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ESCOLA SUPERIOR DE BIOTECNOLOGIA
PORTO

TRADITION, SCIENCE, AND INNOVATION: BIOACTIVE SUBSTANCES TO MITIGATE MICROBIOLOGICAL RISKS OF INNOVATIVE *ALHEIRAS*

Thesis submitted to *Universidade Católica Portuguesa* to attain the degree of PhD in Biotechnology, with specialization in Microbiology

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Inês Gonçalves de Azevedo Moreira

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May 2024

To my family and friends

“The greatest glory in living lies not in never falling, but in rising every time we fall”

Nelson Mandela

RESUMO

A alheira é um enchido tradicional do norte de Portugal produzido principalmente com carnes de porco e aves desfiadas, pão de trigo tradicional, azeite, gordura de porco e especiarias. Como resposta ao aumento da procura por alimentos percecionados como saudáveis e por fontes de proteínas alternativas às proteínas de origem animal, nos últimos anos têm sido disponibilizadas no mercado alheiras com novas formulações ("inovadoras"). As dietas vegetarianas, veganas e flexitarianas estão em ascensão e enchidos produzidos com ingredientes como, por exemplo, bacalhau, cogumelos, tofu, soja, ou vegetais têm surgido em todo o mundo. Neste contexto, este estudo teve como objetivo a caracterização de novas formulações de alheiras, uma vez que o conhecimento das suas características microbiológicas e químicas que determinam a sua segurança é limitado. Foi realizada a caraterização microbiológica e química por técnicas clássicas de 21 alheiras, 14 "inovadoras" e sete tradicionais. *Enterobacteriaceae*, *Enterococcus*, bactérias do ácido lático, leveduras e bolores constituem a microbiota dominante destes produtos. Não foram detetados esporos de *Clostridium* sulfito-redutores, *Escherichia coli*, *Listeria monocytogenes*, *Salmonella* spp. ou *Staphylococcus aureus* em nenhuma das amostras "inovadoras". Não foram observadas diferenças significativas para os valores de pH e de atividade de água entre as alheiras tradicionais e "inovadoras". As concentrações de nitrito, nitrato e aminas biogénicas estavam dentro dos limites aceitáveis para estes produtos em todas as amostras analisadas. Foi encontrado ácido láctico em todas as amostras, mas os ácidos málico e succínico predominaram nas alheiras "inovadoras". Adicionalmente, foram caraterizadas as comunidades microbianas dos produtos por sequenciação de nova geração (NGS). As comunidades bacterianas e fúngicas associadas a cada alheira foram analisadas, respetivamente, por sequenciação das regiões V3-V4 do gene 16S rRNA e do espaçador interno transcrito 2 (ITS2). Foram encontradas diferenças significativas na composição da microbiota entre as amostras, o que se refletiu em grandes diferenças nos perfis das espécies dominantes. Foram identificados mais de 500 taxa, principalmente pertencentes às famílias *Lactobacillaceae* e *Xanthomonadaceae*. Os géneros pertencentes ao grupo das bactérias do ácido lático e *Xanthomonas* foram predominantes nas comunidades bacterianas. No que diz respeito aos fungos, a levedura *Pichia* foi encontrada na maioria das amostras, seguida do fungo filamentoso *Alternaria*. O comportamento dos patogénicos *E. coli*, *L. monocytogenes*, *Salmonella* Enteritidis e *Staph. aureus* em alheiras tradicionais e "inovadoras" (bacalhau e vegetariana) ao longo do prazo de validade dos produtos armazenados a 4 ºC revelou ser variável por testes de desafio. *Salmonella* Enteritidis e *Staph. aureus* não foram detetados na

alheira vegetariana antes do término do prazo de validade. Com o intuito de encontrar soluções de biocontrolo de bactérias patogénicas nestes produtos, foi avaliada a atividade antimicrobiana de 491 bactérias do ácido láctico isoladas de alheiras tradicionais e inovadoras. Seis isolados revelaram atividade contra *L. monocytogenes*, *Enterococcus faecalis*, *Clostridium sporogenes* e *Clostridium perfringens,* possivelmente pela produção de bacteriocinas. Estes isolados foram identificados como *Lactiplantibacillus plantarum* (2), *Leuconostoc mesenteroides* (1) e *Pediococcus acidilactici* (3). Foram detetados ortólogos de vários genes de bacteriocinas de classe II, nomeadamente plantaricina E, plantaricina F, pediocina PA, enterocina X, leucocina A e coagulina A. Estas estirpes não produziram aminas biogénicas, gelatinase ou DNase e não revelaram atividade hemolítica ou produção da enzima lipase. *Lactiplantibacillus plantarum* 9A3 foi o único isolado sensível a todos os antibióticos testados e, como tal, foi selecionado para testes adicionais. As bacteriocinas produzidas por *Lpb. plantarum* (9A3) demonstraram um modo de ação bacteriostático e estabilidade numa ampla gama de condições (temperatura, pH, surfactantes, detergentes e proteases). Em resumo, não foram encontrados microrganismos patogénicos nem perigos químicos nas alheiras "inovadoras", ao contrário das alheiras tradicionais produzidas pelas mesmas empresas, em que são frequentemente encontradas bactérias patogénicas. A caracterização das comunidades microbianas por NGS revelou padrões distintos de diversidade microbiana em alheiras tradicionais e "inovadoras", mesmo quando produzidas nas mesmas instalações e condições. Com este estudo foram obtidas informações sobre a diversidade microbiana nestes produtos, bem como sobre o comportamento de agentes patogénicos em alheira. As bactérias do ácido láctico aumentaram ao longo do período de armazenamento, particularmente nas alheiras tradicionais. Embora cada agente patogénico estudado tenha apresentado um comportamento diferente nas várias formulações, a alheira vegetariana revelou ter características particulares que condicionam a viabilidade dos agentes patogénicos. Nesta matriz, todas as bactérias foram reduzidas a valores inferiores ao limite de deteção da técnica de enumeração no final do prazo de validade. *Lactiplantibacillus plantarum* 9A3 destacou-se como potencial agente de biocontrolo visto apresentar atividade bacteriocinogénica estável contra bactérias patogénicas como *L. monocytogenes* e *C. perfringens* e ausência de fatores de virulência.

Palavras-chave

Alheira; Bacteriocinas; Culturas bioprotetoras; Metagenómica; Produtos inovativos

ABSTRACT

Alheira, a well-known delicacy produced in the north of Portugal, is a valuable part of our country's gastronomic heritage. Traditional *alheiras*, produced mainly with shredded pork and poultry meats, traditional wheat bread, olive oil, pork fat, and spices, are well-studied products and in the past years, some new formulations have emerged. Consumer preferences are constantly changing, so the food industry must proactively develop new products. In addition to traditional fermented sausages, mainly made with meat, other products are being developed to offer alternatives in line with consumer trends to reduce meat consumption. Vegetarian, vegan, and flexitarian diets are on the rise and sausages made from a variety of ingredients, such as codfish, mushrooms, tofu, soya, or vegetables, and other meat analogues are appearing worldwide. The aim of this study was to characterize these products, as little is known about their microbiological and chemical characteristics, and it becomes essential to determine their potential role in food safety. Classical microbiological and chemical analysis were therefore carried out on a universe of 21 *alheiras*, 14 of which were considered innovative and seven traditional. *Enterobacteriaceae*, *Enterococcus*, Lactic Acid Bacteria (LAB), yeasts, and moulds were the prevalent microbiota found in these innovative products. Sulphite-reducing *Clostridium* spores, *Escherichia coli*, *Listeria monocytogenes*, *Salmonella* spp. or *Staphylococcus aureus* were not detected in any innovative samples. No differences were observed between traditional and innovative *alheiras* concerning pH and water activity, while nitrites, nitrates and biogenic amines were within accepted limits for these products. Regarding organic acids, lactic acid was found in all samples analyzed, while malic and succinic acid seemed predominant only in the innovative *alheiras*. In addition, and to find some similarities between different types of *alheiras* and/or different producers, a study was carried out using next-generation sequencing technology to characterize the microbial communities associated with these products. The bacterial and fungal communities associated with each *alheira* were obtained by sequencing 16S rRNA gene V3-V4 and Internal Transcribed Spacer 2 (ITS2) regions of rRNA gene amplicons. Significant differences in the microbiota composition were found between samples, which were reflected by large differences in the profiles of the dominant species. More than 500 taxa were identified, particularly belonging to the families *Lactobacillaceae* and *Xanthomonadaceae,* which were found in all samples. In the analysis of the bacterial communities, genera belonging to lactic acid bacteria and *Xanthomonas* were predominant. Concerning fungi, the yeast *Pichia* was found in almost all the samples, followed by the filamentous fungus *Alternaria*. A challenge test was conducted in order to investigate the

