INSTITUTO POLITÉCNICO DE BRAGANÇA
Escola Superior Agrária

Exploring the Role of Autochthonous Lactic Acid Bacteria in Enhancing Quality and Safety of *Alheira*

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Dissertation submitted to Escola Superior Agrária de Bragança
to obtain the Degree of Master in Biotechnology Engineering

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Bragança
2024

I dedicate this thesis to my mother Renata for her unconditional love and kindness.

ACKNOWLEDGMENTS

I express my sincere gratitude to my supervisors: Dr. Vasco Cadavez and Dr. Ursula Gonzales-Barron for the opportunity they gave me.

I want to thank all the people I've met at CIMO. Special thanks to Dr. Altino Choupina and Dr. Carina Rodrigues for their collaboration and technical support.

I am grateful to the Saint Martyrs Church for their generosity and service. Special thanks to Pe. José Bento and Ivone Calado for all the moments we shared, I treasure them in my heart.

Many thanks to the people from the church youth groups *Jovens Sem Sofá* and *Convivas Fraternos*, your friendship gives me great joy.

I am grateful for having two extraordinary parents who dedicated their life to me. No distance in this world could make our hearts apart.

Thank you, my almighty God, for the blessings You have bestowed upon my life. You have provided me with more than I could ever have imagined.

Totus tuus, Maria.

The research was funded by the Foundation for Science and Technology (FCT, Portugal) financial support through national funds FCT/MCTES (PIDDAC) to CIMO (UIDB/00690/2020 and UIDP/00690/2020) and SusTEC (LA/P/0007/2021) and through funding of the PRIMA projects ArtiSaneFood (PRIMA/0001/2018) and PAS-AGRO-PAS (PRIMA/0014/2022).

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LIST OF ABBREVIATIONS

AMC: Aerobic Mesophilic Count

BLAST: Basic Local Alignment Search Tool

DNA: Deoxyribonucleic Acid

EFSA: European Food Safety Authority

ELISA: Enzyme-Linked Immunosorbent Assay

GlcNAc: N-Acetylglucosamine

HPLC: High-Performance Liquid Chromatography

INE: Instituto Nacional de Estatística

LAB: Lactic Acid Bacteria

man-PTS: Mannose Phosphotransferase System

MUFAs: Monounsaturated Fatty Acids

MurNAc: N-Acetylmuramic acid

NCBI: National Center for Biotechnology Information

PCA: Principal Component Analysis

PCR: Polymerase Chain Reaction

PDO: Protected Designation of Origin

PGI: Protected Geographical Indication

PUFAs: Polyunsaturated Fatty Acids

qRT-PCR: Real-Time Polymerase Chain Reaction

RP-HPLC: Reversed-Phase High-Performance Liquid Chromatography

SFAs: Saturated Fatty Acids

LIST OF SYMBOLS

AU/g: Arbitrary units per gram

AU/mL: Arbitrary units per milliliter

ºC: Celsius degrees

CFU/g: Colony Forming Units per gram

g: Gram

g/L: Grams per liter

h: Hour

Kb: Kilobase

kDa: Kilodalton

Kg: Kilogram

kGy: Kilogray

log: Logarithm

Mpa: Megapascal

Mt: Megaton

µg/g: Microgram per gram

ug/mL: Micrograms per milliliter

mm: Micrometer

mg: Milligram

mM: Millimolar

MIC: Minimum Inhibitory Concentration

M: Molar

ng/mL: Nanograms per milliliter

ppm: Parts per million

%: Percentage

PFU/g: Phage Forming Units per grams

KCl: Potassium Chloride

NaCl: Sodium Chloride

t: Ton

v/w: Volume per weight

Aw: Water activity

ABSTRACT

The *alheira* is a traditional meat sausage produced in the Portuguese region of *Trás-os-Montes*. Lactic Acid Bacteria (LAB) are the dominant microorganisms in *alheira* and can endow it with various technological properties. This study aimed: 1) Isolate and identify LAB from artisanally produced alheiras from the Trás-os-Montes region; 2) Study their phenotypic characteristics and *in vitro* antimicrobial activity; and 3) Differentiate and associate the phenotypic characteristics of the LAB with 16S ribosomal gene sequencing data. Sixty-two LAB isolates were identified and *Enterococcus (E.) faecium* corresponded to 32.3% of isolates, followed by *Leuconostoc (L.) mesenteroides* (19.4%) and *Latilactobacillus (Lb.) sakei* (17.7%), aligning with previous research on traditional Portuguese fermented meat sausages. The phenotypic analysis of LAB isolates indicated diverse acidification capacities, proteolytic activities, and inhibitory effects against foodborne pathogens *Listeria (L.) monocytogenes*, *Salmonella (S.)* Typhimurium and *Staphylococcus (S.) aureus*. LAB play an essential role as biological control agents, generating metabolites with antimicrobial properties, including organic acids, proteases, peptidases and bacteriocins. Overall, lactobacilli displayed high inhibition activity ((inhibition zone radius higher than 10 mm) against the pathogens *S. aureus*, *L. monocytogenes* and *S.* Typhimurium. The mechanisms of pathogenic microorganism inhibition remain to be clarified and will be the subject of future studies. However, these results reveal the significant diversity of LAB found in artisanally produced *alheiras*, as well as their contribution to the microbiological safety and organoleptic characteristics of *alheiras*.

Keywords: antimicrobial peptides; meat products; traditional food.

RESUMO

A alheira é um enchido de carne tradicionalmente produzido na região portuguesa de Trás-os-Montes. As bactérias ácido-lácticas (LAB) são os microrganismos dominantes na alheira e podem conferir-lhe várias propriedades tecnológicas. O estudo teve como objetivos: 1) Isolar e identificar LAB de alheiras de fabrico artesanal produzidas na região de Trás-os-Montes; 2) Estudar as suas características fenotípicas e a sua atividade antimicrobiana in vitro; e 3) Discriminar e associar as características fenotípicas das LAB com os dados de sequenciação do gene ribossómico 16S. Num total de 62 isolados de LAB, *Enterococcus (E.) faecium* correspondeu a 32,3% dos isolados, seguido de *Leuconostoc (L.) mesenteroides* (19,4%) e *Latilactobacillus (Lb.) sakei* (17,7%). O estudo fenotípico evidenciou uma grande variabilidade das LAB na sua capacidade de acidificação, na sua atividade proteolítica e no seu efeito inibitório contra os microrganismos patogénicos de origem alimentar: *Listeria (L.) monocytogenes*, *Salmonella (S.)* Thyphimurium e *Staphylococcus (S.) aureus*. Em geral, os lactobacilos apresentaram elevada atividade de inibição (raio de inibição superior a 10 mm) contra os agentes patogénicos *S. aureus, L. monocytogenes* e *S.* Thyphimurium. Os mecanismos de inibição dos microrganismos patogénicos carecem de explicação, pelo que serão objeto de estudo em trabalhos futuros. Todavia, estes resultados revelam a grande diversidade de LAB encontradas nas alheiras de fabrico artesanal, bem como a sua contribuição para a segurança microbiológica e as características organolépticas das alheiras.

Palavras-chave: péptidos antimicrobianos; produtos à base de carne, alimento tradicional.

I. General Introduction

1.1 Background

Meat is source of high-quality proteins, minerals and vitamins (Price, 1987). In 2022, a Portuguese person on average consumed 118.5 kg of total meat, from which 45.2 kg was from poultry, 42.5 kg from pig and 21.5 kg from cattle meat. The meat consumption by the Portuguese has increased in over 4.0 kg (Fig. 1) compared to the years of 2020 and 2021, this rise in consumption reflects evolving dietary preferences and cultural habits, in which meat remains a significant aspect of the Portuguese diet (Guiné et al., 2021). Not only that, but it also underscores the importance of ensuring the safety and quality of meat products to meet the growing demand.

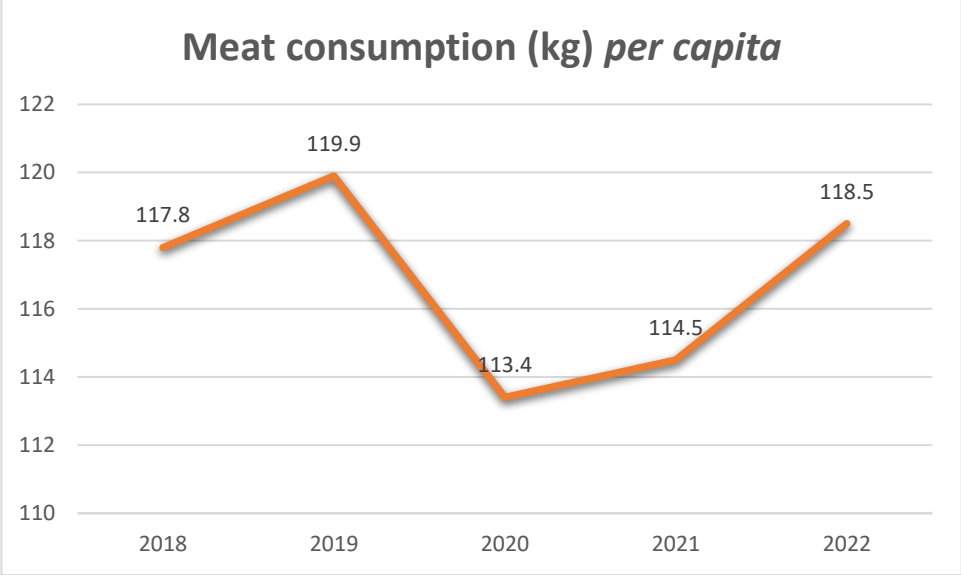

Figure 1. Meat consumption (kg) per capita. Source: Instituto Nacional de Estatística - Estatísticas Agrícolas (2022).

Artisanal foods from the Mediterranean region play a vital role in the economic and cultural landscape of rural areas, fostering local commerce, providing employment opportunities for rural residents, and preserving culinary traditions (Mediterra, 2012). In 2022, meat preservation and the production of meat-based products stood out as the top-performing sector in the food industry, constituting to 19.3% of total sale gains (INE, 2023). This growth is paralleled by a 1.4% increase in national meat production from

2019 to 2022 and a high contribution by the North of Portugal (Fig. 2), from where 73,573 tons of meat was produced in 2022. A substantial portion of this produce – 73,346 tons – was dedicated to sausage production, underscoring the enduring importance of sausages in local culinary traditions.

Sausages are treasured traditional foods that carry a historical narrative. They symbolize the resourcefulness of rural communities in sustaining themselves during hash climatic conditions, particularly winter (Orand-Fehr et. al, 1998). Transforming fresh produce into long-lasting products (by salting, drying, smoking and cooking) was not just a matter of practicality but a cultural legacy. The knowledge embedded in this process contributes to the rich local heritage which results in products with unique flavor and texture.

The appreciation and promotion of traditional products has become essential for the development strategies across European rural regions (Germov, 2008). Recognizing the significance of preserving cultural heritage and fostering economic growth in rural areas, European authorities have implemented regulations aimed at supporting the valorization and protection of products originating from regions renowned for their traditional production methods. These regulations serve as frameworks to ensure the authenticity, quality, and distinctiveness of these products while also safeguarding the livelihoods of local producers and communities.

In Europe, traditional, typical and local food products can be recognized by the community authorities within the framework of quality protection schemes such as Protected Designation of Origin (PDO) and Protected Geographical Indication (PGI). Through rigorous certification processes, products that meet specific criteria related to their origin, production methods, and quality standards are granted PDO or PGI status (OECD, 2000). This recognition not only enhances the market value of these traditional products but also provides consumers with assurance regarding their authenticity and adherence to traditional practices. Additionally, PDO and PGI designations contribute to the preservation of cultural identity and promote sustainable agricultural practices, thereby supporting rural economies and fostering pride within local communities.

Furthermore, PDO and PGI labels serve as valuable tools for promoting regional diversity and enhancing market competitiveness for traditional products. By

highlighting the unique characteristics and heritage associated with products from specific geographic areas, these labels facilitate market differentiation and consumer recognition. Moreover, they create opportunities for rural producers to access new markets, both domestically and internationally, while maintaining a focus on quality and tradition (Partarakis & Zabulis, 2023). As a result, PDO and PGI designations not only contribute to the economic vitality of rural regions but also contribute to the cultural richness and diversity of European gastronomy.

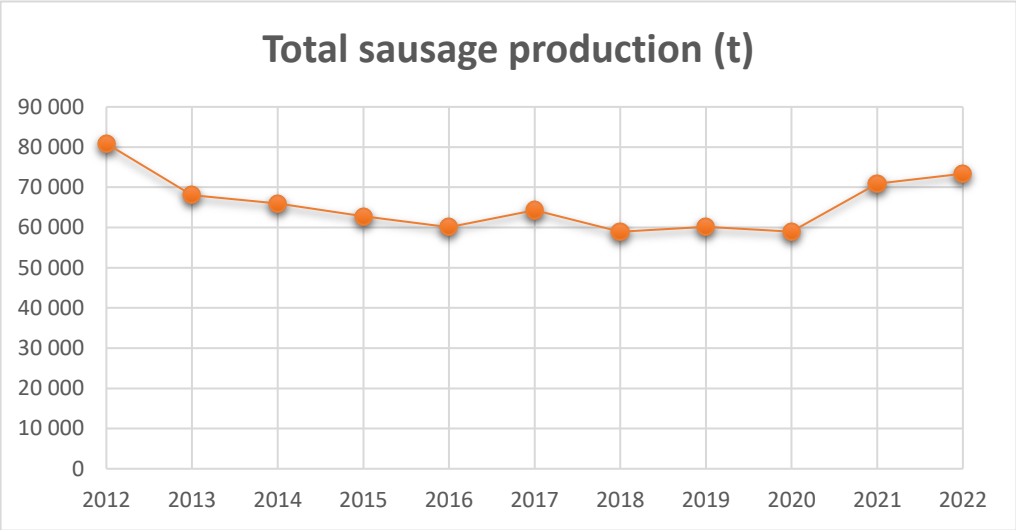

Figure 2. Total sausage production (t) in the North of Portugal for a ten-year period from 2012 to 2022. Source: Instituto Nacional de Estatística - Estatísticas Agrícolas (2022).

The resurgence of interest in traditional foods, fueled by a contemporary shift toward organic choices, has prompted consumers to actively seek meat products possessing enhanced quality, authenticity, and appealing aromatic attributes, coupled with health-promoting properties, due to the perception that traditionally crafted products inherently embody a sense of naturalness and superior quality, contributing to their perceived legitimacy (Michet, 2015; Trichopoulou et al., 2007).

Concurrently, it is acknowledged that small-scale productions, inherent in the traditional methods, often deviate from the standardization seen in industrial processes (Pasquali et al., 2022). This deviation is characterized by a more hands-on approach from staff personnel, introducing a nuanced challenge in maintaining strict control over production parameters. Despite this variability, the allure of authenticity and heightened quality remains a driving force behind the growing preference for traditionally produced meat products in the evolving landscape of consumer choices.

Alheira is a traditional fermented meat sausage made in the Portuguese region of Trás-os-Montes. The production of *alheira* sausage uses various meats which are typically pork and poultry. The final product is a paste that is stuffed in pig/cattle intestinal or cellulose-based casings. Following casing, the *alheira* undergoes a cold smoking process lasting between 2 to 8 days, contributing to its flavor profile while simultaneously reducing water activity (a_w) and adjusting pH levels. (Ferreira et al., 2006). Subsequently, the sausage is subjected to a drying period lasting 1 to 2 days, maintained at a relative humidity of 55% to 65%. This drying stage further enhances the preservation of the sausage while also contributing to its unique texture and taste. The final composition of *alheira* comprises a a_w ranging from 0.9 to 1.0 and a pH level approximately between 5.1 to 5.8 (Patarata et al., 2008).

The sausage (latin *salsus* = meat preserved by salting) preparation is based on seasoned meat and usually have a tangy flavor with a chewy texture (Marchello & Garden-Robinson, 2022), which is consequence of the bacterial lactic acid fermentation performed by lactic acid producing microorganisms that can be added into the meat or inoculated by chance. This process results in a flavorful sausage with distinct sensory characteristics, representing a cherished aspect in the *alheira*.

