitro study, it was possible to obtain a chorizo LAB bank and comprehend the properties of a selection of LAB. It can therefore be concluded from the analyses carried out that four main LAB were obtained (ML2CA (#93), ML4CE (#104), ML5CD (#108) and AG3CC (#18)), which deserve molecular characterisation and identification of the bacteriocins produced, in order to better understand their technological profile and action. Furthermore, the effectivity of

such strains for ensuring the safety of Portuguese chorizo should be evaluated by challenge inoculation studies.

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