behaviour of foodborne pathogens (*E. coli*, *L. monocytogenes*, *Salmonella* Enteritidis and *Staph. aureus*) in traditional and innovative *alheiras* (codfish and vegetarian) along the product shelf-life at 4 ºC. Each target pathogen showed a different behaviour on the *alheira* matrices, but most pathogens were not detected in vegetarian *alheira* before the expiration date. As part of the risk mitigation, 491 LAB were isolated from traditional and innovative *alheiras*, and their antimicrobial activity against several foodborne pathogens was investigated. Six strains revealed antimicrobial activity by possible bacteriocin production against *L. monocytogenes*, *Enterococcus faecalis*, *Clostridium sporogenes* and *Clostridium perfringens*. These strains were identified as *Lactiplantibacillus plantarum* (2), *Leuconostoc mesenteroides* (1) and *Pediococcus acidilactici* (3). Additionally, orthologues of several class II bacteriocins genes were detected, namely Plantaricin E, Plantaricin F, Pediocin PA, Enterocin X, Leucocin A, and Coagulin A. None of these strains produced biogenic amines, gelatinase or DNase, as well as no hemolytic activity or lipase enzyme production was observed. However, only *Lpb. plantarum* 9A3 was sensitive to all the antibiotics tested and was therefore selected for further testing. Bacteriocins produced by *Lpb. plantarum* (9A3) demonstrated a bacteriostatic mode of action and stability across a wide range of conditions (temperature, pH, surfactants, detergents, and proteases). In conclusion, unlike traditional *alheiras*, which often contain pathogens, neither harmful organisms nor chemical hazards were found in these new products, even though they were produced by the same companies. Characterization of microbial communities by WGS revealed distinct microbial diversity patterns in traditional and innovative *alheiras*, even when produced in the same facilities and conditions. While this study offers initial insights into microbial diversity in these products, it sheds light on the behaviour of foodborne pathogens in the *alheira* matrix. Lactic acid bacteria increased throughout the shelf-life, particularly in traditional *alheiras*. Although each target pathogen showed a different behaviour on *alheira matrices* in general, vegetarian *alheira* proved to have particular characteristics that influence the viability of foodborne pathogens, since all pathogens tested were below the detection limit of the enumeration technique at the end of shelf-life in this matrix. *Lactiplantibacillus plantarum* 9A3 emerged as a promising candidate for industrial use due to the production of stable bacteriocins targeting pathogens like *L. monocytogenes* and *C. perfringens* and absence of virulence factors.

Keywords

Alheira; Bacteriocins; Bioprotective cultures; Innovative products; Metagenomics

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LIST OF CONTENTS

ALOA	Agar Listeria according to Ottavani & Agosti
ANOVA	Analysis of Variance
ATCC	American Type Culture Collection
AU	Arbitrary Units
a_w	Water Activity
BA	Biogenic Amines
bp	Base pair(s)
BHI	Brain Hearth Infusion
BPA	Baird Parker Agar
BPW	Buffered Peptone Water
CECT	Colección Española de Cultivos Tipo (Spanish Type Culture Collection)
CFS	Cell Free Supernatant
CFSn	neutralized Cell-Free Supernatant
CFSnC	neutralized Cell-Free Supernatant treated with catalase
CFSnK	neutralized Cell-Free Supernatant treated with proteinase K
CFU	Colony Forming Units
CLSI	Clinical & Laboratory Standards Institute
CNC	Coagulase-Negative Cocci
DNA	Deoxyribonucleic acid
DNAse	Deoxyribonuclease
E.	*Escherichia*
EC	European Commission
EDTA	Ethylenediaminetetraacetic acid
EFSA	European Food Safety Authority
ESB	Escola Superior de Biotecnologia
FAO	Food and Agriculture Organization
FDA	Food and Drug Administration
GHP	Good Manufacturing Practices
GRAS	Generally Regarded As Safe
HACCP	Hazard Analysis and Critical Control Point
HPLC	High-Performance Liquid Chromatography
ISO	International Organization for Standardization

ITS	Internal Transcribed Spacer
kDa	Kilodalton
LAB	Lactic Acid Bacteria
LMO	*Listeria monocytogenes*
Ln.	*Leuconostoc*
Lpb.	*Lactiplantibacillus*
MIC	Minimum Inhibitory Concentration
MRS	de Man Rogosa and Sharpe
nd	not detected
NCTC	National Collection of Type Cultures
NGS	Next-Generation Sequencing
NP	Norma Portuguesa
OTU	Operational Taxonomic Unit
P.	*Pediococcus*
PCR	Polymerase Chain Reaction
QPS	Qualified Presumption of Safety
RPF	Rabbit Plasma Fribrinogen
rRNA	ribosomal Ribonucleic acid
RTE	Ready to Eat
RSM	Response Surface Methodology
SDS	Sodium Dodecyl Sulfate
SDS-PAGE	Sodium Dodecyl-Sulfate Polyacrylamide Gel Electrophoresis
Staph.	*Staphylococcus*
TBX	Tryptone Bile X-glucuronide
TSA	Tryptic Soy Agar
TSB	Tryptic Soy Broth
USA	United States of America
WGS	Whole Genome Sequencing
WHO	World Health Organization
XLD	Xylose Lysine Deoxycholate
YE	Yeast Extract

Alheira, a well-known delicacy produced in the north of Portugal, is a valuable part of our country's gastronomic heritage. Traditional *alheiras*, produced mainly with shredded pork and poultry meats, traditional wheat bread, olive oil, lard, and spices, are well-studied products. In recent years, some new recipes have been developed to meet consumers' demands for healthier, tastier, and higher-quality products. The introduction of innovative products incorporating ingredients like fish, mushrooms, tofu, soya, and vegetables - designated as "innovative" *alheiras* - has increased competitiveness in the market. Although traditional *alheiras* are well characterized either microbiologically or chemically, very few studies report the characteristics of "innovative" formulations of these fermented products, whose popularity has increased in recent years.

The main objective of this project was to increase knowledge of the microbiological risks of "innovative" *alheiras* and to define strategies to mitigate these risks. Metagenomic approaches to evaluate the microbial diversity on "innovative" *alheira* matrices can help to characterize pathogenic organisms and lactic acid bacteria often present in fermented sausages. Simultaneously, classical microbiological and chemical characterization was also performed. These new products require new strategies to mitigate potential microbiological risks, such as using bacteriocinogenic lactic acid bacteria and plant-based preservatives, which have demonstrated biocontrol efficacy without compromising sensory characteristics and meeting marketing pressures for "clean label" products. Studies on their potential use to mitigate have been carried out on "innovative" *alheira* matrices, in an attempt to meet consumer demands and market trends, tradition and safety standards.

This thesis is divided into four parts, comprising seven chapters describing the research during the four years of the doctoral program (Figure 1).

Part I includes Chapter 1, which provides a general overview of the state of the art on non-meat sausages and their worldwide characteristics, including microbiological, physicochemical and functional characteristics but also their sensory traits.

Part II, consisting of chapters 2 to 4, embraces a more-in-depth study of these innovative products, since there is already extensive scientific knowledge on fermentations and behaviour of pathogens in traditional *alheiras,* but little is known about these new types of *alheiras*. Chapter 2 is entirely dedicated to a better understanding of these new products, compared to the traditional ones, using traditional physicochemical and microbiological analysis. Chapter 3 describes the complex microbial diversity of traditional and "innovative"

alheiras via high-throughput next-generation sequencing of 16S and ITS rRNA. The introduction of new approaches to food microbiology and food fermentation, using molecular methods, complements the studies carried out to date and allows the limitations of traditional methods to be overcome. Concerning Chapter 4, a study was conducted to better understand the survival of foodborne pathogens on traditional and "innovative" *alheira* matrices.

Part III entails Chapter 5, in which lactic acid bacteria isolated from innovative and traditional *alheiras* were identified and characterized in order to evaluate their probiotic characteristics and determine a potential candidate for use as a biocontrol agent.