Recent occurrences of foodborne outbreaks within the European Union (EFSA, 2023) have been linked to the consumption of contaminated meats and homemade sausages (Table 1). These outbreaks have been attributed to various pathogens, including *Salmonella (S.)* spp. (*n* = 289), *Clostridium (C.) perfringens* (*n* = 102), *Staphylococcus (S.) aureus* toxins (*n* = 34) and *Listeria (L.) monocytogenes* (*n* = 17), which are disease-associated microorganisms.

The consequences of such outbreaks are severe, resulting in a substantial toll on public health and the economy. Foodborne outbreak incidents have inflicted economic losses, which solely in the United States was estimated to be up to US$90 billion (EFSA, 2023; Scharff, 2018).

Normally, *alheiras* are cooked before consumption by frying, grilling, or boiling. However, cooking temperatures inside the sausage may not be sufficient to kill all the pathogens (Ferreira et al., 2006). Studies on the microbiology of *alheira* have reported contaminated samples in lots obtained from local producers, that were most commonly

found positive for *S. aureus, Salmonella* spp. and *L. monocytogenes* in concentrations higher than 100 CFU/g (Albano et al., 2008; Ferreira et al., 2007; Silva et al., 2019).

The presence of disease-causing microorganisms in meat and sausage products underscores the critical importance of stringent food safety measures throughout the production and distribution chain. Despite efforts to mitigate risks, the persistence of pathogens such as *Salmonella*, *C. perfringens*, *S. aureus*, and *L. monocytogenes* remains a challenge regarding the safety of meat-based foods. Such challenges require continued vigilance and investment in preventive strategies to safeguard public health and minimize economic burdens associated with foodborne illnesses.

Furthermore, the prevalence of foodborne outbreaks linked to contaminated meats is a stark reminder of the multifaceted impacts of inadequate food safety practices. Beyond the immediate health risks posed to consumers, these incidents also have broader societal and economic implications. They result in consumer distrust in food products, disrupt food supply chains, and impose significant costs on healthcare systems and industries. Addressing these challenges requires a comprehensive approach that encompasses robust regulatory frameworks, enhanced surveillance and monitoring systems, and public education initiatives to promote safe food handling practices and awareness of foodborne risks (Warmate & Onarinde, 2023).

Table 1. Foodborne outbreaks, human cases, hospitalizations and deaths by contaminated meat/meat products and by causative agent. Source: EFSA, 2016-2021.

	Outbreaks	**Human Cases**	**Hospitalizations**	**Deaths**
Listeria monocytogenes	17	378	256	35
Salmonella spp.	289	4 693	967	4
Staphylococcus aureus	34	381	116	0
Clostridium perfringens	102	3 738	24	0

Recently, the sausage's microbiome has been shown to consist of a complex interaction between microorganisms, which can create an inhibitory environment (i.e., by the production of metabolites, changes in pH) for the survival of pathogens and can promote growth of beneficial bacteria, such as lactic acid bacteria (LAB), by mutual exchange of nutrients (Taylor et al., 2020).

Classified as Gram-positive and catalase negative, LAB exhibit diverse morphologies, appearing as either rod-shaped or cocci-shaped cells. LAB thrive in environments with low oxygen levels, relying on anaerobic conditions for their growth and metabolic activities, which is the case in *alheira* since the closed casing doesn't allow entrance of air in the cooked meat/bread/condiments paste and the smoking process contributes to the formation of an anaerobic environment. Microorganisms isolated from *alheira* are mainly LAB (Albano et al., 2008; Ferreira et al., 2007).

LAB are capable of producing antimicrobial metabolites which highlights their significance in biotechnological applications for food preservation and underscores their potential as sources of bioactive compounds, including but not limited to organic acids such as lactic acid and acetic acid, short-chain fatty acids, proteases, peptidases, and bacteriocins (Ibrahim et al., 2021). LAB are pivotal in various biological processes and are recognized for their role as biocontrol agents in food preservation. Therefore, the variability of LAB strains across different fermented products contributes to the diversity of microorganisms constituting the product's microbiome, emphasizing the importance of characterizing these microbes for the enhancement of safety and quality control.

1.2 Objectives

This study aims to investigate the population of LAB in artisanal *alheira* produced in different regions of the North of Portugal by establishing the following objectives:

i. Characterize the physicochemical and microbiological composition of artisanal *alheira* sausages by regional producers in the Northeastern Portuguese region of *Trás-os-Montes*.

ii. Screen indigenous LAB by its physicochemical characteristics e.g., acidification potential, lactic acid production, proteolytic activity, and antimicrobial capacity against the foodborne pathogens: *S. aureus, L. monocytogenes*, and *S.* Typhimurium.

iii. Identify LAB with antimicrobial activity against tested pathogens by 16S ribosomal gene sequencing.

The ultimate goal is to identify and utilize LAB with strong antimicrobial properties as biocontrol agents in the production of various types of sausages and potentially other fermented food products, thereby supporting both artisanal producers and the wider food industry. By doing so, this study could be further utilized for research aiming to improve the safety, shelf-life, and overall quality of fermented food products.

1.3 Outline of the Thesis

The thesis is organized in two chapters. The first chapter consists of a literature review on the use of bacteriocins, lactic acid bacteria metabolic products, towards the preservation of meat and meat products and mitigation of foodborne outbreaks. The second chapter describes the practical work with the *alheira* sausages; therefore, it contains the methods, results and main findings of the study.

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II. Chapter 1: Biopreservation Strategies Using Bacteriocins to Control Meat Spoilage and Foodborne Outbreaks

INTRODUCTION

Meat is source of high-quality proteins, minerals and vitamins (Geiker et al., 2021). However, it is especially prone to spoilage, as it undergoes microbial (e.g., microbiological spoilage), chemical (e.g., autolytic enzymatic reactions), and physical (e.g., slime and liquid formation) deterioration, in fact, estimates show that as much as 23% of the annual production in the meat sector is lost and wasted (Karwowska et al., 2021; Luong et al., 2020; Odeyemi et al., 2020).

Research on meat preservation considers not only the extension of the product's organoleptic features, but also its microbiological safety (Ec No 2073/2005). In the domain of biopreservation, natural or controlled microbial communities and their antibacterial products are used as an approach for controlling microbial growth. An integral component of the initial microbial community of meat is lactic acid bacteria (LAB), which rapidly develops under chill stored, post-processed and vacuum packed/modified atmosphere conditions (Nauman et al., 2022).

LAB are classified as Gram-positive, catalase negative, anaerobic, with a varied morphology (rods or cocci) and play a crucial role in fermentation. Moreover, LAB can be used as biocontrol agents by the generation of metabolites with antimicrobial properties against pathogens including organic acids (e.g., lactic acid, acetic acid), short-chain fatty acids, proteases/peptidases, and bacteriocins (Ibrahim et al., 2021).

This review highlights the recent advances in the optimization of bacteriocin use, considering the bacteriocin's structure, and mode of action. Moreover, the strengths and

weaknesses of different techniques for bacteriocin screening, including novel bioengineering methods are described. Finally, we discuss on the advantages and limitations for different bacteriocins mode of application towards preservation of fresh, cured and novel meat products.

GENERAL OVERVIEW

Bacteriocins are small peptides synthesized in the ribosome and can be categorized into four groups, according to their size, structure and function: class I or lantibiotics (<5 kDa), class II (<10 kDa), class III or bacteriolysins/non-lytic (>30 kDa) and class IV (reclassified as bacteriolysins) (Barcenilla et al., 2023; Simons et al., 2020).

These peptides play a crucial role in the competition for colonization sites and are able to influence the dynamics of the microbiome (Umu et al., 2017). For instance, bacteriocinogenic LAB inhibit target bacteria by interacting with the negatively charged cell membrane, a process mediated by their cationic and amphiphilic motifs (Heilbronner et al., 2021; Lei et al., 2019).

I. MODE OF ACTION

The bioactivity of bacteriocins can be either of narrow spectrum – if the inhibition is exclusive to species that are closely related - or broad spectrum, which is the case for lantibiotics (Woraprayote et al., 2016); in addition to presenting either bacteriostatic (inhibition) or bactericidal (killing) effects (Negash & Tsehai, 2020).

The general mode of action of these antimicrobial peptides is given by their specialized functional domains: substrate binding site, translocation and catalytic site (Gillor et al., 2008). The binding domain attaches to specific receptors on bacterial membranes, the translocation domain interacts with specific proteins integral to the cell membrane, and the effector domain executes lethal action: DNA degradation and/or induction of pores in the membrane (Davidson et al., 2008).

i. NISIN

Lantibiotics act by blocking lipid II cycle (Fig. 3), preventing correct cell wall synthesis, and inducing pore formation by interacting with the outer membrane (Diep et al., 2009). Nisin (grey) is composed of 34 amino acids and has a positive charge (+4), which allows it to interact with the anionic lipid II that constitute the peptidoglycan layer in the bacterial cell membrane. The peptidoglycan is formed by a chain composed of N-acetylglucosamine (GlcNAc; green), N-acetyl muramic acid (MurNAc; red), a pentapeptide (not shown), two pyrophosphate molecules (blue) and a prenyl chain (black).

The lipid II mediated pathway of nisin starts by the recognition of lipid II MurNAc and isoprene units. The dehydrobutyrine and alpha-amino butyric acid of nisin then establish a hydrogen bond with the pyrophosphate molecules of lipid II. The assembly of nisin occurs without a canonical secondary structure, where two lanthionine rings fold to form a cage-like structure with the nisin backbone amides and the lipid II pyrophosphate, with a molar ratio of 8:4 for nisin and lipid II, respectively. Finally, the cage-like structure induces pore formation resulting in disruption of the cell membrane.

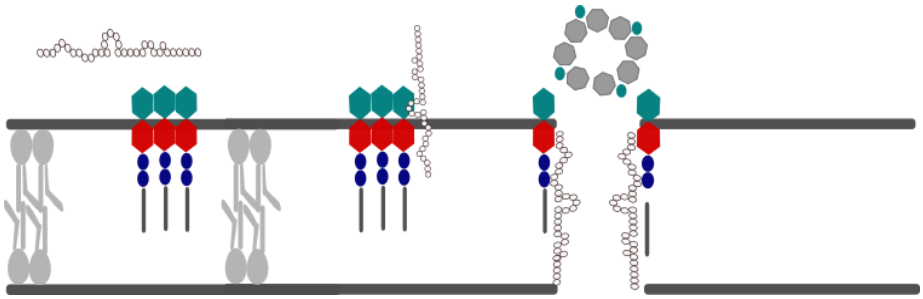

Figure 3. Nisin mode of action, adapted from Hsu et al., 2004.

ii. PEDIOCIN

On the other hand, class II or non-lantibiotic bacteriocins (Fig. 4), divided into four subclasses, utilize the mannose phosphotransferase system (Man-PTS) to permeabilize

the membrane, disrupt proton motive force and deplete ATP pools (Diep et al., 2007). Class IIb bacteriocins activity depends on a two-component, alpha and beta subunits, which folds into alpha-helical structures and insert themselves into target bacterial membranes to alter their permeability, resulting in ion leakage and cell death (Nissen-Meyer et al., 2011; Proutière et al., 2023).

Pediocin PA-1 is composed (Fig. 4) of a hydrophilic and cationic N-terminal (brown) which consists of three-stranded-beta-sheet (containing the pediocin-box) linked by disulfide bridges (black). The region between N- and C- terminal is a flexible region composed of Asp/Asn in the residue 17. The C- terminal is hydrophobic and is organized in an alpha-helix (blue), coupled with a hairpin-like tail (light pink).

The N-terminal beta-sheet of pediocin PA-1 links with the extracellular man-PTS core domain (red). Normally, this transporter protein composed by a v-motif (green) and a core domain (red) switches from position depending on the transport of mannose. The change in conformation occurs by an elevator movement.

The binding occurs with the effector domain of pediocin PA-1 (pediocin-box and positively charged Lysn II and His 12) and the core domain of the man-PTS (Val 7, Cys 9, Cys 14, Tyr 3) establishing a linkage that blocks the elevator movement of man-PTS, with a molar ratio of pediocin PA-1 and man-PTS at 3:3. The hairpin-like tail of pediocin makes pi-stacking interactions with Trp 18 (white) of man-PTS and stabilizes the pediocin-manPTS structure, leading to disruption of the cell membrane (Zhu et al., 2022).

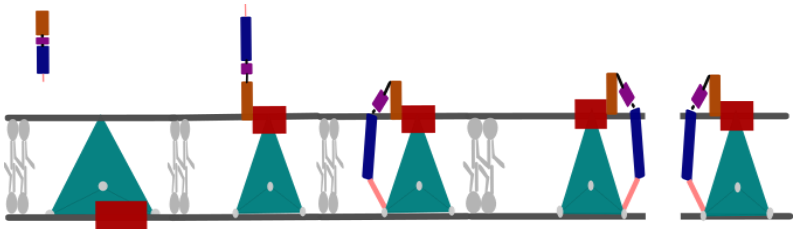

Figure 4. Pediocin-PA-1 mode of action, adapted from Zhu et al., 2022.

II. SCREENING OF BACTERIOCINS

The screening of bacteriocins can be divided into three stages: search for presence of bacteriocin-encoding genes, evaluation of bacteriocin expression and assessment of the bacteriocin antimicrobial activity. For cultivable bacteria, the search for a bacteriocinogenic LAB strain begins with the isolation of autochthonous strains from the meat matrix. Then, the bacteriocin-encoding genes can be amplified by PCR (Polymerase Chain Reaction) and their expression evaluated in real time using RT-qPCR (Real-Time Quantitative PCR). After purification, the bacteriocin's antimicrobial activity can be assessed by antagonism tests (Fig. 5) against indicator microorganisms.

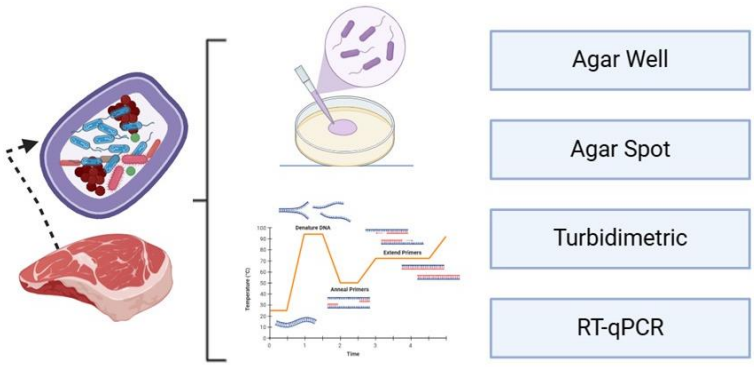

Figure 5. Diagram for different *in vitro* methods for bacteriocin activity screening from foodstuffs.

Several methodologies have been used for the *in vitro* assessment of bacteriocins or bacteriocin-like substances: agar well diffusion (Bungenstock et al., 2020), agar spot (Moraes et al., 2010; Selman et al., 2021), turbidimetric (Piazentin et al., 2022; E. Yang et al., 2018), ELISA (Martínez et al., 2000; Surati, 2020) and RT-qPCR (Balutis, 2014; Dortu et al., 2009; Wan et al., 2015). Moreover, the bacteriocin does not have an antimicrobial effect on the producer strain. These mechanisms are constituted of self-immunity proteins that competitively antagonize the putative bacteriocin receptors by anchoring the membrane surface (antagonism), being embedded in the membrane

(repulsion), or producing metalloproteases that degrade the bacteriocin (Bastos et al., 2015; Deegan et al., 2006).

Culture-based methods towards assessing antimicrobial activity mostly relying on outdated protocols persist on being the most used (Balouiri et al., 2016), in spite of recent advancements in genomics, namely the whole genome sequencing (WGS), RNA sequencing (RNA-seq), and PCR-based techniques. While agar-based tests offer cost-effectiveness and simplicity, particularly at an initial sample screening, they lack the depth necessary to fully explore a strain's bacteriocinogenic potential. Moreover, utilizing different agar-based tests within a single study may introduce intra-study variability and errors due to differing experimental conditions and subjective interpretation of results (Hossain, 2024).