Part IV, comprising Chapters 6 and 7, concludes with the main results and future research developments that this research could offer in the near future.

Preliminary note: This thesis core comprises one review of the state of the art (in press) and four research papers: two published and two under preparation to submit to peer-reviewed international scientific journals.

PART I – STATE OF ART

CHAPTER 1

Non-meat sausages and their characteristics worldwide

(Azevedo *et al*., in press)

PART II – NON-MEAT *ALHEIRAS* CHARACTERIZATION

CHAPTER 2

Non-meat *alheiras* - a safer novel trend

(Azevedo *et al*., 2020)

CHAPTER 3

Evaluation of the microbial diversity of traditional and non-traditional Portuguese *alheiras*

(Azevedo *et al*., submitted)

CHAPTER 4

Behaviour of foodborne pathogens in different *alheira* matrices along their shelf-life (Azevedo *et al*., submitted)

PART III – RISK MITIGATION BIOACTIVE SUBSTANCES SELECTION

CHAPTER 5

Lactic Acid Bacteria isolated from traditional and innovative *alheiras* as potential biocontrol agents

(Azevedo *et al*., 2024)

PART IV – CONCLUSIONS AND FINAL REMARKS

CHAPTER 6	CHAPTER 7
Conclusions	Future research and development

Figure 1 – Structure of thesis outline.

PART I

STATE OF THE ART

CHAPTER 1

Introduction: Non-meat sausages and their characteristics worldwide

Abstract

Consumers' preferences are constantly changing and, consequently, the food industry must be active and perceptive in developing new products. In addition to traditional fermented sausages, mainly made with meat, other products are being produced to offer alternative products in line with consumer trends to reduce meat consumption.

Vegetarian, vegan, and flexitarian diets are on the rise and sausages made with different ingredients, such as fish, seafood, and meat analogues are emerging worldwide. Since these products are becoming market-relevant, and little is known about their microbiological and chemical characteristics, it seems essential to determine their potential role in food safety. This review explores what is being produced as an alternative to fermented meat sausages worldwide and increases knowledge regarding their microbiological, chemical, sensorial and functional properties.

1.1. Introduction

Fermented sausages are part of the daily diet of many individuals around the world. Variations in ingredients and cooking recipes, various processing conditions as well as habits and customs of the different countries lead to the production of diverse types of fermented sausages (Bis-Souza *et al.*, 2019; Flores and Piornos, 2021; Franciosa *et al.*, 2018; Gardini *et al.*, 2001). Traditional sausages, mainly made with meat, fat, spices, water, and salt can have a huge variety depending on their origin. Although these meat products contribute to the intake of different nutrients, potential adverse effects on health have been attributed to their consumption (Bou *et al.*, 2017; Font-i-Furnols and Guerrero, 2022b; Godfray *et al.*, 2018; Holck *et al.*, 2017; Leroy *et al.*, 2022). Apart from their variety, consumers' preferences are constantly changing. Therefore, the food industry must be active and perceptive in developing new products allowing alternative nutritional options to be offered (Adise *et al.*, 2015; Flores and Piornos, 2021; Pernu *et al.*, 2020; Sha and Xiong, 2020).

Vegetarian, vegan, and flexitarian diets are on the up and sausages made with different ingredients, such as fish, seafood, and meat analogues are emerging worldwide. Using modified technologies, companies are presenting innovative meat-like products. A meat analogue is generally made from vegetarian ingredients, and most of them are soy-based or gluten-based, but also made from pulse proteins (Font-i-Furnols and Guerrero, 2022a; P. Kumar *et al.*, 2017). Meat analogues' texture, flavour, colour, and others depend entirely on the ingredients used. Century-old recipes have been modified using materials such as wheat gluten, soy protein, mushrooms, pulses, and flavouring additives to produce a final product tasting like meat or seafood. It has been a considerable challenge for food producers to develop products that resemble meat while being, at the same time, appealing, healthy and tasteful alternatives (Hoogstraaten *et al.*, 2022; Tziva *et al.*, 2020).

Overall, meat substitutes may be classified as plant-based (such as soy, gluten and pulses), cell-based (*in vitro* or cultured meat), and fermentation-based (mycoprotein). Recent developments incorporated other protein sources, such as microalgae proteins extracted from Spirulina and insects' proteins (Altmann *et al.*, 2018; Onwezen *et al.*, 2021; Trotta *et al.*, 2022).

1.2. Non-meat sausages trend

Non-meat sausages are claimed to be healthier since they generally contain less animal fat or cholesterol, salt and oil, and calorie quantities can be easily adjusted using the same rules used for making traditional sausages. The biggest problem associated with this kind of

product lies in the fact that it is quite challenging to mimic meat flavour (Smart and Pontes, 2022; Chen *et al.*, 2022; McClements and Grossmann, 2021). However, it must be accepted that if the meat is not used, the product will have a different taste and will never compete with traditionally made meat products. This does not mean that this product still presents its own character, and it does not need to mimic or compete with meat sausages since, overall, these products are manufactured for specific niche markets (Boukid, 2021; Chen *et al.*, 2022; He *et al.*, 2020).

By definition, a traditional sausage is composed of meat stuffed into a casing, together with some other ingredients, such as bread, water, olive oil, and spices (Silva *et al.*, 2019). Being a popular alternative to meat products, vegetarian sausages are available on the market with ingredients including typically a plant or fungal protein source. Soy, wheat, chickpea, pea protein, mycoprotein, and some other plants, such as corn, potato, pepper, onion, garlic, and so on, mixed with herbs, spices, salt, vegetable oils, and additives (clotting agents, stabilisers, pH regulator and antioxidants) are some ingredients that can be found in these substitute products (Pernu *et al.*, 2020; Tremlova *et al.*, 2022). Most commercialised vegetarian sausages, such as veggie hot dogs, are emulsified since it is easy to place all ingredients into a grinder or a mixer machine and then stuff the resulting paste into a casing. Control texture in case of not emulsified sausages into a food processor is difficult. Still, when the texture is under control, it is fairly easy to add an infinite number of materials, and hundreds of recipes can be created (Bedin *et al.*, 2018; Birke Rune *et al.*, 2022; Yesuraj *et al.*, 2022).

Many controversies have revolved around these meat alternatives since public opinion declares that the word “meat” should not be used in these products' labelling. Different names are being studied in order to meet consumers' opinions, including “rolls”, “patties” and “portions” for products like sausages, burgers and steaks, respectively. In the USA, the National Cattlemen's Beef Association is in conflict since February 2018 about whether plant-based products should be titled as “meat” since they do not originate from animals, which may confuse the consumer. For example, in France, the use of meat-like products, such as “vegetarian sausage”, was excluded from vegetarian food labelling once it was known that it might delude consumers. All products made from soy, tofu, and some other vegetables which are considered meat substitutes may not include words such as “burger”, “sausage” or “ham” on their label (Sha and Xiong, 2020). In December 2019, Plant-Based Foods Association (PBFA) published Voluntary Standards for the Labeling of Meat Alternatives in the United States. However, Food and Drug Administration (FDA) is still

trying to establish label guidelines for plant-based foods, which were expected to be published as drafts or finals by the end of December 2022.

1.3. Non-meat sausages worldwide

In addition to consumer trends, factors such as cost, availability, environmental sustainability, suitability of new products and their functional proprieties are the main drivers for the demand for new protein sources (Haque *et al.*, 2016; Lima *et al.*, 2022; M. Kumar *et al.*, 2022). Nowadays, more and more proteins of different origins and their functionalities, such as water and oil holding abilities, solubility, emulsification, foaming, and gelation are being studied since they are vital for meat analogue structure development. At present, the main sources of vegetable proteins incorporate oilseeds (soybeans, canola, cottonseed, peanut, sunflower seed, sesame, safflower, and flaxseed), cereal (wheat, corn, rice, barley, oats, sorghum, and grain amaranth), pulses (beans, chickpeas, cluster beans, lentils, lupines and peas) and leaf (alfalfa, lucerne, tobacco, mulberry bush, grass, sugar cane, sugar beet, and clovers) (Fu *et al.*, 2023; Kyriakopoulou *et al.*, 2018; Zahari *et al.*, 2022).

Taking a cursory look around World Wide Web, hundreds of non-meat sausages can be found, and it can be recognised that there have never been as many plant-based sausages on the market as there are today. Table 1.1 presents examples of plant-based sausages produced worldwide. This table was compiled based on a web search using the keywords "vegan/vegetarian sausages/products/brands in "country name" and only those with fermentative ingredients, such as yeasts and starter cultures, were included.

1.4. Meat analogues used in non-meat sausages

1.4.1. Soy protein

Due to their unique properties and low prices, at the moment, most meat analogues are based on soy protein. Other oilseed crops and proteins generated through fermentation, based on several substrates and microorganisms, are also being integrated into meat analogues manufacturing (Dekkers *et al.*, 2018; Hashempour-Baltork *et al.*, 2020; Sun *et al.*, 2021). For over 50 years, soy protein has been used as a nutritional and functional food ingredient (Jooyandeh, 2011; Qin *et al.*, 2022; Ravani and Sharma, 2022). Soy proteins are considered a good source of some essential amino acids, offering innumerable functional benefits and a healthy diet. Additionally, soy proteins are believed appropriate for most diets since they contain no cholesterol and are lactose-free (Bond, 2010; Guo, 2009; He *et al.*, 2020; Jooyandeh, 2011; Kapoor *et al.*, 2014; Singh and Sit, 2022). Soy-based analogues

are mainly produced to mimic traditional foods in appearance, colour, flavour, and texture (Bohrer, 2019; Hoogstraaten *et al.*, 2022; McClements and Grossmann, 2021).

1.4.2. Wheat protein

Wheat protein gluten, a by-product obtained from starch isolation from wheat flour, is frequently used due to its binding, dough-forming and leavening quality (Chen *et al.*, 2022; Day *et al.*, 2006; Godschalk-Broers *et al.*, 2022). Gluten, the dough-forming protein of wheat flour, is the key to the exceptional quality of wheat to generate leavened products. Although known for almost 300 years, gluten has only been used as a unique vegetable protein of commercial significance in the past five decades. Since the need for plant-sourced proteins with different functional properties is quickly growing, the high protein content, exclusive viscoelastic characteristics, thermosetting, and water absorption properties of wheat gluten offer food scientists and technologists opportunities for innovative product design (Chen *et al.*, 2022; Day, 2011). Being rich in glutamine and cheaper than soy, gluten is finding its way to inclusion in meat analogues (Kumar *et al.*, 2017). The main problem associated with wheat proteins is gluten-related disorders due to people with genetically predisposed conditions. For example, celiac disease is a chronic inflammatory intestinal disease triggered by gluten and is estimated to affect 1% of people worldwide (Anzani *et al.*, 2020; Elli *et al.*, 2015; Gazikalović *et al.*, 2021; Pi *et al.*, 2022). New gluten-free products are being developed in order to work around this health challenge (Jnawali *et al.*, 2016; Starowicz *et al.*, 2022; Szpicer *et al.*, 2022). Wheat can be found in a large number of sausages (Table 1.1), whether in flour, starch, protein, or gluten form, usually combined with other sources of vegetable protein such as peas or soy.

1.4.3. Pulses' proteins

About 20 leguminous species are commonly used as dry grains for human nutrition. Peas, lentils, lupine, chickpea, and some other beans play a vital role in the human diet due to their high protein content and beneficial nutritional value and also because they are low cost and popular (Chandler and McSweeney, 2022; Klupšaitė and Juodeikienė, 2015). Along with wheat protein gluten, the most promising for meat analogue application is still pea protein due to its high-moisture extrusion (Berrios *et al.*, 2022; Kim *et al.*, 2021; Vatansever *et al.*, 2020). Several studies indicate that these proteins present weaker gelling capacities than soy, with the exception of chickpeas (Berghout *et al.*, 2015; Grasso *et al.*, 2022; Osen *et al.*, 2014). Providing energy, dietary fibre, protein, minerals, and vitamins required for human health, they are the best-known pulse proteins for emulsification, foam stabilisation, and gel formation. Playing an important role in food formulation and

processing, they are highly recommended as a potential supplement in a vast number of food applications. The pulse protein concentrates or production of isolates is booming since they enhance the nutritional value of food products (Fernando, 2022; Nadeeshani *et al.*, 2022). Table 1.1 shows that some brands use exclusively, for example, pea protein, flour, or starch, either combined with soy or wheat. Overall, the products described in Table 1.1 indicate that most meat alternatives are prepared with soy, wheat and/or pea protein combinations to get the desired structure.

1.5. Non-meat fermented sausages microbiological characteristics and their role in food safety

Whether they are traditional or innovative, the characteristics of fermented sausages are known to be highly dependent on the ingredients and raw materials' quality. Also, due to particular processing conditions, ripening and existing microbial population, all of these products have a specific microbiota (Belleggia *et al.*, 2022a; 2022b; 2022c; Ferrocino *et al.*, 2022; Yang *et al.*, 2022).

In this chapter, the major aim is to know the emerging worldwide meat alternatives to traditional sausages and understand more about their microbial ecology. In addition, it is also intended to understand their potential role in food safety since they are becoming market-relevant, and little is known about their microbiological and chemical characteristics (Azevedo *et al.*, 2020; Bedin *et al.*, 2018; Silva *et al.*, 2019).

In Italy, Bedin *et al.* (2018) developed suitable recipes to prepare food products that mimic the shape and texture of traditional würstel and mortadella, focusing on the Italian market. For this, they faced several challenges, such as finding ways to maintain the similar characteristics of the traditional foods while accomplishing the consumer's requests and enlarging the market share of the food industries. To avoid confusion and misunderstanding, and since the primary goal of this research was to mimic these products, with vegan-allowed ingredients and proteins of vegetal origin, the authors renamed them as "mimic-mortadella" and "mimic-würstel". Microbiological analyses were carried out to determine the level of safety of the novel products, and food safety of the "mimic-würstel" and "mimic-mortadella" was respected in alignment and agreement with the food regulations when compared to the traditionally processed meat-based products (Borch *et al.*, 1996). In the sector of traditional salami production, bacterial contamination usually occurs after slaughtering since the internal muscles of the healthy animals are uncontaminated until that moment. Animal muscles' chemical and physical composition nurture the development and colonisation of an

elevated type of microorganisms, mostly bacteria; even though some are necessary for fermentation and aroma production, but some are human pathogens. These non-meat products respected food safety regulations when compared to their meat analogues. Still, the greatest challenge was trying to fulfil consumers' requests as well as eliminate issues related to shelf-life and expected texture as a result of animal protein substitution. Related to the challenges faced in this study, the authors first concentrated on using different proteins as the basis to create a clear structure, identifying the wheat gluten proteins as the best solution since this plant-based substitute is accepted in vegan formulations and helps to preserve the elasticity of the product. Both non-meat products obtained were marketable regarding texture and organoleptic properties. In conclusion, even though the biggest challenge is to handle consumer's requests, but on the other hand, animal protein replacement can raise questions related to shelf-life and expected texture.

In Portugal, the authors of a preliminary study (Silva *et al.*, 2019) characterised microbiologically nine different formulations of *alheira* from five different producers. Isolates obtained were characterised using various phenotypic and biochemical tests, and their susceptibility to different antimicrobials and the presence of virulence factors were also investigated. Although lactic acid bacteria were the predominant microbiota, pathogenic bacteria such as coagulase-positive staphylococci, *Listeria monocytogenes* (LMO) and *Salmonella* spp., and other indicator organisms were also found. Several virulence factors were detected among different groups, presenting a high incidence of β-hemolytic isolates, and also resistance to several antimicrobials leading to a large number of multiresistant isolates. In short, microbial hazards of traditional *alheira* were also found in these new formulations, which is not surprising since the possibility of cross-contamination always exists. That way, it is crucial to warn to safely cook these products since some common cooking practices of *alheira* may not guarantee a sufficient inside temperature that can eradicate pathogens potentially present (Campos *et al.*, 2013; Felício *et al.*, 2011). Given the sufficient environment for bacterial contact, horizontal transmission of antimicrobial resistance genes or virulence determinants is a matter of concern. The results obtained in the study of Silva *et al.* (2019) refer to the need to analyse higher numbers of products and inform consumers of the need for safe cooking time/temperatures as a correct preparation of these products.