In the case of unculturable microbes, the use of metagenomics tools is utilized since it’s estimated that 99% of microorganisms are not possible to culture in isolation (Ayrapetyan & Oliver, 2016). The DNA of the food sample is extracted, followed by library preparation and then sequencing of all of the DNA present in the sample. This sequencing data enables the visualization of the microbial community composition within the food matrix, providing insights into the relationship between meat preservation and 16S rRNA diversity analysis, as the microbiota continually changes during storage (Dorn-In et. al, 2024).

Shotgun metagenomics enables the discovery of the microbial profile of samples, allowing for the detection of spoilage and pathogenic organisms, as well as differentiation at the strain level (Srinivas et al., 2022). This method can be used to monitor the supply chain for agents of concern, providing valuable insights that incentivize food manufacturers to invest in preventative control measures (Imanian et al., 2022).

The development of new *in silico* techniques have made it possible to analyze bacteriocins in a high-throughput manner (Nedyalkova et al., 2024). In contrast with classical assays for antimicrobial activity determination, the current methods for predicting bacteriocin gene clusters are high-speed and can be automated. BAGEL is a web mining tool that uses whole-genome sequence data to analyze the technological potential of bacterial strains (van Heel et al., 2018).

Mining genomes with automated software (Sowers et al., 2023; Wosinska et al., 2022) for identification of bacteriocins reveal gene loci that can be functional or not. The data generated from this type of analysis has the potential to be exploited by bioengineering, including *de novo* design of novel bacteriocins (Deo et al., 2022; Kordi et al., 2024).

Recent increase in RNA-seq data, which describes the presence and quantity of RNA in a biological sample, demonstrates that RNA-seq can be used to follow survival of target bacteria in the presence of the bacteriocin, by measurement of the expression of genes in food systems. Moreover, it is useful for analyzing the influence displayed by various environmental conditions on gene expression and fine-tune them for achieving optimal conditions (Yang et al., 2024).

Representative genomes on the NCBI platform have been used in comparative genomics to predict peptide expression and secretion by the bacteria (Marques et al., 2023). These tools in combination with molecular dynamics analysis allow automated assessment of the binding mechanism of action performed by bacteriocins (Rodrigues Blanco et al., 2022; Leslie et al., 2021; Walsh, 2017). The computational tools include docking software and three-dimensional structure modelling of the putative peptides (Das et al., 2021; Frederix et al., 2018; Krishnamoorthi et al., 2022; Nain et al., 2020; Palmer et al., 2021; D. Wang et al., 2023; B. Xin et al., 2020).

The development of these novel methodologies enables the fast discovery of structural and functional characteristics of specific amino acid residues, which can be associated with binding sites in the bacterial membrane (Bindu & Lakshmidevi, 2021; Chen et al., 2022; Marques et al., 2023; Oftedal, 2023; Shastry et al., 2020). By understanding the mode of action of bacteriocins, given the varied nature of their structures, strategies to enhance and potentiate the bacteriocin's antimicrobial activity can be further developed (Amarh et al., 2023).

Lantibiotics, for instance, are subjected to post-translational processing, featuring compact structures with modified amino acids (e.g., lanthionine bridges, beta-methyllanthionine, and dehydrated amino acids) and thioether ring structures. In the case of nisin (Fig. 6), its structure comprises a globular chain consisting of lanthionine and dehydrated serine residues. These elements undergo post-translational modifications and proteolytic cleavage during the peptide processing phase, contributing to the unique structure of nisin.

Lactocin S, however, is prone to oxidation due to the sulfide bonds in its alpha and beta rings which results in its inactivation; therefore, the synthesis of this bacteriocin occurs under anaerobic conditions, which could be improved by replacement of the sulfide bonds with hydrocarbon chains in analogs of lactocin S leading to more oxidative stability (Ross et al., 2012; Tsukano et al., 2024).

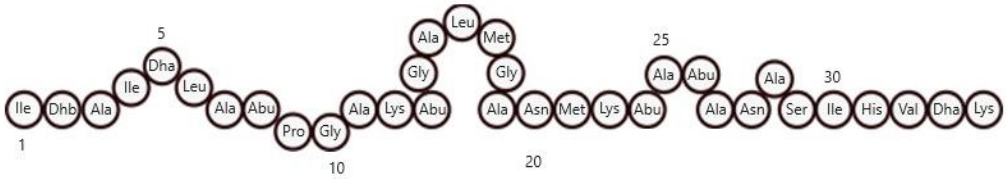

Figure 6. The amino acid composition of nisin adapted from Hsu et al., 2004.

Lantibiotics active site include the amino acids residues such as the catalytic site and the substrate biding site, with the F(ND)L(DEN)(LVI) motif (Fig. 7) being conserved across different bacteriocins from this class. There is a gap on the literature on the correlation between the conserved motifs of bacteriocins and their mode of action considering their 3D structure and its influence on the bacteriocin's activity. Figure 7 depicts predicted 3D structures produced by the Alpha Fold algorithm and visualized with Chimera X (Jumper et al., 2021; Meng et al., 2023; Varadi et al., 2022), N- and C-terminus indicated in the figure, region highlighted in red indicates the conserved region FNDLV, the conserved region of the three peptides appears differently across the space on the 3D structure format.

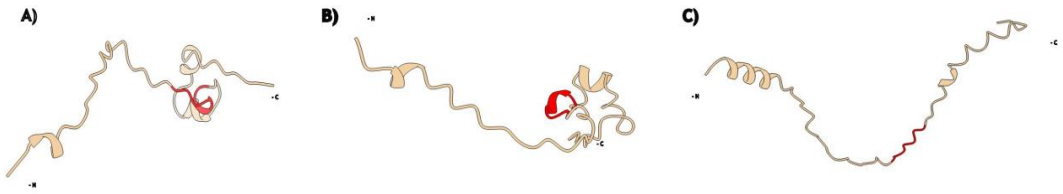

Figure 7. Lantibiotics three-dimensional primary structures. A) Nisin A (P13068); B) Nisin Z (P29559); C) Lactocin S (P23826).

Class IIa bacteriocins are characterized by a conserved sequence at the N-terminal (YGNGV), also known as pediocin-box (Fig. 8), a sequence related to strong anti-listerial activity first described in pediocin PA-1 (Cui et al., 2012). Class IIb is composed of two-short chains. Class IIc are circular bacteriocins that lack the leader peptide sequence and are dependent on the general secretion pathway (sec) for transportation across the cytoplasmic membrane (Choi et al., 2023; Perez et al., 2014). Class IId bacteriocins are typically constituted of NGY residues at the N- terminus and central YxVTK motifs (Yoo et al., 2023); this class covers the remaining single-peptide and non-pediocin-like bacteriocins (Iwatani et al., 2011).

Class II (pediocin-like) suffers cleavage of a leader peptide (Fig. 8) in the N- terminus in order to turn into a mature bacteriocin, which can be seen from the representation of class IIa bacteriocins isolated from meat aligned by the CLUSTAL W algorithm (Drider et al., 2006; Lee & Kim, 2011; Thompson et al., 1994). Sequences were fetched from UniProt. The pediocin-box is highlighted in a black square, hydrophobic (blue), positive charge (red), polar (green) glycine (orange), prolines (yellow), aromatic (cyan) residues. Three-dimensional primary structures of Class IIa bacteriocins (Fig. 9), generated using the Alpha Fold algorithm and visualized with Chimera X shows that the conserved region YGNGV exhibits distinct spatial arrangements within the 3D structures of the different Class IIa bacteriocins. This variation suggests that although the sequence is conserved, the spatial positioning and orientation of this region can differ significantly from one peptide to another (Fig. 9). Such differences in the three-dimensional conformation could influence how these bacteriocins interact with their target receptors, potentially affecting their antimicrobial activity and specificity (Jumper et al., 2021; Meng et al., 2023; Varadi et al., 2022) .

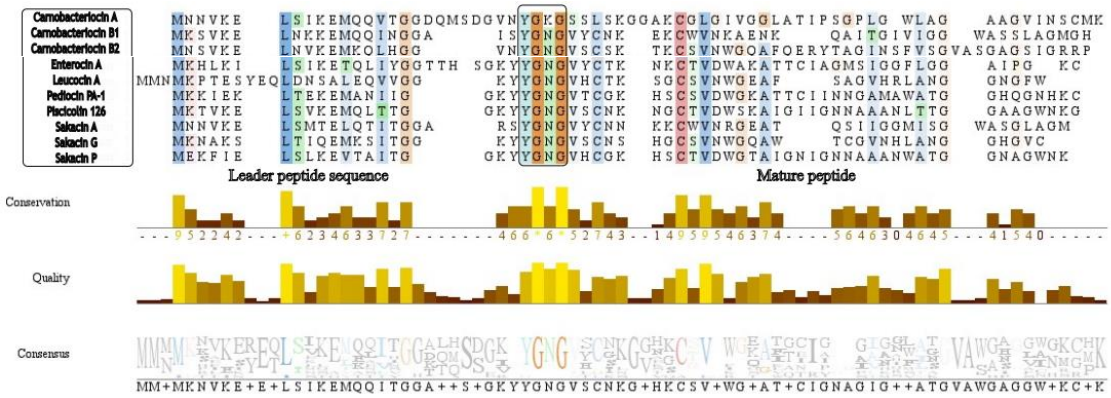

Figure 8. Representation of class IIa bacteriocins isolated from meat aligned by the CLUSTAL W algorithm and visualized in Jalview (Waterhouse et al., 2009).

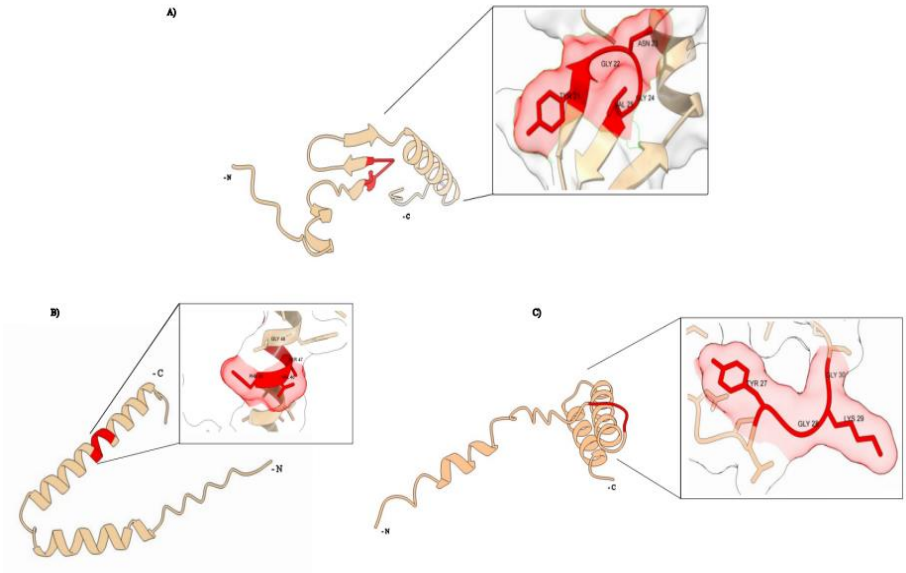

Figure 9. Three-dimensional primary structures of Class IIa bacteriocins. A) Pediocin PA-1 (P29430); B) Divergicin 750 (Q46597); C) Carnobacteriocin A (P38578).

Bacteriocins exhibit robust resistance to high temperatures and low pH due to their specific amino acid composition, a high number of disulfide bridges, and ion pairs (Szilágyi & Závodszky, 2000). The solubility of these peptides increases at low pH due to a net charge change that facilitates greater diffusion through bacterial membranes (Yu et al., 2023). Lantibiotics, in particular, demonstrate strong resistance under extreme conditions. Additionally, owing to their proteinaceous nature, bacteriocins are susceptible to proteolytic enzymes—such as pancreatin complex, trypsin, and chymotrypsin—found in the gastrointestinal tract (Aljohani et al., 2023). The characteristic nature of bacteriocins can be determined by testing their sensitivity to an array of proteolytic enzymes, producing a pattern of protease sensitivity (Bromberg et al., 2004).

WGS has made it possible to identify conserved open reading frames and understand the organization of gene loci encoding the bacteriocin and its immunity genes, in addition, it allowed to predict the promoter and terminator sequence of the peptide from

the DNA data by predicting the RNA-polymerase biding motif, which can be useful for improving expression of bacteriocin encoding genes (Ruiz Puentes et al., 2022).

Promotion of bacteriocin synthesis can be obtained by constitutive expression of genes or by regulating gene expression as a response to the metabolite production from competing strains (Ng & Bassler, 2009; González & Keshavan, 2006). The bacteriocin synthesis gene clusters have been found to be located in the chromosome and in mobile elements such as plasmids and/or transposons (Achemchem et al., 2005; Lahiri et al., 2022). These clusters encode genes for the expression of the bacteriocin itself, enzymes, self-immunity regulators of bacteriocin production and are organized in operons and/or regulons that undergo rapid evolution and are susceptible to high rates of horizontal transfer and spontaneous loss (Almeida-Santos et al., 2021; Mørtvedt & Nes, 1990; Noda et al., 2018).

A series of databases have been developed specifically for information on bacteriocins, for instance, the open-access database BACTIBASE (http://bactibase.hammamilab.org) with information on bacteriocins based on published literature extracted from PubMed (Hammami et al., 2010). LABiocin (https://labiocin.univ-lille.fr/) a database of LAB bacteriocins containing 517 entries extracted from literature searches on Scopus, PubMed/Medline and ScienceDirect with articles published up until 2017 (Kassaa et al., 2019). These databases provide valuable information on structure, amino acid sequence, gene sequence, purification, and physicochemical characteristics of bacteriocins; homology search is an additional feature comprised in these databases which allow for sequence alignment using algorithms such as BLAST (McGinnis & Madden, 2004).

MEAT APPLICATION

Gram-positive bacteria, particularly LAB, are the most studied source of bacteriocins from the meat environment (da Costa et al., 2019). The bacteriocins isolated from LAB in meat and meat products belong to different species, for instance, *Lactobacillus sakei*

from vacuum-packed lamb meat (Holck et al., 1994), *Carnobacterium piscicola* from spoiled meat (Jack et al., 1994), *Leuconostoc gelidum* from processed packaged meat (Hastings et al., 1991), *Leuconostoc carnosum* from packaged meat (Raimondi et al., 2021), *Enterococcus faecium* from dry fermented sausages (Casaus, et al., 1997), *Carnobacterium divergens* from vacuum-packed meat (Zhang et al., 2019) and *Carnobacterium maltaromicus* from vacuum-packed chilled meat (Quadri et al., 1994).

In the last 30 years, bacteriocins have been screened and applied in meat for controlling microbial decay and spoilage, acting as natural inhibitors and extending the shelf life of meat products (da Costa et al., 2019). However, there are still challenges associated with this approach, including the variability in peptide function depending on the nature of the meat matrix – especially in more fibrous matrices, effective inhibition of target microorganisms, resistance development and compatibility with surrounding LAB (Sionek et al., 2024).

Regarding the nature of the meat matrix, challenges arise when applying bacteriocins in meat products primarily due to the hydrophobic nature of the meat and its instability at neutral pH, its interaction with phospholipids derived from meat products and other emulsifiers make it difficult to distribute and solubilize the bacteriocin at pH values higher than 6.0. For instance, in fresh meat, three glutathione molecules are able to conjugate with one nisin molecule resulting in activity loss. However, it is possible to regulate the amount of free sulfhydryl groups present in the matrix, such as with the process of cooking the meat which reduces the free sulfhydryl groups and prevents the formation of the nisin-glutathione complex (Rose et al., 2002).

Strains resistant to a specific class of bacteriocins express immunity genes that confer protection against antimicrobial peptides from different classes, for instance, nisin

resistant strain of *L. monocytogenes* has been reported to show cross resistance to pediocin PA-1 and leuconocin S (Crandall & Montville, 1998; Darbandi et al., 2021).

To overcome the resistance development related to bacteriocins in food systems, it is advisable to apply a combined strategy of peptides in multi-hurdle strategies, for instance, in combination with additives, pH and atmosphere control, recipe modifications with spices and condiments, natural extracts and essential oils as ingredients (Kaur et al., 2013; Soltani et al., 2021).

When classifying bacteriocins isolated from fresh and processed meat products, two main classes based on biochemical structure emerge: lantibiotics and pediocin-like peptides (Woraprayote et al., 2016). There are different methods regarding their practical application as biocontrol agents (Fig. 10) and each come with their set of advantages and disadvantages; for instance, crude preparations are tasteless, colorless and odorless, however, their activity may be limited by a narrow spectrum reach, limited diffusion in solid matrices, and cross-resistance generation (Morata, 2015; Urso et al., 2006). In the section below, we describe the types of bacteriocin application regarding meat preservation.

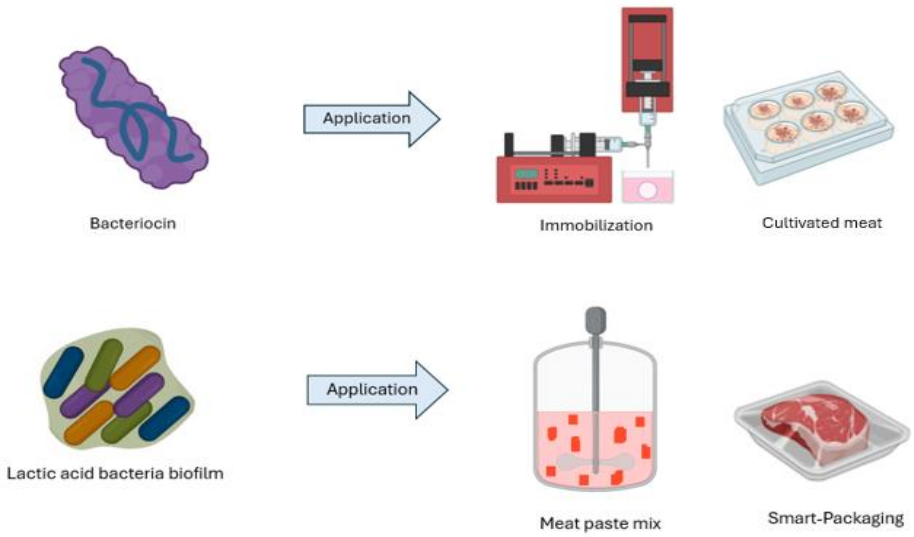

Figure 10. Diagram of lactic acid bacteria biofilm and bacteriocin for different kinds of applications in processing and conservation of meat and meat products.

i. MIXED STARTERS

The use of LAB as an inoculum is the most commonly used mode of application for preservation of foodstuffs. For meat preservation, bacteriocins can be applied as an inoculum of pure or mixed cultures (Baillo et al., 2023), as a crude bacteriocin preparation (Xin et al., 2023) and as a purified or semi-purified formulation (da Costa et al., 2019).

A selection of two autochthonous LAB strains (6.3 log CFU/g) isolated from spontaneously fermented Spanish sausages (salchichón) – *Lactiplantibacillus paraplantarum* BPF2 (producer of leucocin K) and *P. acidilactici* ST6 (producer of pediocin PA-1) were used as starter and reduced levels of rancidity in aroma and taste and improved the intensity and the persistence of the sausage's characteristic flavor (García-López, Barbieri, et al., 2023).

Commercial bacteriocin products such as Nisaplin© (Aplin and Barrett Ltd.) and Bactoferm© (Chr. Hansen AS) often consist of a mixture of crude preparations and organic acids in bioactive powder form. These products offer a mix of lyophilized

starters for use in meat processing industries, such as for the production of salami, pepperoni, dry and cured meats, which are frequently used combined in multi-hurdle strategies with curing, drying and smoking preservative methods (Soltani et al., 2021).

Traditional Iberian cold-smoked fermented sausages underwent testing to assess the antilisterial effect of Bactoferm F-LC©. While Bactoferm F-LC© exhibited bacteriostatic activity at 10ºC, *L. sakei* CTC494 showed a more pronounced and rapid inhibition of *Listeria*. This resulted in a significant reduction of the pathogen by 2 log counts (Ortiz et al., 2014).

The application of mixed starters is beneficial because it combines strains that shorten fermentation time with those that enhance the meat's organoleptic properties. Additionally, strains that produce antimicrobial metabolites can be included to combat hazardous and pathogenic bacteria, thereby increasing the food's microbial shelf life.

However, compatibility between strains remains a significant issue, as the antimicrobial metabolites produced by biocontrol strains can be detrimental to the survival of strains promoting fermentation. Therefore, the choice of strains must consider their competitiveness with surrounding bacteria, the expression levels of bacteriocin synthesis, and their compatibility with the fermenting culture (Zacharof & Lovitt, 2012).

ii. PURE CULTURE

A novel multi-hurdle strategy was developed to extend the shelf life of the Portuguese fermented sausage *alheira*, combining mild high-pressure processing (300 MPa, 5 min at 10 ºC), 0.1% (v/w) *Pediococcus acidilactici* (producer of pediocin PA-1), and 0.1% (v/w) phage Listex. This approach displayed no significant differences in color, texture or lipid peroxidation between unprocessed and minimally processed samples (Komora et al., 2023). In this study, homogenization was manually performed by gently

massaging the sample for approximately 3 minutes, which did not impact the texture. Although the acidifying capacity of LAB can increase the firmness of sausages, the selected LAB in this case was not a strong acidifier, which may explain why the texture remained unchanged.

In contrast, The *E. lactis* Q1, producer of enterocin P, added as a pure culture (10^7 CFU/g) on raw beef reduced *L. monocytogenes* counts by 6 log units after one week of cold storage and improved the sensorial characteristics such as color, odor and appearance, especially since Enterococci are good acidifiers, particularly in meat products (Ben Braïek et al., 2020). When comparing mixed and pure cultures, mixed cultures offer greater advantages due to their diverse LAB, which can be fine-tuned for better product quality. Moreover, while pure LAB cultures are effective in reducing pathogen growth in meats, their efficacy is enhanced when used alongside other hurdle strategies.

iii. CRUDE PREPARATION

The application of the peptide (1,280 AU/g) onto the surface of ham resulted in a 1.74 log reduction of *L. monocytogenes* counts ($p<0.05$); therefore, potentially increasing the ham shelf life to one month in refrigerated storage (4 ºC). Incorporating the peptide in the meat paste produced an inhibition of background spoilage bacteria resulting in significantly lower counts ($p<0.01$). However, *L. monocytogenes* strains were reported to develop resistance to plantaricin UG1 in subsequent generations (Enan, 2006).

A combination of lactocin and high hydrostatic pressure treatments increased *L. monocytogenes* cell death in chilled vacuum-packed pork loin slices (Dallagnol et al., 2017) by a 6 log CFU/g reduction. By applying 200 AU/mL of the pure bacteriocinogenic culture in the salami batter, *L. monocytogenes* was reduced by 2 log CFU/g (de Souza Barbosa et al., 2015).

Pediocin PA-1 reduced the counts of *L. monocytogenes* inoculated in raw chicken meat from 5 log CFU/g to 3.8 log CFU/g when stored at 4 ºC for one month, however re-growth was observed after this period, which could be due to the actions of proteases derived from the meat (Kiran & Osmanagaoglu, 2014).

Choosing between a pure culture of bacteriocin-producing bacteria and a crude bacteriocin preparation involves a nuanced assessment of their respective advantages and disadvantages. A pure culture offers the benefit of continuous bacteriocin production, potentially increasing antimicrobial activity over time and reducing initial preparation costs. However, using live bacteria can lead to compatibility issues, where different strains may inhibit each other's growth and compete for nutrients, resulting in inconsistent efficacy. Additionally, regulatory concerns arise with live cultures, particularly in food applications, due to potential health risks and spoilage.

In contrast, a crude bacteriocin preparation provides predictable and controlled antimicrobial effects, as the activity of the bacteriocins is well-characterized and targeted against specific bacteria. This method bypasses compatibility issues and is generally more acceptable from a regulatory perspective. However, the process of purifying bacteriocins is labor-intensive and costly, with challenges in achieving effective concentrations across different food matrices and target microorganisms. Moreover, maintaining the stability of crude bacteriocin preparations requires careful handling and storage. Ultimately, the choice depends on the specific application, balancing cost, regulatory considerations, and the need for precise antimicrobial activity.

iv. ENCAPSULATION

Challenges with the bacteriocin adsorption in the matrix can be addressed by using immobilized preparations, such as encapsulation on gel coatings, films, silica particles,

or liposomes (Gálvez et al., 2007). Encapsulation of bacteriocins involves incorporating these antimicrobial peptides into a protective matrix or carrier to enhance their stability, control their release, and improve their effectiveness in various applications.

The application of encapsulated bacteriocins combined with citrus extract, and thyme essential oil led to a synergistic antimicrobial activity for meatballs protection against *L. monocytogenes* and all tested LAB; however, there was no effect observed on the inhibition of *S.* Typhimurium (Sarmast et al., 2023). Moreover, the anti-listerial activity of cell-adsorbed bacteriocins combined with oregano essential oil had a synergistic effect on the reduction of *L. monocytogenes* counts and delayed the growth rebound by two weeks in pork meat during storage at 4 ºC (Ghalfi et al., 2007).

Smart packaging represents an advanced approach to packaging design, integrating technology to augment the functionality, safety, and user experience of packaged products. A recent advancement in this domain is the incorporation of bacteriocins into packaging materials, utilizing natural antimicrobial agents to enhance food safety and preservation. Typically, this method employs crude bacteriocin preparations rather than inoculum. Nevertheless, significant gaps remain in the current field, particularly concerning the varying characteristics of different matrices for the establishment of effective smart packaging solutions.

- EDIBLE FILMS

For instance, whey protein-based edible films, enriched with the cell-free supernatant of *Lactobacillus sakei* strains were applied in beef, resulting in a decrease from 3.5 log ± 0.2 CFU/g to 0.3 ± 0.1 log CFU/g of *Escherichia coli* counts after 36 h of refrigerated storage. Moreover, sensory evaluation of grilled beef wrapped with the antimicrobial films demonstrated no significant differences in flavor and color as assessed by the panelists, the overall acceptability was high (Beristain-Bauza et al., 2017).

- POLYTHYLENE-BASED FILMS

A plantaricin solution applied in an active package made with polyvinylidene chloride film in pork fresh meat inhibited *L. monocytogenes* growth by 1.4 log CFU/g after 7 days of cold storage (Xie et al., 2018). Plantaricin BM-1 solution was used to soak polyethylene-based films applied in meat artificially inoculated with *L. monocytogenes* and exerted antimicrobial activity that inhibited the pathogen growth during storage for 120 days at 25ºC (Zhang et al., 2017).

Curvaticin 32Y produced by *L. curvatus* 32Y isolated from dry sausages has been shown to reduce viable counts of *L. monocytogenes* by 1 log when applied in a polythene film by soaking, spray-coating as a preservative for artificially inoculated pork steaks (Mauriello et al., 2004). Moreover, in bioactive packaging made of sawdust particles and poly lactic acid biocomposite film, the adsorption of pediocin PA1-AcH enhanced raw sliced pork meat protection against *L. monocytogenes*, with counts reduced by ~ 2 log units after storage at 4ºC for 14 days (Woraprayote et al., 2013).

- CELLOPHANE COATING

Nisin has been shown to have greater antimicrobial potential in meat preparation when used in combination with organic acids and salts; for instance, nisin reduced the total aerobic bacteria counts in 0.1 log CFU/g from veal meats when applied in a cellophane coating packaging and extended the shelf life of chopped meat under refrigerated storage (Guerra et al., 2005).

- ALGINATE MATRIX

As a component in antimicrobial packaging, nisin had an inhibitory effect against microbial decay and extended the shelf life of refrigerated chicken meat up to 15 days more than the control when applied incorporated in an alginate matrix (Carrión et al.,

2023). A packaging composed of alginate films and containing immobilized viable enterocin-producing *E. faecium* Smr18 reduced *S.* Typhimurium counts by 3 log CFU/g in chicken meat after 34 days at cold storage (Rashid et al., 2023).

CULTIVATED MEAT

Meat grown from animal cells in a laboratory setting is called 'cultivated meat' (CM). CM appears as a new solution to several safety issues with livestock farming such as the zoonotic transfer of viruses and infection through human consumption (Ramani et al., 2021). The development of CM is dependent on the retrieval of animal cells by biopsy, creation of a bank of cells, growth and differentiation by reprogramming stem cells into skeletal muscle cells, harvesting the cells and processing them into tissues.

CM meat can become contaminated by bacteria, fungi and viruses, which is managed by the addition of antibiotics during cell growth, as well as by the addition of cryoprotectants during cell storage. Moreover, risk for contamination during further downstream processing is expected to be similar as in case of traditional meat products (Broucke et al., 2023).

CM production systems are considered to be more sustainable and safer in comparison to the conventional meat production systems, but it may have a completely different risk profile, such a risk coming from antibiotic application *in vitro* to promote growth of cells still consist of a gap in the field and much attention would require to be paid to the safety of added substrates and other compounds of the culture medium to the human health.

As it will be easier to keep control of pathogenic contamination in cultured meat production, CM is associated with fewer risks with respect to microbial contamination. The application of LAB as starters for cultivated meat production is expected to influence the final product similarly than it already affects the meat that incorporates

starter cultures, having a positive effect on organoleptic features and on microbial safety (Kolodkin-Gal, I., Dash, O., & Rak, R. 2024).

CONCLUSION AND FUTURE PERSPECTIVES

Meat is particularly susceptible to spoilage, posing a significant challenge in reducing losses and waste of products. In this context, biopreservation emerges as a natural alternative for extending the shelf life of meat products by managing their inherent microbial communities. LAB are the dominant group present in meat and produce a variety of metabolites with antimicrobial effects, such as bacteriocins, which are also stable in extreme temperatures and pH.

While culture-based methods remain prevalent in assessing microbial inhibitory activity due to their cost-effectiveness and simplicity, they do not explore the potential of uncultured strains. Recent advancements in genomics, such as whole genome sequencing, RNA sequencing, and PCR-based techniques, offer more comprehensive insights into antimicrobial mechanisms. Moreover, computational tools, including docking software and three-dimensional structure modeling, now enable automated assessment of bacteriocin binding mechanisms. This advanced understanding of bacteriocin structures and their modes of action holds promise for developing strategies to enhance and optimize their antimicrobial efficacy in practical applications.

However, challenges with the use of bacteriocins, such as their proper diffusion through the meat matrix and the potential inhibition of closely related LAB, could reduce their effectiveness when used alongside starter cultures. The development of higher efficiency bacteriocin diffusion from packaging surfaces or application sites into meat is influenced by the texture and the thoroughness of mixing between the bacteriocin and the meat, which significantly influences its distribution within the meat matrix.

Looking forward, advancements in 3D printed and cultivated meats are likely to profit from integrating LAB and bacteriocins to improve their safety and functionality. Continued research into bacteriocins is crucial for advancing meat safety and expanding functional meat product quality in the future.

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III. Chapter 2: Genetic Identification and Technological Potential of Indigenous Lactic Acid Bacteria Isolated from *Alheira*, A Traditional Portuguese Sausage

Introduction

Mediterranean artisanal foods play a significant role in the development of rural regions, allowing and stimulating local commercialization, employment of the rural population, and preservation of local heritage. *Alheira* is a naturally fermented meat sausage traditionally made in the Portuguese region of *Trás-os-Montes*. The production of *alheira* uses various meats (most commonly pork and poultry meat) that are comminuted to form a heterogeneous batter that is then stuffed in pig, cattle intestinal or cellulose-based casings (Albano et al., 2008; Ferreira et al., 2007; Patarata et al., 2008).

In addition, the *alheira* sausage undergoes an intermittent cold smoking process lasting 2–8 days that reduces water activity (a_w) and pH. The sausage then goes through a drying process (1-2 days) carried out at 55-65% relative humidity. The final product composition is usually of 35% water, 25% protein, 40% lipids, a_w in a range of 0.9 – 1.0 and an approximate pH of 5.1 – 5.8 (Albano et al., 2008; Ferreira et al., 2007; Patarata et al., 2008; Campos et al., 2013).