Following the abovementioned study, Azevedo *et al.* (2020) characterised more innovative products concerning their microbiological and chemical safety. Therefore, fourteen innovative products and eight corresponding meat analogues, acquired in the Portuguese

market and made from codfish, mushrooms, tofu, soy, and vegetables, were analysed. *Enterobacteriaceae*, *Enterococcus*, lactic acid bacteria, yeasts, and moulds, were the main bacteria found in innovative *alheiras,* but no sulphite-reducing *Clostridium* spores, *Escherichia coli*, *L. monocytogenes*, *Salmonella* spp. nor *Staphylococcus aureus* were detected. In this study, the authors concluded that no biological threat was detected in these innovative products even though they were produced by the same companies. Still, some of the traditional analogues have shown concerning microbiological status in terms of food safety due to the presence of *E. coli* and *L. monocytogenes*. Due to this, an important aspect to consider in future confirmation studies is understanding why traditional products are more contaminated than innovative *alheiras*. Although produced in the same facilities and eventually under the same conditions, it is undisputed that some ingredients used in their manufacture are different, and meats used in traditional *alheiras* might be more contaminated. Also, producing conventional *alheiras* in higher amounts can be one of the answers to diverse contamination types found and, consequently, to different associated hazards (Azevedo *et al.*, 2020).

In Belgium, Geeraerts *et al.* (2020) explored bacterial diversity in a selection of packaged meat product alternatives. Bologna sausage, Chorizo, and Salami produced without meat, in a total of fifteen samples (eight vegetarians, six vegan imitations of meat products and one insect sample), were acquired at different supermarket groups and one bio-shop in Brussels. In this study, bacterial counts ranging from <2.0 to 8.7 log colony forming units per gram (CFU/g) were detected in these vegetarian and vegan imitations of fermented meat sausages, with some differences between the products. The bacterial load of the edible insect product was generally higher than those on vegan and vegetarian products. Several isolates were collected using different selective agar media, classified, and identified using (GTG)-5-PCR fingerprinting, followed by gene sequencing. *Latilactobacillus sakei* was the main isolate collected from vegetarian products, while *Lb. sakei*, *Enterococcus faecium* and *Carnobacterium divergens* were mainly found in vegan products. The insect product mostly contained *Ent. faecium*, *Macrococcus caseolyticus*, and *Cronobacter sakazakii*. These LAB species, specifically *Lb. sakei* and *Ent. faecium*, were the most prevalent microorganisms found, both of which have been associated with food spoilage (Geeraerts *et al.*, 2018; 2017; Dušková *et al.*, 2016; 2015). Despite these food quality and safety concerns, *Lb. sakei* has the potential use as a starter culture, and *Ent. faecium* can be a bioprotective culture as well as flavour-producing in meat products (Barbosa *et al.*, 2014; Favaro and Todorov, 2017; Kaškonienė *et al.*, 2017). As for future studies, the authors emphasised the need to evaluate

how this divergent variability in bacterial loads can be explained. They also intend to find if, when prolonged storage is needed, whether the bacteria encountered may lead to spoilage or food safety concerns. Also, related to food safety, the occasional presence of relatively high enterococci levels deserves extra attention. Even though they are lactic acid bacteria, they are also important nosocomial pathogens and might cause bacteremia, endocarditis, and other infections. Some strains are resistant to many antibiotics and studies conducted on the incidence of virulence factors among enterococcal strains isolated from foods revealed that some harbour virulence traits. The use of metagenomic, meta-transcriptomic, and meta-metabolomic analyses is suggested as a way to offer additional information and to gain better insight into the variable microbial communities that are correlated with this category of food products (Franz *et al.*, 2011).

In Finland, a significant food safety concern is emerging since *Clostridium botulinum* is being found in numerous outbreaks in the raw materials used for preserved vegetables. Vegetarian sausages are becoming popular as alternative meat products, and their preparation involves limited additives and salt for products with several months of shelf-life. In the study of Pernu *et al.* (2020), 74 (8 frozen and 66 chilled) packaged vegetarian sausage samples from seven producers were acquired in Finland and Germany. These vacuum-packaged products' shelf-life varied from less than two weeks to six months, and some of these products contained detailed instructions for cooking. In some cases, these instructions included heating temperature and time, but some products had just a suggestion of heating method without time indications, and other products were advised to be served either heated or cold. The main ingredients used were soy (soy protein or tofu), wheat protein, vegetable oil, sugar, spices, salt, and corn, wheat, or potato starch, and additives such as pH regulators, emulsifiers, stabilisers, and antioxidants were also commonly included but not identified in more detail. *C. botulinum* is a major food safety concern due to its ability to produce a highly potent neurotoxin and resistant endospores. This pathogen was found in 32% of the analysed vegetarian sausages suggesting that these products can be a source of botulism (Pernu *et al.*, 2020). Vegetarian sausages have become a popular source of plant protein and alternatives to meat products. While vegetarian sausages have not been linked to botulism, numerous outbreaks due to preserved vegetables suggest a frequent occurrence of *C. botulinum* spores in the raw material (Date *et al.*, 2011; Jalava *et al.*, 2011; King *et al.*, 2009; Lindström *et al.*, 2006; Read and Grundy, 2012; Sobel *et al.*, 2004). The main food safety concern is that it might grow and produce toxins at refrigeration temperatures. For this reason, the safety of these sausages relies mainly on heat treatment and chilling storage. The product formulation

of vegetarian sausages involves limited NaCl and preservatives, and the shelf-life may be of several months. The highest cell counts (1200 spores/kg) were observed for *C. botulinum* Group II in products with a remaining shelf-life of 6 months at the time of purchase, leading to the conclusion that vacuum-packaged vegetarian sausage products frequently contain *C. botulinum* spores and may possess a high risk of *C. botulinum* growth and toxin production (Pernu *et al.*, 2020). Even though chilled storage below 3 °C and thorough reheating before consumption are warranted, moderate heat treatments will not eliminate *C. botulinum* Group I and Group II spores, and long shelf-life may support spore germination, outgrowth and toxinogenesis (Graham *et al.*, 1997). Therefore, vegetarian sausage products' safety relies on multiple hurdles controlling growth and toxin production and certainly not simply on toxin inactivation during cooking. The authors suggest subsequent challenge studies where product packs might be inoculated, and shelf-life tests will be mandatory to determine the growth and toxic potential of *C. botulinum* in vegetarian sausages and the length of safe shelf-life (Pernu *et al.*, 2020).

1.6. Non-meat fermented sausages' physicochemical characteristics

Similar to what happens in the microbiological evaluation of these innovative products, studies concerning chemical characterisation are also scarce. A study conducted by Bayram and Bozkurt (2006) revealed that bulgur is proper as a meat replacement for the production of a vegetarian sucuk, a dry, spicy and fermented sausage that is eaten from the Balkans to the Middle East and Central Asia. Bulgur-sucuk main quality parameters were similar to those of meat-sucuk, such as moisture content and pH decrease along the ripening period. The initial moisture content decreased to almost half the value during the ripening period. This might be due to water evaporation as a result of the low relative humidity of the ripening cabinet and low pH. In order to control fermentation, taste, texture and colour, the bulgur-sucuk pH was crucial. The considerable pH decrease in the first three days of ripening was mainly due to the bacteria's organic acid production. The high pH values found in the sucuk initial batter allow the growth of most bacteria, yeasts and moulds, and so pH must be reduced. Acid production will be highly conditioned by the type and amount of added carbohydrates, lactic acid bacteria present, drying level and temperature. This product should find good acceptance among consumers due to its lack of nitrite/nitrate, cholesterol, dangerous microorganisms, chemical compounds, animal fat and low price. Also, the probability of nitrosamines, biogenic amines (BA) and cancerous compound formation or presence seems to be very low in this product (Bayram and Bozkurt, 2006).

The use of starfruit dietary fibre concentrate as a different ingredient in Vienna sausages was investigated by Vivar-Vera *et al.* (2018). Different amounts of starfruit fibre were used in a restricted mixture layout to assess trim force, shrinkage, colour, residual nitrite, moisture, and polyphenol contents on sausages. This study demonstrated that this ingredient in mixture with pork or turkey meat has a reducing effect on nitrites, moisture, trim force, and shrinkage, although an increase in polyphenol concentration was observed as the starfruit fibre amount was rising. Optimisation was achieved with a mixture of pork/turkey and between 7.4 to 8.4% of starfruit fibre. The developed product possesses low residual nitrite content, shrinkage, red colour, high total dietary fibre content, and antioxidant polyphenols. In conclusion, it seems clear that Vienna-type sausages enhanced with starfruit fibre are an excellent approach to achieving prospective functional meat products with high antioxidant dietary fibre content and reduced nitrites.