These conditions characterize a microbiologically unstable product that allows the growth of pathogenic organisms such as *Staphylococcus aureus*, *Salmonella enterica* and *Listeria monocytogenes*, whose growth can be hampered by the process of fermentation. The fermentation occurs by the action of acid producing bacteria that

lower the pH of the product, these bacteria occur naturally, but can also be added as starter cultures (Albano et al., 2008).

The *alheira* manufacturing process and final composition can vary considerably between regional producers and even within different batches of a factory. These variations stem from differences in the initial formulation, particularly in the quantity and type of meat (such as various carcass parts and different animal meats) as well as variations in fat and bread content. Other quality factors associated with differences in *alheira* composition are the duration of fermentation and extent of drying (Coelho-Fernandes et al., 2020).

Lactic Acid Bacteria (LAB) can be described as gram-positive, catalase-negative and ferment glucose to lactic acid – through either homofermentative or heterofermentative pathways (Doyle et al., 2013). The conventional method for identifying LAB species involves utilizing phenotypic attributes such as determining morphology, fermentation patterns, and lactic acid isomer profiles; or genetic methods, such as 16S rRNA sequencing – a gene which contains both well-conserved and less-conserved regions used to sort different genera and species; or species-specific genes to discriminate those that are closely related (Albano et al., 2008; Moracanin et al., 2013).

LAB associated with food are generally restricted to the genera *Carnobacterium, Enterococcus, Lactobacillus, Lactococcus, Leuconostoc, Oenococcus, Pediococcus, Streptococcus, Tetragenococcus, Vagococcus*, and *Weissella* (Doyle et al., 2013). LAB have been found to be the dominant microorganisms in *alheira* (Coelho-Fernandes et al., 2020; Albano et al., 2008). Importantly, LAB strains, utilized as starter cultures or those developing spontaneously during meat fermentation, must display specific and discernible metabolic attributes. These encompass the capacity to generate acids and

aromatic compounds; curb the propagation of harmful microorganisms; enzymatically degrade proteins (Doyle et al., 2013). These attributes stand as imperative safeguards, ensuring the elevated quality and safety of the final product.

The objectives of this study were: 1) to conduct a phenotypic characterization of LAB isolated from *alheira*, encompassing technological features such as *in vitro* proteolytic activity, production of L-lactic acid, acidifying potential and antimicrobial activity against *Staphylococcus (S.) aureus*, *Salmonella (S.)* Typhimurium and *Listeria (L.) monocytogenes*; and 2) to identify genotypically the LAB species and reveal any associations with desired phenotypic characteristics.

Materials and Methods

Sampling

Fifty-eight *alheira* sausages were obtained from thirteen artisanal establishments located in the towns of *Bragança* (BST, BF, QP, TB), *Mirandela* (CM, FN), *Mogadouro* (BPM), *Vimioso* (BV), *Chaves* (C), *Vinhais* (CSM, AG, AMO) and *Valpaços* (SM, MP) which belong to the Portuguese northeastern region of *Trás-Os-Montes* (Fig. 11). The sausages were acquired unpacked at traditional markets and underwent physicochemical and microbiological analysis. It is worth mentioning that *alheira* sausages were obtained from artisanal producers that do not employ starter cultures in their elaboration.

Figure 11. Map of the geographical location of different alheira collection sites. Source: Instituto Nacional de Estatística (INE).

Physicochemical analysis

The casings enveloping the sausages were removed, and the contents were then cut in small pieces (25 g) and were homogenized in 225 mL buffered peptone water and ten-fold dilutions were prepared thereof. Physicochemical analysis of the sausage included measurement of pH, with a probe (Hanna Instruments, HI5522, Woonsocket, RL, USA) and a_w with an Aqualab meter (4TE Decagon, WA, USA), measured in triplicate. Additional determinations of moisture, dry matter and ashes, were made according to ISO 1442:1997 (direct drying method), ISO 937:2023 and ISO 936:1998, respectively, and the results were expressed in dry matter basis (%). Finally, measurements of carbohydrates (CHO), fat and protein contents (%) were carried out in duplicate and expressed in dry matter basis (%).

Microbiological analysis

Aerobic Mesophilic Count (AMC) was determined in Plate Count Agar (Liofilchem, Teramo, Italy) at 35°C ± 1°C for 48 ± 2 h. Previous reports on AMC demonstrate its

positive correlation with LAB counts (Coelho-Fernandes et al., 2020; Magalhaes et al., 2011; Esteves et al., 2008; Zefanias, 2020).

S. aureus was counted on Baird-Parker Agar supplemented with Egg Yolk Tellurite incubated at 35 °C for 48 h, following ISO 6888-1:2021 and *Clostridium (C.) perfringens* was determined using Tryptone Sulfite Cycloserine Agar with egg yolk supplement and incubated at 35 °C for 24 h, in accordance with the Compendium of Methods for the Microbiological Examination of Foods (Dwivedi et al., 2013).

Additionally, *S. enterica* search was performed using tetrathionate broth (610183, Liofilchem, Teramo, Italy), Rappaport-Vassiliadis broth (610175, Liofilchem, Teramo, Italy), Hektoen enteric agar (610021, Liofilchem, Teramo, Italy), Bismuth sulfite agar (610301, Liofilchem, Teramo, Italy) and Xylose-Lysine-Deoxycholate (DSHB3011, Alliance Bio Expertise, Bruz, France), according to the Bacteriological Analytical Manual (BAM) for *Salmonella* detection in food matrices from Food and Drug Administration (FDA) (Andrews et al., 2023), following the additional step of serological confirmation described in the BAM – with the *Salmonella* Latex Kit for higher specificity (Liofilchem, Teramo, Italy). Samples were assigned 1 if positive and 0 if negative, for the statistical analysis. Determinations were carried out in duplicate.

LAB isolation

In order to isolate LAB from the *alheira* sausages, one-mL volumes from the ten-fold dilutions were incorporated in De Man, Rogosa and Sharpe (MRS) and M17 selective agars, overlayed with agar 1.2%, and incubated at 30 °C for 48 h (Faria et al., 2021). Five typical colonies on MRS and M17 agar (each) were selected for purification and incubated at 30 °C for another 48 h in the respective media – a sampling plan which did

not seek the major fermentation driver, but to evaluate diversity within samples. Finally, isolates were confirmed by catalase (3% hydrogen peroxide) and gram tests, as well as morphologic observation by microscopy. The confirmed isolated LAB were cryopreserved in 25% glycerol at -80 °C until further testing.

Phenotypic characterization

a) Antimicrobial activity

Antimicrobial activity of 335 LAB isolates was tested against three foodborne pathogens: *S. aureus subsp. aureus* ATCC 6538, *S. enterica subsp. enterica* serovar Typhimurium ATCC 43971 and *L. monocytogenes* WDCM 00019 using the agar spot method. Each LAB strain reactivated in MRS or M17 broth after an overnight culture was spotted (3 µl) on solidified MRS or M17 agar plates divided in four spots per plate, respectively. Then, the plates were covered with 10 mL of BHI broth with 0.75% (w/v) bacteriological agar seeded with 1 mL of each bacterial indicator strain (separately) at approximately 8 log CFU/mL – pathogenic strains were revived in 10 mL Brain Heart Infusion (BHI) broth for 16 h at 37 °C. The bacterial strains of *L. monocytogenes* went through an additional step of activation in 5 mL of BHI for 16 h at 37 °C. Cultures were then successively inoculated until reaching the concentration mentioned above. After solidification, plates were incubated at temperatures of 37 °C for 16 h or 10 °C for 10 days (Magalhaes et al., 2011). The inhibition diameter was measured (mm) in duplicate and a control without addition of the LAB was used to validate the results. Sixty-two presumptive LAB with highest antimicrobial capacity at both temperatures were selected. On this subset of strains, the following phenotypic assays were carried out: proteolytic activity, acidifying capacity and L-lactic acid production.

b) Proteolytic activity

For the standard determination of general exocellular proteolytic activity, overnight cultures were spotted (3 µl) onto the surface of milk agar (composed of 10% (w/v) skim milk powder and 2.5% (w/v) agar) and incubated at 35 °C for 4 days. Proteolytic activity was measured as the diameter of the clear zones around each LAB colony (Franciosi et al., 2009).

c) Acidifying capacity

To quantify the general acidifying capacity, each isolate was reactivated separately in MRS or M17 broth overnight (30 °C, 24 h). Then, a loop of culture was placed in 10 mL of sterile reconstituted skim milk supplemented with yeast extract 0.3% (w/v) and glucose 0.2% (w/v) for two successive subcultures (30 °C for 24 h). Sterile reconstituted skim milk (100 mL, initial pH 6.7) was then inoculated with 1 mL of the 24 h activated culture. For the acidification profiling, pH changes were determined using a pH meter (Hanna Instruments, model HI5522, USA) equipped with a HI1131 glass penetration probe during incubation at 30°C during 8 h (t = 0, 3, 6, 8 h), and after 24 h. For every strain, pH data was fitted to a decay curve to characterize acidification capacity (Faria et al., 2021). The following descriptors were extracted from the fitted curves: ΔpH03: pH decrease between t = 0 h and t = 3 h; ΔpH06: pH decrease between t = 0 h and t = 6 h; ΔpH36: pH decrease between t = 3 h and t = 6 h; and pH6: pH at t = 6 h.

d) L-lactic acid production

To quantify the L-lactic acid (g/L) produced by the LAB, isolates underwent a revival process in 10 mL of MRS or M17 broth, followed by an incubation at 37 °C for 24

hours, to ensure growth. Subsequently, the inoculum was transferred to MRS or M17 agar plates, corresponding to the isolation media, for obtaining isolated colonies from the initial pure culture stock. These plates were then placed in an anaerobiosis jar and incubated at 30 °C for 48 hours. Two isolated colonies were carefully selected and combined with 5 mL of saline solution. The measurement of absorbance at 625 nm was done once turbidity reached an estimated 0.5 on the McFarland scale. Samples were adjusted to fall within absorbance values of 0.08-0.13. Following this, 100 µl of the adjusted samples were transferred to 4 mL of MRS broth and incubated at 30 °C for 4 hours. After the incubation period, 1 mL of the culture underwent centrifugation for 5 minutes at 13,000 rpm, and the resulting pellet was discarded. Subsequently, 10 µl of the supernatant was added to 500 µl of deionized water and vortexed. The concentration of L-lactic acid in g/L was determined using the Kit Nzytech L-lactic (NzyTech, Portugal), UV method, following the manufacturer's instructions. The concentration obtained is the sum of the free and esterified lactic acid, based on the spectrophotometric measurement of NADH formed through the combined action of L-lactate dehydrogenase (L-LDH) and D-alanine aminotransferase (Noll et al., 1988).

Genotypic identification

DNA extraction

Pure genomic DNA was obtained using the GF-1 Bacterial DNA Extraction Kit, in order to obtain a DNA library of isolates (Vivantis, Malaysia). LAB isolates were grown in MRS or M17 broth for 24 h at 37 °C, and 3 mL of bacterial culture was centrifuged at 10,000 x g for 2 min to obtain a pellet (Fernandes et al., 2022). The pellet was resuspended in 80 µl of R1 Buffer and treated with 20 µl of lysozyme (50 mg/mL; Vivantis, Malaysia) for 30 min at 37 °C. The cells were centrifuged at 10,000 x g for 3

min to form the pellet, which was resuspended in 180 µl of R2 Buffer and 3 µl of Proteinase K (10 mg/mL; Vivantis, Malaysia) and incubated in a dry bath at 65 °C for 40 min. Then, 3 µl of RNAse A (20 mg/mL; Vivantis, Malaysia) was added and incubated at 37 °C for 10 min, and 372 µl of BG Buffer homogeneous solution was added to the sample and incubated at 65 °C for 20 min. Washing of DNA was done with a clean glass filter membrane, to where it was transferred absolute ethanol (200 µl) and sample (558 µl); and centrifuged at 10,000 x g for 1 min. The membrane was washed with 650 µl of Wash Buffer and centrifuged at 10,000 x g 1 min two times for removal of residual ethanol. Pure DNA in the membrane was eluted in 30 µl pre-heated TE buffer for 2 min, centrifuged at 10,000 x g for 2 min, and stored at -20 °C, as recommended by the manufacturer.

16S rRNA amplification

The primers used for amplification of the 16S rRNA gene (Hou et al., 2018; Mohania et al., 2008) were 27f 5'- AGA GTT TGA TCC TGG CTC AG -3' and 1492r 5'-CTA CGG CTA CCT TGT TAC GA-3' at 5µM (IDT, Belgium), 1X PCR-Buffer (Frilabo, Portugal), 200 µM of each dNTP in a mix (Frilabo, Portugal), 1.25 U of DFS-Taq DNA Polymerase (ThermoFisher Scientific, Portugal), 10 ng/µL of template DNA, adjusted to a 50 µL reaction. The PCR cycle was 94 °C for 2 min, followed by 30 cycles of 94 °C for 10 sec, 62 °C for 20 sec and 72 °C for 1 min (Dwivedi et al., 2013). An 80 mL agarose gel 1% (w/v) prepared with 1X TAE Buffer and stained with 4.7 µL Ethidium Bromide was used to load the samples - 4 µL of PCR product and 1 µL 5X bromophenol blue (Frilabo, Portugal); and 1 Kb DNA (Frilabo, Portugal) Ladder (0.1µg/µL) . Electrophoresis was run at 100 V for 45 min, and the fragments (~1.5 Kb) were visualized in ChemiDocTM (BioRad, Portugal). DNA bands were cleaned up

using GF-1 PCR Clean-up Kit (Vivantis, Malaysia). The volume of samples was adjusted to 100 µL with nuclease-free water and mixed with 500 µL of PCR Buffer. The sample was loaded to a glass filter membrane and centrifuged at 10,000 x g for 1 min; then the membrane was washed with Wash Buffer (750 µL) and centrifuged at 10,000 x g for 1 min two times to remove residual ethanol. Pure DNA in the membrane was eluted in 30 µl TE buffer for 2 min, centrifuged at 10,000 x g for 2 min, and stored at -20 °C. For subsequent sequencing reactions, the concentration (260 nm) and purity of the amplicon (2 µl) was measured with the 260/280 nm absorbance ratio (~1.8).

16S rRNA sequencing

Sequencing reactions were conducted in the facilities of Mountain Research Center (CIMO). The BigDyeTM Terminator v3.1 ready reaction mix was used (ThermoFisher Scientific, Portugal) with 27f and 1492r primers at (3.2 µM), 5X Sequencing Buffer and nuclease-free water to a final volume of 7 µL and mixed with 3 µL of purified amplicon (Hou et al., 2018). Samples were assessed in forward and reverse reactions. The parameters for the sequencing reaction were 96 °C for 1 min and 25 cycles of 96 °C for 10s (denature), 62 °C for 5 s (anneal) and 60 °C (extend) for 4 min. For removal of interferences with base calling, samples were purified with 60 µL of SAM/BigDyeXTerminatorTM bead solution (ThermoFisher Scientific, Portugal) and vortexed at 1,800 rpm for 20 min. Capillary electrophoresis carried out in the SeqStudio Genetic Analyzer (Applied Biosystems, Portugal) was run at 12,000 V for 25 s and the final results were analyzed using the Sequencing Analysis Software v7.0 (Applied Biosystems, Portugal).

Data analysis

Species identification

Sequences from both directions were aligned using the Benchling software to obtain a consensus sequence. Results were aligned with sequences from the National Center for Biotechnology Information (NCBI, USA) using the rRNA/ITS – 16S ribosomal RNA sequences database run with the Basic Local Alignment Search Tool (BLAST) algorithm optimized for highly similar sequences (Altschul et al., 1990). Finally, sequences with identity equal or higher than 97% were accepted as the best match for the LAB isolate at the species level but could not be used to separate closely related species (Mattarelli et al., 2014).