Mousavi *et al.* (2019), attempted to establish the impact of quite a few percentages of tofu on nutritional values and physicochemical components of sausages towards development as a non-meat ingredient. Sausages prepared with 25, 50 and 75% of tofu were studied, and higher moisture and residue content was observed in 75% added tofu. Still, protein content results revealed that as tofu levels increased, the protein content decreased. The best sausage creation included 25% of tofu since it exhibited an exceptional nutritional composition with the highest protein content and lowest fat content compared to the other formulations.

The objective of the study performed by Kamani *et al.* (2019) was to partially and completely replace chicken meat with plant proteins in sausage preparations and then compare their characteristics with the whole meat sample. The outcomes demonstrated that mixed plant proteins, such as soy protein isolate and gluten, improved emulsion stability, and minimised cooking loss and shrinkage, but poor elasticity and gel quality were also observed.

Azevedo *et al.* (2020) also performed chemical characterisation of innovative and traditional *alheiras*. Concerning pH and water activity values, no differences were observed between innovative and their traditional analogue, and nitrites, nitrates, and biogenic amines were found to be within acceptable limits for these kinds of products. Lactic acid was found in all analysed samples, but malic and succinic acid seemed to be predominant in innovative products.

Sha and Xiong (2020) argued about the many supposed advantages of plant-based meat alternatives compared to animal meat and meat products. The authors claimed that meat alternatives often hold more salt than the products they intend to substitute, and by this, it has been a challenge to reduce sodium content, promoting health. Another hurdle described

is related to the lack of a clean label, where a large number of ingredients are found in these innovative products, including preservatives, stabilisers, and colourants that are not usually added in meat products (Berman, 2019). This raises the question of whether these products are indeed more nutritious and healthier than analogous meat products.

Recently, Priya *et al.* (2022) developed a vegan sausage using novel ingredients such as jackfruit and banana florets. This meatless sausage showed good sensory and physic-chemical properties, demonstrating to be rich in fibre and protein and proved to be a good alternative in various value-added products helping in the environmental reduction effect of our food system.

1.7. Non-meat fermented sausages' sensorial traits

In the study of Bayram and Bozkurt (2006), Bulgur-sucuk consumer satisfaction scores related to its sensory properties, like flavour, colour and cutting ease, were evaluated by qualified panellists. For the formulation of this product, olive oil was used in order to obtain a healthy vegetarian sucuk. In meat products, fat is a major contributor to the flavour, texture, mouthfeel, and juiciness of the final product. Fat allows mixture ease, enabling continuous moisture, which is essential for proper fermentation and aromatisation of fermented products. Fat reduction may influence product suitability since it promotes sensory properties. In several studies, olive oil and some other vegetable oils have been included as being suitable in various meat products replacing animal fat in order to reduce saturated fatty acid and cholesterol content in these products (Bloukas *et al.*, 1997; Kayaardi and Gök, 2004; Kılıç and Özer, 2017; Muguerza *et al.*, 2002; Öztürk-Kerimoğlu *et al.*, 2021). This ingredient, bulgur, can be found in more than 250 different types of food due to its physical, textural, nutritional and functional properties. Its chewiness, adhesiveness, water-holding capacity, resistance to mould contamination and insect attack, appealing taste, easy preparation, and long shelf-life makes it a very good option for a meat substitute.

Tahmasebi *et al.* (2016) investigated sesame and walnut paste, corn flour, and pigeon pea flour effects, using response surface methodology (RSM), to establish the ideal formulation of sausages, made using the above ingredients, in order to maximise emulsion stability, minimise cooking loss and improve textural properties. When increasing pigeon pea and corn flour amounts, high emulsion stability and textural properties, such as hardness, gumminess, and deformability, were observed. Nevertheless, when these flours increased proportions were combined with walnut paste, a slight reduction was observed in cohesiveness, and the results showed high levels of hardness. These vegetable ingredients

improved gel-network formation while revealing enhanced emulsion stability parameters and high textural properties promotion. Microstructure images of sausages using different formulations revealed interactions between the protein matrix and the various vegetable ingredients and considering fat globules, which can also affect the parameters. The scanning electron microscopy images showed compact structures with low sponginess due to combining vegetable ingredients and their interaction with meat protein during cooking, which generates a constant and stable protein matrix. Through these results, they concluded that RSM is a promising method to improve sausage formulation for the addition of vegetable ingredients**.**

Neville *et al.* (2017), developed hybrid meat analogues where a fraction of meat was substituted by other sustainable protein sources. Consumers tested unique formulations of hybrid beef burgers and pork sausages in order to obtain their satisfaction scores analogues with both meat and meat-free commercial products. Samples of commercial meat, meat-free and hybrid products were presented to consumers and sensory attributes perceived were identified. The results showed that hybrid products were appreciated by consumers, although hybrid sausages presented better general acceptability when compared to hybrid burgers. Meat-free products showed substantially lower acceptability when compared with meat and hybrid products, where no meaningful discrepancies were observed. They also found that imitating a "meaty flavour" and "meaty colour" in hybrid products is vital to increase tolerability among consumers mainly used to meaty foods. It seems clear that this hybrid model eases to link the tolerability gap among mainly meat consumers when it comes to meat and meat-free products. This hypothesis, through enhancing the experience of consumers with meat substitution, may support the conversion of meat-eaters to a meat-reduced diet. The authors intend to follow up on their studies in order to understand if, although these hybrid products were found to be acceptable, the consumers intend to buy them.

As previously described in the study of Vivar-Vera *et al.* (2018), supplementation of Vienna-type sausages with starfruit fibre in a mixture of pork and turkey meat allowed for attaining likely functional meat products, sensory suitability for the consumer and contributing to the daily proposed intake levels of fibre. Good taste, colour and texture qualities were described when pork/turkey was mixed with starfruit dietary fibre concentrate in Vienna sausages.

The main challenge Bedin *et al.* (2018) faced in their study was to find a proper recipe for würstel and mortadella imitations. Trying to create a good structure, they used different proteins and recognised that wheat gluten was the best solution found not only because it is

accepted in vegan formulations but also because it helps to keep elasticity. The developed products are viable in terms of texture but also in organoleptic and sensory texture evaluation. Overall, although the requests of consumers can be fulfilled, animal protein substitution can cause concerns related to shelf-life and expected texture.

In Mousavi *et al.* (2019) study, as previously reported, among all treatments, 25% of tofu was suggested to be incorporated in sausages. According to sensory evaluation, 25% of added tofu showed global approval qualities and high scores related to colour, texture, juiciness and flavour. High-fat content found in 50 and 75% formulations was due to the low emulsification qualities of soy lecithin to mix fat and water. During the cooking process, this formulation became oily, enhancing the sausage's instability.

Analysing the sensory evaluation results in Kamani *et al.* (2019) study, free-meat sausages were highly appreciated in terms of texture, odour and colour and global approval. The global acceptance of chicken meat substitution with plant proteins is promising, and an 80 to 100% replacement might be considered. Nevertheless, further studies must be done in order to improve gel-forming characteristics, which is the greatest obstacle in meat-free sausage production.

1.8. Non-meat fermented sausages' functional characteristics

Like microbiological and chemical traits of non-meat fermented sausages, little is known about these innovative products related to their functional characteristics. From what is known, traditional fermented sausages have a long tradition in consumers' habits, and these products have their specific microbiota typical of the region/area from where they are produced. Even though many typically fermented meat products are still manufactured with traditional technologies, not using starter cultures, in some industries, the use of starter cultures is becoming more frequent since it provides an additional tool in foodborne pathogens prevention as well as enhancing the competitiveness of the starter organisms (Franciosa *et al.*, 2018). The introduction of starter cultures has become essential in such a way that allows shortened ripening period, ensures colour development, enhances flavour, and improves food safety (Ren *et al.*, 2022; Y. Li *et al.*, 2023). Actually, a starter culture should be qualified enough to allow fermentation, colonise the product and dominate over other microorganisms from the beginning until the end of the process (Cocolin *et al.*, 2006; Gradinarska *et al.*, 2022; H. Li *et al.*, 2022). However, using commercially available starter cultures may result in a loss of organoleptic characteristics with consequent deprivation of flavour and aroma. For this reason, in some countries, there are still artisanal sausages that