Phenotypic characterization of LAB

Data were divided into three subsets, by foodborne pathogen species: *L. monocytogenes*, *S.* Typhimurium and *S. aureus*. Principal component analysis (PCA) of each subset was performed to assess the contribution of the antimicrobial, proteolytic and acidifying capacities to the differentiation of isolates. The function principal from the psych package was used in R software (version 4.3.0, R Foundation for Statistical Computing, Vienna, Austria), where a varimax-rotated solution for two principal components was obtained. Projections of the sample scores onto the span of the principal components were produced by using the function prcomp from the factoextra package. Heatmaps were created using the pheatmap function (Kolde, 2019) to find relationship patterns within the samples. The legend of the variables in the PCA are the following: Mean inhibition diameter (ID) in mm at 10 °C for *L. monocytogenes* (ID_10 °C Listeria), *S.* Typhimurium (ID_10 °C Salmo), *S. aureus* (ID_10 °C Staphy) and at 37

°C (ID_37 °C Listeria), (ID_37 °C Staphy) and (ID_37 °C Salmo). pH values: pH6 (after 6 h), Δ03 (drop between 0 h and 3 h), Δ06 (between 0 h and 6 h), Δ36 (between 3 h and 6 h). Proteolytic activity (ProteolyticAct) in mm and Concentration of L-lactic acid (LAC) in g/L.

Results and Discussion

Genetic identification of Lactic Acid Bacteria Isolates

The results of sixty-two isolates of LAB identified by 16S gene sequencing are shown in Table 2. Considering the results at the species level, *Enterococcus (E.) faecium* was the most common, accounting for 32.3% of the total, followed by *Leuconostoc (L.) mesenteroides* (19.4%), *Latilactobacillus (Lb.) sakei* (17.7%), *Lactiplantibacillus (Lb.) plantarum (6.5%)*, *Pediococcus (P.) pentosaceus (4.8%)* and *Weissella (W.) viridescens* (1.6%).

Reports on the microbiological composition of *alheira* have demonstrated that the genetic classification of LAB isolated from this fermented sausage is heterogeneous. According to a previous study on the evaluation of *alheira* sausages collected from different production plants, the most common species were *Lactobacillus (L.) plantarum* (72 out of 90) and *E. faecalis* (87 out of 159) (Albano et al., 2009).

Table 2. Identification results of 62 isolates of lactic acid bacteria by 16S gene sequencing. The main identity expressed is the mean percentage and in brackets are the [minimum; maximum] individual values.

N. isolates	Species	GenBank	Identity (%)
20	*Enterococcus faecium*	NR_115764.1	99.2 [98.4;100.0]
6	*Lacticaseibacillus paracasei*	NR_025880.1	100.0 [100;100.0]
2	*Lactiplantibacillus herbarum*	NR_145899.1	99.5[99.3;99.6]
3	*Lactiplantibacillus plajomi*	NR_136785.1	98.9 [98;99.8]
4	*Lactiplantibacillus plantarum*	NR_042394.1	98.5 [97;100.0]
11	*Latilactobacillus sakei*	NR_115172.1	99.8 [99.6;100.0]
4	*Leuconostoc mesenteroides*	NR_074957.1	98.5 [97;100.0]
8	*Leuconostoc mesenteroides subsp.*	NR_157602.1	99.9 [99.8;100.0]
3	*Pediococcus pentosaceus*	NR_042058.1	99.5 [99.4;99.6]
1	*Weissella viridescens*	NR_040813.1	100.0

In addition, our findings reveal *L. mesenteroides* as one of the most common LAB (12 out of 62). Previous reports on the *alheira* sausage composition have indicated that the counts of LAB identified as belonging to the *L. mesenteroides* species corresponded to only 4 out of 283 isolates (Albano et al., 2009). Such variations can be attributed to the sausage's production – recipe and ingredients, fermentation – smoking/drying and storage methods.

Interestingly, *L. mesenteroides* species have been demonstrated to display technological attributes, for example, a strain isolated from a traditional Serbian sausage made with garlic, sweet pepper, fat and pork exhibited the capability to produce bacteriocins with antilisterial activity, suggesting a potential role of this species in enhancing the safety and quality of cured meat products (Moracanin et al., 2013).

Moreover, the LAB classified as *Lb. sakei* had a relative abundance of 18% within *alheira* isolates. This specific species was found to be the dominant (40% relative

abundance) and the major fermentation driver in a metagenomics assessment of the *Salame Piemonte* meat sausage, an Italian sausage with a short maturation period (Franciosa et al., 2021). However, members of the *Lb. sakei* species are usually found in low numbers in the *alheira* composition (Albano et al., 2009). Such a difference could be attributed to the meat used for the preparation of the sausage: while *alheira* meat is cooked, the meat used for *Salame Piemonte* manufacturing is raw.

Identification results indicate there are strains within the same species associated with different producers. Small-scale meat processing typically involves using meat that is produced and transformed on-site, leading to greater variability among producers**.** By definition, an artisanal food manufacturing and processing have a high hands-on workload, this manual aspect of the food manufacturing leads to a less standardized final product. Such a difference implies a potential variation in the manufacturing conditions within batches, denoting that conditions within *alheira* sausages from a particular producer do not lead to a uniform collection of strains (Ferreira et al., 2006).

This phenomenon was observed previously in a study where the heterogeneous metagenomic content between three batches of spontaneously-fermented dry sausages that were submitted to standard production parameters – recipe, ingredients and ripening yielded distinct microbial profiles. The source of the differences was derived from the natural microbial composition of the meat employed in the sausage-making (Franciosa et al., 2021). Since there are no standard manufacturing processes in the production of artisanal *alheira* sausages, small differences are likely to appear between batches from the same producer. Further evaluation of the genomic content from individual strains might reveal specific niche adaptations

Alheira physicochemical and microbiological analysis

Correlations of the physicochemical and microbiological analysis of a total of 58 *alheira* sausages are compiled in Fig. 12a; and a subset of 22 selected samples from which LAB with high antimicrobial activity were isolated are shown in Fig. 12b. The heatmaps offer a comprehensive visualization of the interrelationships among the assayed attributes. Regarding the protein content as an example, the producers with lower amount of meat in the recipe used a high amount of fat, a typical case are the sausages manufactured by the AG producer that had a mean protein content of 13.15% and a mean fat content of 48.86%, whilst BV producer sausages were composed of 28.64% of meat and 34.75% of fat. Previous research on the nutritional value of *alheira* sausages have reported an average protein and lipid content of 15% and 35%, respectively (Ferreira et al., 2007; Patarata et al., 2008; Albano et al., 2007; Campos, 2013; Coelho-Fernandes et al., 2020; Zefanias, 2020).

Moreover, the composition of the fat in the *alheira* was previously classified as monounsaturated fatty acids (MUFAs) and saturated fatty acids (SFAs), although polyunsaturated fatty acids (PUFAs) could be detected in lower amounts (Campos et al., 2013). Overall, the fat composition reflects the animal's diet which is more likely to include fatty components, this is particularly common in swine (Price, 1987). The carbohydrates composition was predominantly high across the different producers, which is a consequence of the use of moistened bread in the sausage preparation, these proportions vary according to the amount of meat and fat on the producer's recipe (Patarata et al., 2008). The mean carbohydrates content of *alheira* in this study was 39.40% (n=58), when compared to previous reports this value was considerably higher: 19.46% (n=40) (Patarata et al., 2008), 21.5% (Campos et al., 2013) and 15.2% (Ferreira et al., 2006).

The *alheira* is considered a high moisture food product. The mean value of a_w in the current study was 0.986 ± 0.008 (n=58), which causes great concern regarding safety and quality since a high a_w value makes the environment favorable for the growth of foodborne pathogens and spoilage microorganisms with minimum a_w up to 0.98, which is the case for *Salmonella* spp. 0.94, *Staphylococcus* spp. 0.86, *Clostridium botulinum* 0.94, *L. monocytogenes* 0.97 (Bintsis et al., 2017). The mean pH of the sausages was 4.25 ± 0.23, the highest pH was 4.68 and the lowest was 3.88; previous reports have demonstrated similar values of pH (4.92) (Patarata et al., 2008). The low pH is a consequence of the fermentation promoted by the LAB naturally occurring in the sausage (Doyle et al., 2013). Moreover, a low pH value (5.5 or less) result in decrease of water binding capacity and provide conditions that are less favorable to spoilage (Price, 1987).

The ash content has been previously shown to be highly correlated ($r = 0.88$, $p < 0.001$) with NaCl that is used during the seasoning of the meat. In our results, the mean ash content was 3.59 ± 0.87%, comparable to another reference study that found 2.21 ± 1.20% (Campos et al., 2013). Additional hurdles used in the making of *alheira* are the exposure to the products of wood combustion that provide protection against spoilage microorganisms. Such by-products confer a bacteriostatic activity mainly driven by formaldehyde – which is the main component of the smoke, aliphatic acids, other aldehydes and phenolic compounds (Price, 1987). These antioxidant compounds contribute to the preservation of the meat by protecting lipids from oxidation.

The microbiological quality of these sausages was determined, and the mean AMC results were 8.80 ± 1.58 log CFU/g. Similar results were obtained from previous research, such as 8.42 ± 1.26 (Coelho-Fernandes et al., 2020), 8.5 ± 0.6 (Magalhães et al., 2011) and 8.28 ± 0.67 (Esteves et al., 2008). AMC is a generic test for mesophiles –

that grow at temperatures in the range of 25 to 40 ºC. A research study (Ferreira et al., 2006) compared the *alheira* microbiological analysis results with the guidelines of microbiological quality for ready-to-eat foods (Gilbert et al., 2000). However, this guideline states microbiological quality limits of AMC from smoked sausages were classified as not appropriate for measurement of poor hygienic quality. In fact, high microbial counts were previously thought to be involved with *alheira* spoilage, however a high AMC in *alheira* did not reflect organoleptic signs of deterioration (Esteves et al., 2008). Taking into consideration the naturally fermented nature of *alheira*, a high AMC is expected. Moreover, previous reports have demonstrated the positive correlation of AMC with LAB counts – the dominant microflora (Coelho-Fernandes et al., 2020; Esteves et al., 2008). In this study, the microbiological counts of *Lactobacillus spp.* were 9.83 ± 1.03 log CFU/g and *Lactococcus spp.* were 9.22 ± 1.32 log CFU/g. AMC has been shown to double in *alheira* from the immediate preparation and filling manufacture step to the final smoked dry sausage (Magalhães et al., 2011).

Regarding the microbiological safety, the mean counts of *Staphylococcus spp.* were 2.46 ± 1.40 log CFU/g, with a minimum value of 0.699 log CFU/g and a maximum value of 6.06 log CFU/g. Eleven out of fifty-eight sausages were considered unacceptable and potentially hazardous since *Staphylococcus* spp. counts were superior to 4 log CFU/g (Gilbert et al., 2000). Similar results have been previously reported in *alheira* analysis, mainly attributed to the manual handling of the sausage components during filling. The pathogen occurrence could also arise from cross-contamination from initially contaminated meat carcasses, equipment or utensils (Magalhães et al., 2011; Esteves et al., 2008; Zefanias et al., 2020).

The mean *Clostridium* spp. counts were 1.07 ± 0.94 log CFU/g with minimal values of 0.699 log CFU/g and the maximum was 4.127 log CFU/g. Eight out of fifty-eight

sausages were unsatisfactory in terms of microbiological safety once that the colony counts had values higher than 2 log CFU/g. Moreover, one sausage was considered potentially hazardous with a 4.13 log CFU/g pathogen load. These results are to be viewed with caution in the context of public health (Gilbert et al., 2000). Previous reports on *alheira* microbiological safety have shown mean values of 1.2 ± 0.5 log CFU/g which were in the acceptable range (Coelho-Fernandes et al., 2020). Pathogenic microorganisms including *C. perfringens*, *Salmonella* spp. and *S. aureus* are the most prevalent in *alheira* sausages (Esteves et al., 2008). The sporulated form of *C. perfringens* is resistant to extreme temperatures, similar to the enterotoxin produced by *Staphylococcus aureus.* The high levels of contamination observed after analysis of samples are related to poor hygienic and sanitary manufacturing conditions.

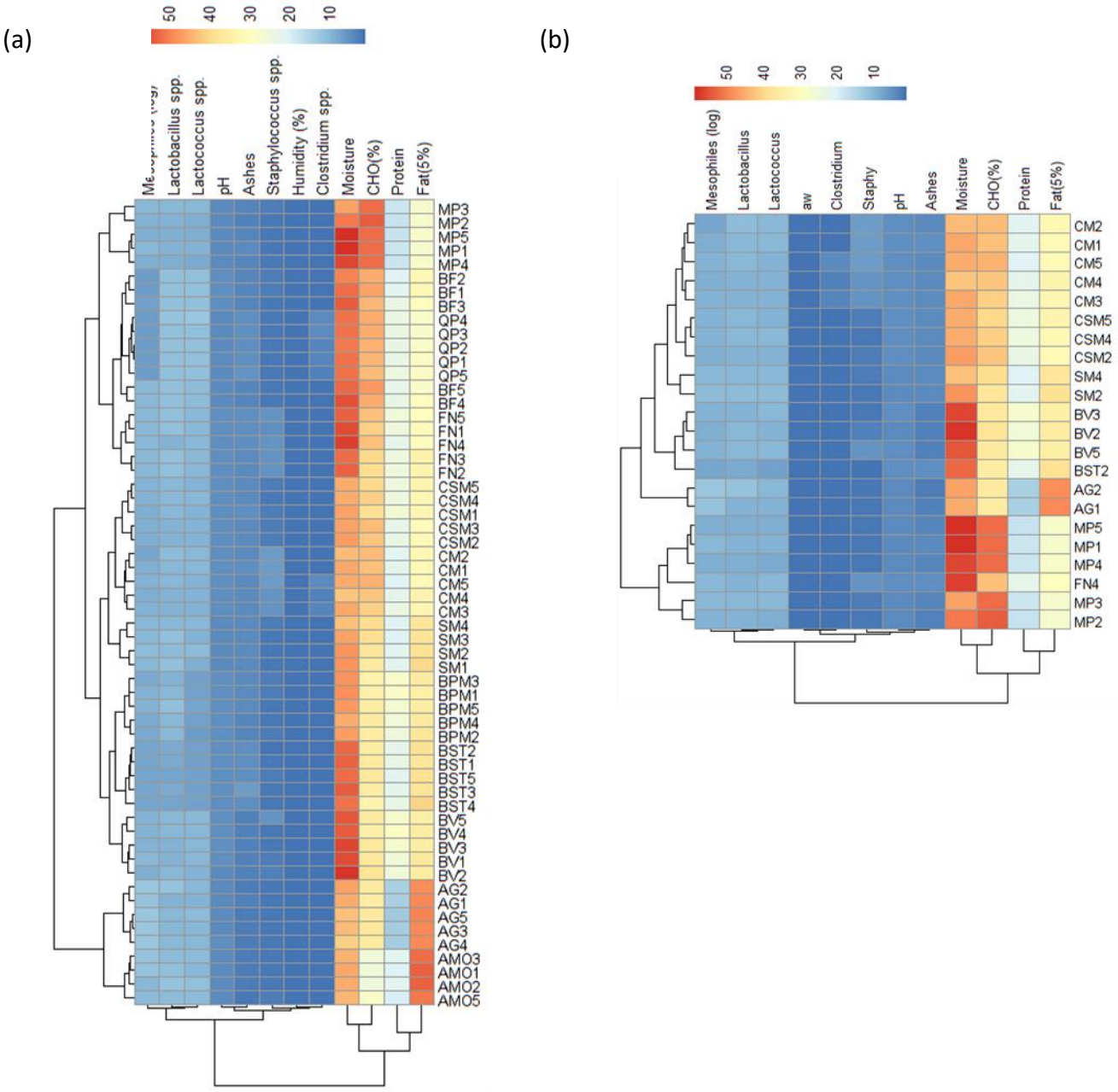

Figure 12. Heatmap of physicochemical and microbiological data of (a) total (n=58) and (b) selected (n=22) alheira sausages (n=59). Legend: The variables are described in Data Analysis section.