are made relying on unknown microbiota since they are preferred by most consumers. These spontaneously fermented sausages possess unique characteristics and are frequently superior when compared to regulated fermentations inoculated with industrial starters (Franciosa *et al.*, 2018). During the fermentation process, two broad groups are mainly prevalent, lactic acid bacteria (LAB) and coagulase-negative cocci (CNC). Lactic acid bacteria are usually more numerous than CNC during fermentation and ripening, staying more stable in cured products. Facultatively heterofermentative lactobacilli, such as *Lb. sakei* and *Lb. curvatus*, generally prevails, and within CNC, *Staphylococcus xylosus* fastidiously dominates (Aquilanti *et al.*, 2016). In the study of Geeraerts *et al.* (2020), the authors described some of these microorganisms as being dominant in some alternative meat products. In vegetarian products, *Lb. sakei* was the main isolate that remained, while *Lb. sakei*, *Ent. faecium* and *Carnobacterium divergens* were mainly found in vegan products. *Enterococcus faecium*, *M. caseolyticus* and *C. sakazakii* were the main microbiota in insect products. These microbial cultures can increase fermented products' safety by means of rapid matrix acidification or due to the production of antimicrobial substances such as bacteriocins. These compounds comprise a group of peptides with bactericidal or bacteriostatic activity against some food spoilage and food poisoning bacteria such as *Bacillus* spp., *Clostridium* spp., *Staphylococcus* spp. and *Listeria* spp. Nisin, pediocin, sakacin, curvacin, plantaricin and bacteriolysins are some examples of bacteriocins that are effective in several pathogenic species control, such as *L. monocytogenes*, *S. aureus*, *Campylobacter* spp., *E. coli*, *C. perfringens* and *Bacillus cereus* (Arbulu *et al.*, 2022; Ferrocino *et al.*, 2022; Laranjo *et al.*, 2019). This means that, similar to traditional fermented sausages, bacteriocin research in innovative products is also feasible, and we might identify novel bacteriocins and bacteriocin-producing strains for specific applications. The main compromise is finding the best-suited bacteriocin/bacteriocin-producer to control spoilage/ pathogenic microorganisms often present in this kind of product. Bacteriocin-producing lactic acid bacteria active against *L. monocytogenes*, *Ent. faecalis* and *Clostridium sporogenes* were found in innovative *alheiras* (Azevedo *et al.*, 2024).

1.9. Probiotics in non-meat fermented sausages

According to Pimentel *et al.* (2021), probiotic products represent an appealing economic point for vegan consumers that demand products with health benefits. Probiotics are microorganisms that, when consumed in adequate amounts, will benefit the host (FAO/WHO, 2002). In the study of Pimentel *et al.* (2021), the effect of probiotics on the

technological and sensory properties of various vegan products was explored, and a summary of several other studies conducted in order to evaluate probiotics' health benefits can be found. These probiotic products may improve the immune system (Agraib *et al.*, 2022; Kristensen *et al.*, 2016; Yadav *et al.*, 2022), manage diabetes (Ahmadian *et al.*, 2022; Madempudi *et al.*, 2022; Razmpoosh *et al.*, 2019), show anticarcinogenic properties (Dasari *et al.*, 2017; Pop *et al.*, 2022), and improve the overall well-being. Based on these benefits, the food industry is being compelled to produce new products containing probiotics and food researchers to study their effects on human health and their specific characteristics. Besides their effectiveness and proven healthiness, probiotic cultures can easily adapt to different food matrices, so the development of products that are nutritionally stable and/or add value should be encouraged (Açik *et al.*, 2020; Behera and Panda, 2020; Grom *et al.*, 2020; Tangyu *et al.*, 2019).

For years, probiotics were mainly centred on dairy products, but the rise of lactose intolerant, vegan and hypercholesterolemic individuals demanded drastic changes (Cosme *et al.*, 2022; Nguyen *et al.*, 2019). Based on vegetables such as almonds, coconut, oats, rice, chickpeas, and soybeans, the food industry was able to suggest vegetable matrices as potential probiotic agents (Rincon *et al.*, 2020). According to Min *et al.* (2019), fermented foods are selected as non-diary food matrices once studies have shown that fermentation allows superior viability of probiotic culture survival. In developing innovative probiotic non-dairy products, it is essential to consider factors such as viability and stability of the probiotic bacteria, resistance to pH, temperature and other stresses, adequate water content, carbohydrate consumption, and metabolite production from fermented or non-fermented foods. Even though non-dairy food matrices have shown the ability to sustain the growth of probiotics, being good alternatives to offer benefits without milk proteins, lactose, cholesterol, or saturated fat, at present, most probiotic food markets are dairy-based (Cosme *et al.*, 2022).

Probiotics may modify product composition, colour, and acidity, but the main problem relies on the survival of the probiotic culture, which is dependent on processing steps, food matrix, and storage conditions. Also, even when products with proper probiotic survival, physicochemical characteristics, technological properties, and sensory acceptance are obtained, the strain source may compromise vegan status since most of those presently available are not isolated from vegetable matrices (Pimentel *et al.*, 2021).

Probiotics used in food products require evidence of their effectiveness and safety. This might be achieved through scientific studies that validate a history of safe use, lack of adverse incidents, non-existence of virulence and pathogenic issues, absence of substances

and/or metabolites that might cause human health risks, absence of antibiotic resistance, and susceptibility to at least two antibiotics (FAO/WHO, 2006). A relatively large collection of lactic acid bacteria (LAB) species are registered as Generally Recognized as Safe (GRAS) and include the most used probiotic species in supplements or food products (Gu, 2019). Along with *Bifidobacterium* genus, species of *Lactobacillus* are the leading used probiotic cultures. *Saccharomyces* yeast and some *Streptococcus* and *Bacillus* species are also used, and their incorporation into non-dairy foods has been studied (Abid *et al.*, 2022; Alves *et al.*, 2016).

1.10. Prebiotics in non-meat fermented sausages

A lot is heard about probiotics' value and how they are crucial to our gut microbiome and good digestion promotion, but prebiotics are as important as probiotics to gut health. Therefore, not being informed about them can lead us to unconsciously not consume plenty in our diet, which will weaken any probiotic action. Prebiotics are food elements that allow beneficial microorganisms, such as bacteria and fungi, towards beneficial growth or functional action. These nutritional prebiotics are usually nondigestible fibre compounds not digested in the gastrointestinal tract's upper part and promote beneficial bacteria growth by acting as a substrate. They must withstand host digestion and adsorption before fermentation by more than one species of the local microbiota (Preidis *et al.*, 2011; Preidis and Versalovic, 2014). Prebiotics, predominantly polysaccharides and oligosaccharides, are abundant in high-carbohydrate foods. These compounds may be found in many fruits, vegetables, and whole grains, such as apples, bananas, berries, tomatoes, asparagus, green vegetables, leeks, soybeans, wheat, peas, and beans. Through label reading, they might be found if words such as galactooligosaccharides, fructooligosaccharides, oligofructose, chicory fibre, and inulin are searched. Proven benefits include, besides feeding our good gut bacteria, support of calcium, faster food fermentation during digestion, and favors gut cells to be healthy (Câmara *et al.*, 2020).

Dietary fibres' value in health is well known, and some benefits are directly associated with prebiotic use. Seeds, nuts, and mushrooms are good sources of prebiotic compounds, which have enhanced industry interest in these ingredients due to their possible use as non-meat protein sources. Several studies have evaluated meat substitution by non-meat protein sources in meat products. Leonard *et al.* (2019), used up to 36% of the total ingredient composition of lupin flour for meat substitution within beef sausage and concluded that carbohydrate levels were increased, but fat and protein content decreased. Lupin flour

increase also improved emulsion stability and cooking yield while the strength of the textural structure became weaker. After sensory analysis, they observed that they could incorporate lupin flour up to 12% of the total content with no significant effect on consumers' acceptability. At higher amounts (up to 36%), a negative impact was observed in consumers' acceptability in flavour and texture terms.

Another study by Kamani *et al.* (2019), investigated the suitability of different plant proteins to wholly and partially substitute chicken meat in sausage. They determined that 100% replacement with plant proteins is promising since sensory evaluation demonstrated that the overall acceptability of meat-free sausages was like the full meat product. Despite this, they suggested more studies and future research that might improve gel-forming characteristics, which have been shown to be the main obstacle in meat-free sausage manufacturing.