Lactic acid bacteria phenotypic and genetic analysis

The phenotypic and genetic features of sixty-two LAB isolates are shown in Fig. 13, the phenotypic analysis of LAB indicated diverse acidification capacities. For instance, *E. faecium* (n=20) had the steepest (0.516) pH drop between time intervals 3 and 6 h, while *Lb. herbarum* had a pH drop of 0.056 during the same period. Previous research

on the acidifying ability of enterococci has demonstrated that the more rapid acidifiers were from strains of food origin (Sarantinopoulos et al., 2001). However, the collection of strains selected in this study appear to behave as slow fermenters since the reduction of pH was achieved by only 0.3-0.9 units.

In the case of *alheira*, the volatile acids derived from the smoke have an additional role in reducing the pH, which influences the metabolic traits of fermenter LAB. The rate of pH fall has an equally if not more important role than the final pH in determining the physical properties of the meat. The glycolysis performed by LAB in the *alheira* positively influences the preservation of the product by lowering the pH. A low pH value and a_w at the beginning of the processing minimizes the proliferation of pathogens and detrimental bacteria (Ferreira et al., 2007).

In our study, *E. faecium* was the most abundant LAB. However, the presence of enterococci in the *alheira* and other traditional Portuguese fermented sausages has been pointed out as controversial due to enterococci's behavior as opportunistic pathogens. In fact, *E. faecium* isolated from these sausages were associated to resistance to antibiotics such as erythromycin (36.4%) and tetracycline (2.3%). This resistance could hinder effective treatment to nosocomial infections associated with enterococci (Barbosa et al., 2009).

Regarding the inhibitory effects displayed by the assayed LAB against foodborne pathogens, lactobacilli presented a high (<10 mm) inhibition diameter (ID) against the pathogens *S. aureus*, *L. monocytogenes*, and *S.* Typhimurium. The isolates identified as *Lb. plajomi* and *Lb. plantarum* could inhibit *L. monocytogenes* at 10 °C with ID of 21.62 and 19.77 mm, respectively. However, when tested under a higher temperature (37 ºC) the isolates with antilisterial activity were identified as *Lb. herbarum* (ID=11.79

mm) and *Lb. sakei* (ID=11.35 mm). These findings indicate a variation on the most effective antilisterial strain according to the temperature, the inhibition could then be associated to the action of enzymatic compounds. In fact, the production of antibacterial compounds is a natural way of preserving the meat and can be produced in combination with organic acids (Albano et al., 2007; Albano et al., 2009; Azevedo et al., 2024). Moreover, the results of lactic acid production indicate that the inhibitory action of organic acids is likely to have contributed to the antilisterial activity displayed by these lactobacilli strains which yielded 0.4-0.5 g/L of lactic acid.

Similar patterns were observed in LAB antimicrobial activity against *S. aureus*. Anti-staphylococcal activity was mainly promoted by *Lb. plajomi* (ID=11.91 mm) and *L. herbarum* (ID=11.29 mm) at 10 °C. While at 37 °C inhibition of antimicrobial growth was promoted by *Lb. paracasei* (ID=9.06 mm) and *Lb. plantarum* (ID=8.29 mm). Antimicrobial activity of *L. mesenteroides* against *S.* Typhimurium remained consistent at both temperatures. At 10 °C, *Lb. herbarum* (ID=11.21 mm) and *L. mesenteroides* (ID=10.89 mm) exhibited the highest inhibition. Similarly, at 37 °C the inhibition was displayed especially by *L. mesenteroides* (ID=12.89 mm) and by *Lb. plantarum* (ID=11.56 mm).

The application of LAB as protective cultures is proven effective in prolonging the shelf life of meat and meat products (Doyle et al., 2013; Albano et al., 2007; Albano et al., 2009; Azevedo et al., 2024). Previous reports have demonstrated the use of LAB from *alheira* as biocontrol agents. For instance, a partially purified bacteriocin produced by *Lb. plantarum* 9A3 was added to a culture of *L. monocytogenes* during its exponential phase and effectively repressed pathogenic growth by 3.57 log CFU/mL for a 12 h period (Azevedo et al., 2024). Moreover, a *P. acidilactici* HA-6111-2 producer of pediocin PA-1 presented antimicrobial activity against *L. innocua* when added (10^9

CFU/mL) as an inoculum in an artificially contaminated *alheira* paste with 10^6 CFU/mL of *L. innocua*. The bacteriocinogenic LAB could inhibit the growth of the pathogen from an initial population of 4.5 log CFU/g to 2 log CFU/g after 54 days at cold storage (Albano et al., 2009).

Regarding the proteolytic activity displayed by LAB, it has been previously observed that the production of proteases influences changes in texture and aroma of the meat (Kieliszek et al., 2021). This influence can be negative, for instance, the products of protein degradation such as mercaptans, amines and fatty acids result in foul odors and flavors indicative of the development of putrefaction and rancidity (Price, 1987). In the case of *alheira*, the ingredients (meat, spices, condiments) are boiled with water at 100 ºC for 30 min. During this process, many physicochemical and sensorial changes occur in the meat, for instance, the pH increases, the collagen softens, and the typical aroma develops by the release of volatile sulfur containing compounds, generated by the degradation of amino acids such as cysteine and methionine (Zefanias, 2020; Price, 1987). Other amino acids such as leucin, phenylalanine and isoleucine have been associated with the acidic flavor (Toldrá, 1998).

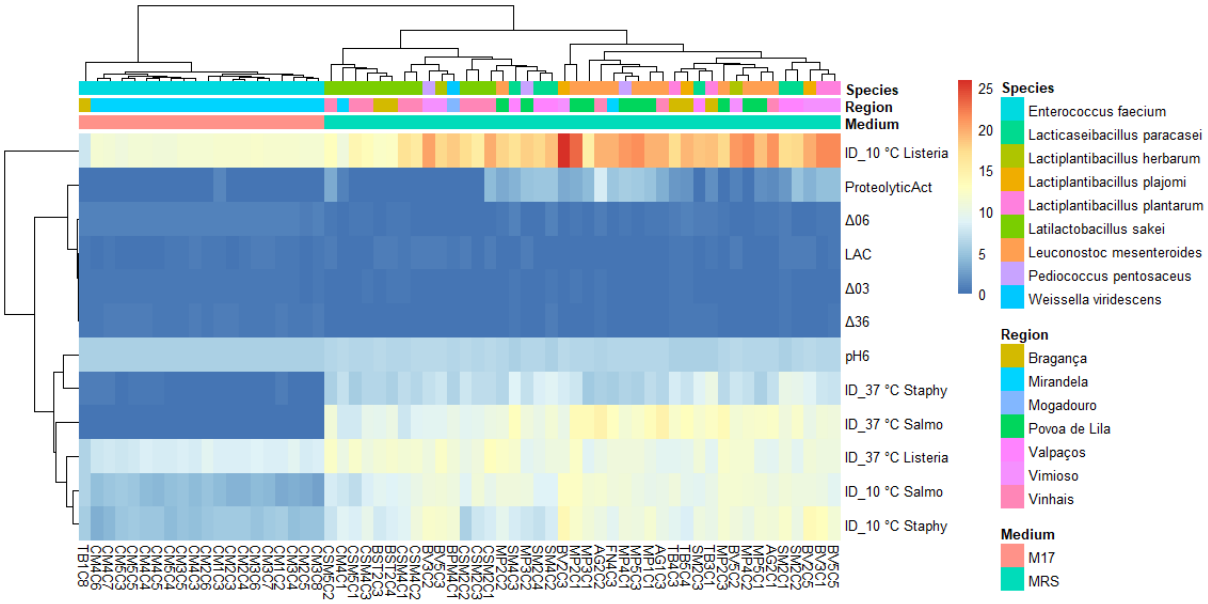

Figure 13. Heatmap of physicochemical characterization data of lactic acid bacteria (LAB) isolated from alheira and species identification of LAB isolates. Legend: See Data Analysis section.

Previous reports on the proteolytic activity displayed by lactobacilli indicate that most species can bring about proteolysis, but to different extents (Kieliszek et al., 2021). Our findings demonstrate that the highest proteolytic activity (3.46 mm) was promoted by *Lb. paracasei* strains, while *Lb. herbarum* had the lowest value (0.41 mm) among lactobacilli. In a previous study, 133 LAB isolated from a Chinese fermented sausage were screened for proteolytic activity (Cao et al., 2019). Most of the strains (63.16%) could effectively hydrolyze myofibrillar proteins and 57.74% sarcoplasmic proteins. These strains were identified as *Lb. plantarum*, *Lb. pentosus* and *Lb. fermentum*. Moreover, proteolytic enzymes have been used to improve meat sensorial attributes, shorten maturation time and delay lipid oxidation (Kieliszek et al., 2021).

To undertake a more comprehensive investigation into the variability of physicochemical attributes exhibited by LAB, a PCA was employed. Isolates were annotated with their species-level genetic identification. The dataset was segregated to

account for the presence of the three foodborne pathogens that were the subject of analysis, yielding three distinct PCA maps (depicted in Table 3).

Table 3. Map of the first and second principal components of the tested technological properties of lactic acid bacteria (LAB) isolated from alheira sausage and species identification of LAB isolates. Legend: See Data Analysis section.

Variables	*S.* Typhimurium		*L. monocytogenes*		*S. aureus*	
	PC1	PC2	PC1	PC2	PC1	PC2
ProteolyticAct	0.538	0.609	0.494	0.653	0.508	0.612
pH6	0.942	-0.232	0.960	-0.159	0.950	-0.175
ΔpH03	-0.896	0.212	-0.903	0.137	-0.901	0.173
ΔpH06	-0.956	0.225	-0.968	0.152	-0.961	0.173
ΔpH36	-0.927	0.218	-0.940	0.149	-0.931	0.162
LAC	-0.068	-0.771	-0.032	-0.783	-0.032	-0.812
ID_10C	0.884	0.209	0.758	0.387	0.834	0.218
ID_37C	0.872	0.299	0.784	-0.099	0.834	0.143
% of variance	66.3	16.2	62.4	16.1	64.4	15.2
Cumulative % of var.	66.3	82.447	62.4	78.5	64.4	79.6

For each map, two principal components were retained (based on their eigenvalues surpassing 1), accounting for a substantial portion of the variation (approximately 80%). In the context of Fig. 14, an analysis was conducted on a subset of LAB with an antimicrobial activity against *S.* Typhimurium at 37 °C and 10 °C (PC1: 66.2%; PC2: 16.2%).

The variables ΔpH36, ΔpH06, pH6 and the inhibition diameter at both temperatures presented the highest loadings, this component primarily measured the antimicrobial activity and acidifying capacity of the LAB. Moreover, there was a stronger positive correlation with the variables ΔpH03 (0.212) and proteolytic activity (0.609) within the second component compared to the lactic acid (-0.771) yield (Table 3).

A similar pattern was observed for *S. aureus* (Fig. 15; PC1: 62.4%; PC2: 16.1%) and *L. monocytogenes* assessments (Fig. 16; PC1: 64.4%; PC2: 15.2%). However, the influence of different temperatures on inhibition diameters was more evident in the

latter, as indicated by the second component, at 37 ºC (0.387) and 10 ºC (-0.099). The overarching acidifying capacity was considerably attributed to the action of *E. faecium*.

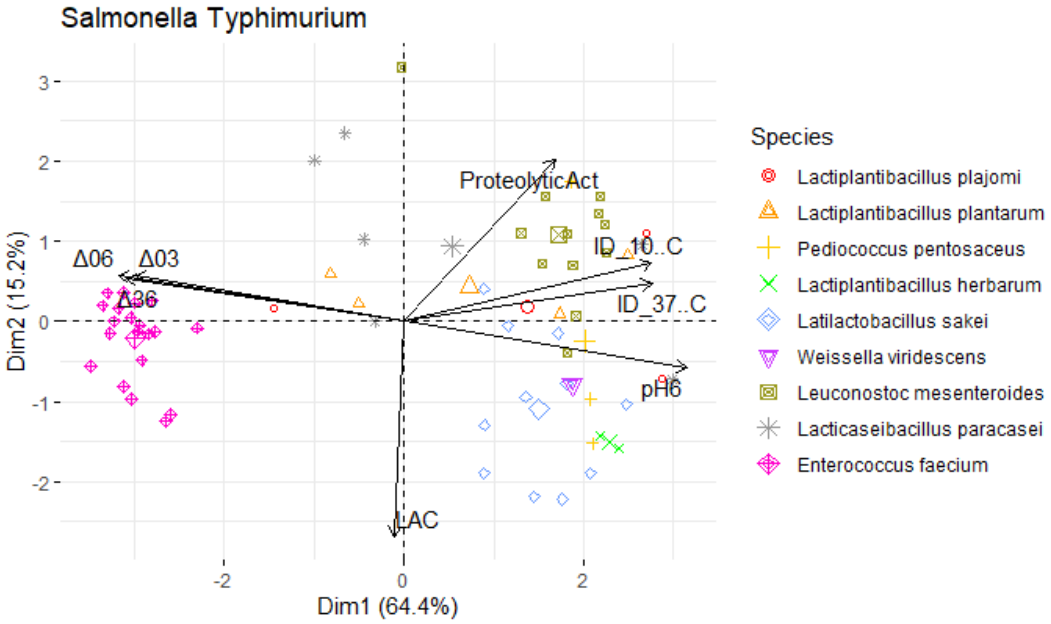

Figure 14. Map of the first and second principal components of the tested technological properties of lactic acid bacteria (LAB) against *Salmonella* Typhimurium isolated from alheira sausage and species identification of LAB isolates. Legend: See Data Analysis section.

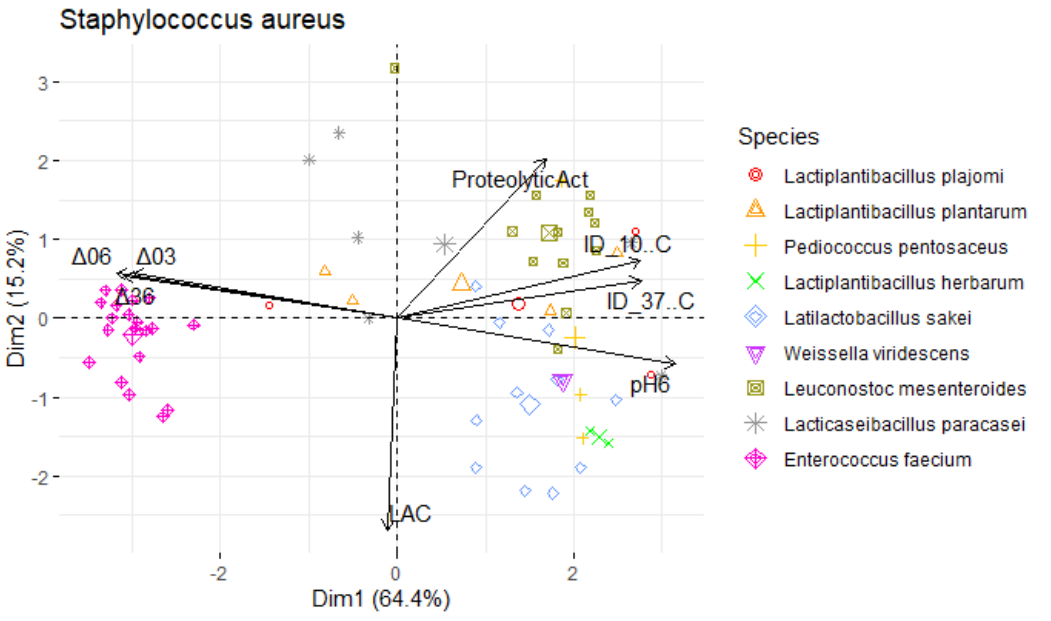

Figure 15. Map of the first and second principal components of the tested technological properties of lactic acid bacteria (LAB) against *Staphylococcus aureus* isolated from

alheira sausage and species identification of LAB isolates. Legend: See Data Analysis section.

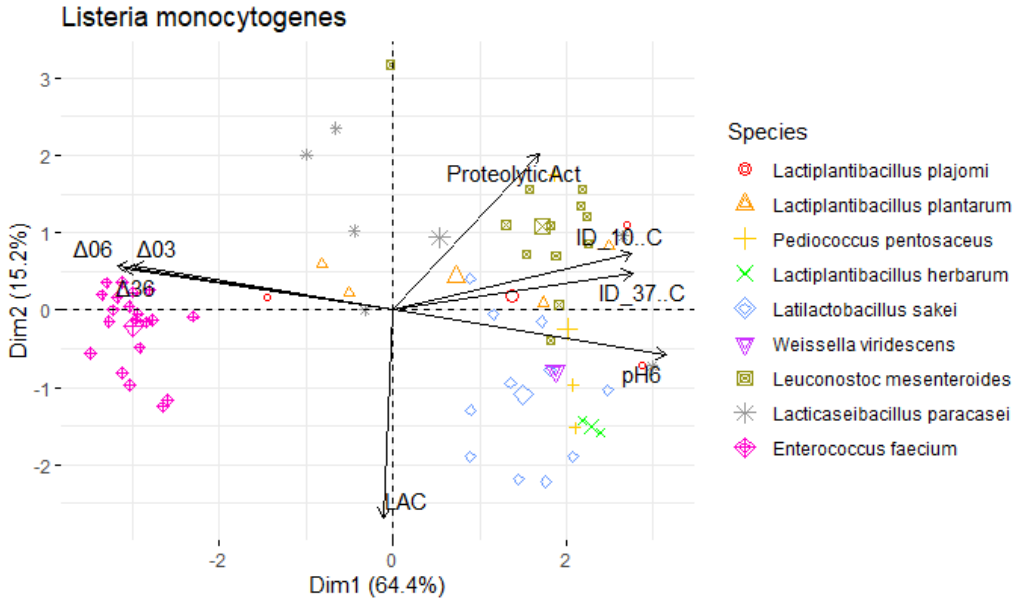

Figure 16. Map of the first and second principal components of the tested technological properties of lactic acid bacteria (LAB) against *Listeria monocytogenes* isolated from alheira sausage and species identification of LAB isolates. Legend: See Data Analysis section.

Conclusions

The careful selection of lactic acid bacteria as starter cultures or biological food-grade preservatives in meat sausages is pivotal for regulating the fermentation process – ensuring product safety by inhibiting foodborne pathogens while contributing to the desired organoleptic characteristics, enhancing consistency and quality. The absence of a standardized production processes for the *alheira* sausage may lead to a microbiologically unstable product, causing variations in quality. The implementation of management practices for food production with a standardized approach focused on enhancing quality and safety could potentially elevate the market choices for artisanal products. These products, integral to Mediterranean culture, biodiversity and economy, require thorough investigation and protection. The observed batch-to-batch variations in

the microbial profiles, indicate that conditions within *alheira* sausages from a specific producer do not lead to a uniform collection of strains. These variations have implications for product consistency and may need further investigation into genetic heterogeneity. A standardized approach could include the use of an ad-hoc starter culture. In this study, sixty-two LAB isolates were identified, with *E. faecium* (32.3%), *L. mesenteroides* (19.4%), and *Lb. sakei* (17.7%) as the most prominent species, consistent with previous research on Portuguese fermented meat sausages. Phenotypic analysis showed diverse acidification capacities, proteolytic activities, and inhibitory effects against *L. monocytogenes*, *S.* Typhimurium and *S. aureus* mainly displayed by lactobacilli. Upcoming research will work on further characterizing the mechanism of inhibition expressed by these strains (e.g., bacteriocins, organic acids, proteases/peptidases), and explore their use as fermentation drivers. This study gathers important data for understanding the composition of *alheira* and its intrinsic properties, which in the future could be applied in the development of new pure cultures for preservation of fermented meat sausages.

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IV. Overall Conclusion and Future Perspective

The application of biotechnology presents significant potential for the agricultural and farming sectors. Throughout this thesis elaboration, insights were gained into the pivotal role that local producers with small-scale farms play in the economies of Mediterranean countries, as well as in the preservation of their cultural heritage through traditional food production. The research focus during the Master's program centered on elucidating the microbial composition of a Portuguese fermented sausage known as *alheira*, while concurrently exploring the technological capabilities of lactic acid bacteria (LAB), which constitute a major component of the *alheira* microbiome.

The meat in the *alheira* sausage is processed in smokehouses through fermentation promoted by the action of LAB. For many years, LAB were associated with the deterioration of meat, but we now know that they also contribute to its preservation. Through the production of acids and aromatic compounds, and the degradation of proteins through their proteolytic activity, they are essential for obtaining a final product with the characteristic sensory and nutritional properties.

LAB play an essential role as biological control agents, generating metabolites with antimicrobial properties, including organic acids, proteases, peptidases and bacteriocins. Bacteriocins are small peptides synthesized on the ribosome of LAB and can be applied as purified or semi-purified preparations, which have no color, smell or taste. Their potential in food preservation is characterized by their microbial inhibition action. Bacteriocins can also be applied as bacteriocinogenic cultures, which can improve the sensory and nutritional profile of meat. The selection of the strain should be based on its competitiveness with the surrounding bacteria, the levels of bacteriocin expression and its compatibility with the fermentation culture. Incorporating bacteriocins into packaging is a promising alternative and can be used in various packaging matrices. Therefore, the use of bacteriocins in the production of traditional meat sausages can help increase their microbiological safety.

The main microbiological hazards that may occur in meat products are foodborne pathogens e.g., *Salmonella spp., Listeria monocytogenes*, as well as the toxins of

Staphylococcus aureus, *Clostridium perfringens* and *Clostridium botulinum*, which are the consequence of inadequate hygienic and manufacturing practices or final products' microbial instability, as they are often produced with variable, less standardized productive processes.

The management of *alheira* production was proposed in this study to establish a more standardized approach aimed at improving quality and safety. This was achieved through physico-chemical and microbiological analysis of sausages, as well as the isolation and screening of LAB from various rural sites. Phenotypic and genetic characterization of these isolates was conducted to evaluate the properties of LAB and select the most suitable strains for enhancing the quality and safety of the product.

Traditional foods, with their characteristics honed over centuries, play a significant role in the economic, agrarian, gastronomic, and cultural development of the Mediterranean region. Despite their esteemed status, it's important to acknowledge that disease-associated microorganisms can be found in meat and homemade sausages, posing potential risks to consumer health. Deciphering the mode of action of LAB against foodborne pathogens, particularly through the production of antimicrobial peptides, presents an intriguing avenue for future research. Exploring the role of LAB as biocontrol agents offers promising opportunities for enhancing the safety and quality of traditional fermented foods. Understanding the mechanisms underlying the antimicrobial activity of these peptides could lead to the development of novel strategies aimed at fortifying the safety and quality of these cherished culinary traditions.

V. Annexes

ANNEX I: List of publications and conference presentations

a. Publications

Fernandes, N., Achemchem, F. Gonzales-Barron, U. and Cadavez, V. (2024). *Biopreservation Strategies Using Bacteriocins to Control Meat Spoilage and Foodborne Outbreaks*. Italian Journal of Food Safety. Accepted manuscript, waiting publication.

Fernandes N, Faria AS, Carvalho L, Choupina A, Rodrigues C, Gonzales-Barron U, Cadavez V. (2024). *Genetic Identification and Technological Potential of Indigenous Lactic Acid Bacteria Isolated from Alheira, a Traditional Portuguese Sausage*. *Foods*. 2024; 13(4):598. https://doi.org/10.3390/foods13040598

Silva, B. N., Fernandes, N., Carvalho, L., Faria, A. S., Teixeira, J. A., Rodrigues, C., Gonzales-Barron, U. and Cadavez, V. (2023) *Lactic acid bacteria from artisanal raw goat milk cheeses: technological properties and antimicrobial potential*. Italian Journal of Food Safety, 12(4). doi: 10.4081/ijfs.2023.11559.

b. Conference presentations

Fernandes, N.; Faria, A.; Carvalho, L.; Choupina, A.; Rodrigues, C.; Gonzales-Barron, U.; Cadavez, V. *Genomics and technological features of lactic acid bacteria isolated from alheira; a traditional fermented sausage produced in Portugal*. 2nd International Conference on Quality and Management Sciences 2023, 13th-15th September 2023. Poznan, Poland.

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Approaches: Book of Abstracts (p. 87). Instituto Politécnico de Bragança. https://doi.org/10.5281/zenodo.8067526

Fernandes, N.; Loforte, Y.; Cadavez, V.; Gonzales-Barron, U. *Meta-Analysis of the Inhibitory Effects of Indigenous Lactic Acid Bacteria Supernatant from Dairy Origin Against Foodborne Pathogens.* ArtiSaneFood – Biopreservation and Risk Modelling Approaches: Book of Abstracts (p. 87). Instituto Politécnico de Bragança. https://doi.org/10.5281/zenodo.8067526

Faria, A.S.; Fernandes, N.; Cadavez, V.; Gonzales-Barron, U. *Assessment of the Bioprotective Capabilities of Lactic Acid Bacteria Isolated from Artisanal Alheira, a Portuguese Fermented Sausage*. ArtiSaneFood – Biopreservation and Risk Modelling Approaches: Book of Abstracts (p. 87). Instituto Politécnico de Bragança. https://doi.org/10.5281/zenodo.8067526

Loforte, Y.; Fernandes, N.; Gonzales-Barron, U., & Cadavez, V. (2023). *A Meta-Analysis of the in vitro Inhibitory Effects of Lactic Acid Bacteria Isolated from Dairy Products against Food-borne Pathogen.* ArtiSaneFood – Biopreservation and Risk Modelling Approaches: Book of Abstracts (p. 87). Instituto Politécnico de Bragança. https://doi.org/10.5281/zenodo.8067526

Carvalho, L.; Fernandes, N.; Silva, B.N.; Gonzales-Barron, U.; Cadavez, V. (2023). *Phylogenetic Analysis of Lactic Acid Bacteria species from Cheese: a Comparison between Taxonomy and Physicochemical Characteristics*. ArtiSaneFood – Biopreservation and Risk Modelling Approaches: Book of Abstracts (p. 87). Instituto Politécnico de Bragança. https://doi.org/10.5281/zenodo.8067526

Fernandes, N.; Faria, A.; Carvalho, L.; Choupina, A.; Rodrigues, C.; Cadavez, V.; Gonzales-Barron, U. *Molecular Identification Of Lactic Acid Producing Bacteria Isolated From Alheira, A Traditional Portuguese Fermented Sausage*. Proceedings of the 3rd International Electronic Conference on Foods: Food, Microbiome, and Health - A Celebration of the 10th Anniversary of Foods' Impact on Our Wellbeing, 1–15 October 2022, MDPI: Basel, Switzerland, doi:10.3390/Foods2022-13035

Fernandes, N.; Faria, A.; Carvalho, L.; Choupina, A.; Rodrigues, C.; Cadavez, V.; Gonzales-Barron, U. *Molecular and phenotypic features of lactic acid producing*

bacteria isolated from Alheira – Portuguese traditional meat product. Oral Presentation. Advances in food bio-preservation, ISAFP 2022 held on 2nd-4th October 2022 at Tunis Science City, Tunisia.

Fernandes, N.; Faria, A.; Carvalho, L.; Choupina, A.; Rodrigues, C.; Cadavez, V.; Gonzales-Barron, U. *16S rRNA Sanger Sequencing of microbial population diversity from Portuguese fermented sausage – Alheira*. Poster. IV Congresso das Escolas Superiores Agrárias (CNESA). Santarém, PT, 2022.

Faria AS, Rodrigues G, Miranda RB, Bonilla-Luque OM, Carvalho L, Fernandes N, Prieto MA, Cadavez V, Gonzales-Barron U. *Preliminary Assessment of Microbiological and Physicochemical Properties of Artisanal "Chouriço de Carne" Fermented Sausages, Manufactured in Northern Portugal*. Biology and Life Sciences Forum. 2023; 26(1):56. https://doi.org/10.3390/Foods2023-15070

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Fernandes, N., Achemchem, F. Gonzales-Barron, U. and Cadavez, V. *Impacto das bacteriocinas produzidas por bactérias ácido-lácticas isoladas de carne e de enchidos cárnicos na segurança microbiológica do fumeiro tradicional*. Jornadas do Porco Bísaro. Associação Nacional dos Criadores de Suínas da Raça Bísara (ANCSUB) e Associação Portuguesa de Engenharia Zootécnica (APEZ). No dia 9 de fevereiro de 2024, Vinhais.

Fernandes, N.; Faria, A.; Carvalho, L.; Choupina, A.; Rodrigues, C.; Cadavez, V.; Gonzales-Barron, U. *Identificação molecular de bactérias ácido-lácticas isoladas de alheiras artesanais do Nordeste de Portugal*. Jornadas do Porco Bísaro. Associação Nacional dos Criadores de Suínas da Raça Bísara (ANCSUB) e Associação Portuguesa de Engenharia Zootécnica (APEZ). No dia 9 de fevereiro de 2023, Vinhais.

5.2. ANNEX II: Leaflet of the main findings and recommendations for ensuring the microbiological safety of traditional sausages to regional producers (written in Portuguese)

Processo produtivo da Alheira

Na alheira, a carne cozida, após um processo de salga, é misturada com o pão, o azeite, o alho, a salsa e outros ingredientes.

É nesta etapa de mistura que as bactérias ácido-lácticas são inoculadas, naturalmente ou através de culturas de arranque.

Potenciais perigos microbiológicos na produção da Alheira

- **Receção da carne** – presença de microrganismos prejudiciais ou parasitas. Causas: falha na preparação do produto pelo fornecedor ou no transporte.
- **Receção do pão** – presença de fungos. Causas: pão mal acondicionado.
- **Receção dos condimentos e tripas** – presença de microrganismos patogénicos. Causas: embalagens deficientes ou danificadas.
- **Armazenagem da carne e demais ingredientes** – crescimento de microrganismos patogénicos. Causas: contaminação cruzada.
- **Salga e Demolha** – contaminação da carne. Causas: quantidade insuficiente de sal; utilização de água contaminada.
- **Cozedura e Desfia** – sobrevivência de microrganismos resistentes ao calor. Causas: tempo e temperatura inadequados.
- **Enchimento** – crescimento de microrganismos patogénicos. Causas: contaminação pelos operadores ou utensílios; temperatura favorável ao desenvolvimento microbiano.
- **Fumagem/Secagem** – sobrevivência de microrganismos patogénicos. Causas: tempo e temperatura insuficientes.
- **Embalamento** – contaminação. Causas: embalamento incorreto, rutura da embalagem, presença de gases.

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Food Safety and Quality Analytics

Centro de Investigação de Montanha

INSTITUTO POLITÉCNICO DE BRAGANÇA Escola Superior Agrária

44º - Feira do Fumeiro Vinhais

Guia do Produtor de Enchidos Tradicionais

As Bactérias Ácido-Lácticas

Vinhais – 2024

O que são as bactérias ácido-lácticas?

As bactérias ácido-lácticas (LAB) são um grupo de microrganismos que crescem em condições anaeróbicas, isto é, quando não há oxigénio.

Estas bactérias estão presentes em diferentes nichos ecológicos, como o solo, o ar, os alimentos e até dentro de nós.

Durante muitos anos, as LAB foram associadas à deterioração da carne, mas hoje sabemos que também contribuem para a sua preservação.

Qual é o efeito das LAB no fumeiro?

No fumeiro, o micro ambiente sem oxigénio é óptimo para o crescimento das LAB.

Nestas condições, as LAB utilizam os nutrientes da carne para produzir ácido láctico.

Este processo é conhecido como **fermentação**, sendo essencial para a obtenção de um produto final com as propriedades sensoriais e nutricionais características do fumeiro tradicional.

Na produção dos enchidos tradicionais, estas bactérias provêm dos condimentos, dos temperos e da própria carne.

Como posso utilizar as LAB no meu produto?

Podemos adicionar LAB através de culturas de arranque, que são compostas por LAB previamente selecionadas.

A utilização das LAB, ao inibir o crescimento de microrganismos prejudiciais, aumenta o tempo de prateleira dos produtos.

Além disso, ao observar o seu conteúdo genético, percebemos que cada produtor possui um conjunto único destas LAB.

Fatores como:

- os ingredientes utilizados, o tipo de carne, a duração da fumagem e da secagem

influenciam o perfil de LAB encontradas nas alheiras, as quais são fontes de diversidade microbiológica que importa estudar e conhecer, pois estas determinam as suas características organolépticas.