In the study of Wang *et al.* (2019), using *Lentinula edodes*, also known as Shiitake mushroom, to substitute lean pork meat in sausages has proven to be a feasible option. Moisture contents, total dietary fibre, and total phenolic contents were superior when using this altered sausage. Also, methionine, glutamic acid, and cysteine were enhanced compared to the control. Nevertheless, this alternative reduced the protein content and energy levels of the modified sausages. The use of mushrooms induced a slight darkening and softening of the sausages texture. From this sensory perspective, the best formulation was substituting 25% of lean pork meat with *Lentinula edodes*. Through these results, investigators concluded that this substitute is a potential alternative for reducing lean pork meat in sausages.

In the study by Gad EL Rab *et al.* (2019), where the main aim was to produce a new low-cost product with noteworthy nutritional value, two formulas replaced beef meat in sausages with 10% milled flaxseeds and 20% chickpea flour. According to sensory evaluations, these replacements were found to be the best palatable proportions. Both preparations were evaluated for physicochemical qualities, consumer acceptance, mineral content, and chemical composition, but also amino and fatty acids profiles. Results have proven that flaxseeds and chickpeas incorporation considerably decreased cooking damage percentage while boosting cooking profit percentage and water holding capability. The sensory assessment revealed that, in general acceptability, no significant differences were reported between the control sample and the two prepared formulas. Flaxseeds' addition enhanced raw fibre %, carbohydrates and unprocessed fat as well as dietary minerals calcium (Ca), iron (Fe), zinc (Zn), and phosphorus (P) while increasing fatty acids profile and methionine levels. In comparison, the chickpea alternative reduced fat content while improving raw fibre and carbohydrate content as well as linolenic acid and total essential amino acids percentage.

Additionally, using flaxseeds and chickpeas in beef sausage preparation reduced the final costs by 8.3 and 16%, respectively.

1.11. Conclusion

Due to the increase in demand for vegetarian, vegan, and flexitarian diets over the past years, consumers' search for products with high functional and nutritional values increased substantially. Looking for products not derived from animals (i.e., vegetarian and/or vegan) has increased globally. Additionally, the current abundance of products based on beans, seeds, and nuts is mainly due to consumers' demands for plant-based alternatives not only for dietary reasons, lifestyle, or health concerns but also due to the concerns associated with environmental sustainability. This dynamic process and change in consumer preferences have made the overall population more health-conscious and have demanded beneficial health-targeted value of food and its sustainability in the food chain. As a result, the food industry is compelled to advance functional foods, providing innovative foods that possess higher quality while adding value based on their functional properties. Despite the known advantages, the biggest hurdle associated with these innovative products lies in the fact that meat alternatives are currently much more expensive when compared to their meat counterparts, representing an economic challenge to vegetarian and vegan consumers.

Despite that, meat analogues present a more sustainable method of production when compared to traditional meat production systems requiring smaller natural sources volumes. Several studies have been made incorporating a broad range of ingredients to improve the physicochemical, nutritive, textural, and sensory properties of these meatless products. In the near future, more studies should be conducted in order to increase recognition and support of these products through consumer acceptability evaluation and improve perception about the beneficial effects of consuming meat analogues. New formulations and innovations related to these products should be supported since meat analogues are increasingly considered a potential alternative to real meat.

Several studies related to the microbiological characteristics of these innovative products show that their microbiota is highly dependent on ingredients, raw materials quality, processing conditions, ripening, and existing microbial population. Although there are few studies related to the chemical characterisation of these products, which indicated that the parameters were within acceptable limits for these kinds of foodstuffs, there are many challenges to overcome. Currently, these non-meat alternative products are still poorly studied. For this reason, several studies should be taken into consideration, not only to verify

their functional and nutritional value but also to confirm that they are safe from a microbiological and chemical point of view.

PART II

NON-MEAT *ALHEIRAS* CHARACTERIZATION

CHAPTER 2

Non-meat-based *alheiras* – a safer novel trend?

Abstract

In response to nutritional and health concerns, the food industry has begun to offer a wider variety of products that reflect changing consumer preferences. In addition to traditional *alheiras*, made with pork and/or poultry meats, other varieties of *alheiras* ("innovative") made from codfish, mushrooms, tofu, soy, and vegetables were launched on the Portuguese market. The objective of this study was the characterization of these products, giving particular attention to their microbiological and chemical safety. Therefore, fourteen different products were analysed. *Enterobacteriaceae*, *Enterococcus*, lactic acid bacteria, yeasts and moulds, were the prevalent microbiota of "innovative" *alheiras*. Sulphite reducing *Clostridium* spores, *Escherichia coli*, *Listeria monocytogenes*, *Salmonella* spp. or *Staphylococcus aureus* were not detected in any sample. No differences were observed between traditional and "innovative" *alheiras* concerning pH and water activity values while nitrites, nitrates and biogenic amines were found to be within accepted limits for these kinds of products. In terms of organic acids, lactic acid was found in all analysed samples while malic and succinic acid seemed to be predominant only in "innovative" *alheiras*. In conclusion, unlike traditional *alheiras* which often contain pathogenic agents, no harmful organisms nor chemical hazards were found in these new products, even though produced by the same companies.

2.1. Introduction

Consumers' interest in traditional and local foods has increased worldwide in recent years, especially in Europe (Albayrak & Gunes, 2010; Balogh, Békési, Gorton, Popp, & Lengyel, 2016; Guerrero *et al.*, 2009; Kühne, Vanhonacker, Gellynck, & Verbeke, 2010; Pieniak, Verbeke, Vanhonacker, Guerrero, & Hersleth, 2009; Verbeke & Roosen, 2009). It is believed that local products are more authentic, nutritious, fresher, and tastier, and buying them from local producers will support and contribute to the local and national economy (Chambers, Lobb, Butler, Harvey, & Bruce Traill, 2007). Also, consumers are increasingly aware that food is connected with their health status and this growing awareness associated with progress in nutrition science provides the food industry with opportunities to develop a range of new products (Hung, Verbeke, & de Kok, 2016; Marcos, Viegas, de Almeida, & Guerra, 2016; Ojha, Kerry, Duffy, Beresford, & Tiwari, 2015).

Alheiras are traditional, smoked, naturally fermented meat sausages, produced in the North of Portugal. In recent years, different formulations of *alheiras* made with ingredients other than pork and/or poultry meats, such as fish, veal, lamb, mushrooms, tofu, soy and vegetables (in this manuscript denominated "innovative" types) have become available, as a way to improve competitiveness through innovation.

Even though there is already extensive scientific knowledge on fermentation and behaviour of pathogens in traditional *alheiras* (Barbosa, Gibbs, & Teixeira, 2010; Felício, Hogg, Gibbs, Teixeira, & Wiedmann, 2007; Ferreira *et al.*, 2011), to our knowledge detailed characterization of these "innovative" products is not available in the scientific literature. Despite traditional fermented meat products are considered relatively safe due to their low pH, low water activity (a_w), high salt level, presence of nitrate and nitrite, competition with endogenous microbiota, addition of spices, herbs and smoke compounds, several studies demonstrated that they can harbour pathogenic bacteria (Anal *et al.*, 2020; Ferreira *et al.*, 2006; Ferreira, Barbosa, Silva, & Felício, 2007; Ferreira, Barbosa, Silva, & Vendeiro, 2007; Prado, Sampayo, González, Lombó, & Díaz, 2019; Siriken, Pamuk, Özakin, Gedikoglu, & Eyigör, 2006; Talon, Leroy, & Lebert, 2007). Foodborne pathogens like *Listeria monocytogenes*, *Escherichia coli*, *Salmonella* spp., *Clostridium botulinum* and *Staphylococcus aureus* can overcome hurdles imposed during processing and cause serious human infections and intoxications and some outbreaks have been reported worldwide (Arnedo-Pena *et al.*, 2016; Castellano, Holzapfel, & Vignolo, 2004; Cocolin *et al.*, 2007; Colak, Hampikyan, Ulusoy, & Bingol, 2007; Ethelberg *et al.*, 2009; Kuhn, Torpdahl, Frank, Sigsgaard, & Ethelberg, 2011; Sartz *et al.*, 2008; Schimmer *et al.*, 2008).

"Innovative" *alheiras* are produced by the same companies that produce the traditional ones, so it is predictable that many of the problems previously associated with these traditional products, could also be associated with these new products. Therefore, the main objective of this study was the microbiological and chemical characterization of "innovative" *alheiras* with particular reference to factors that might influence product safety.

2.2. Material and methods

2.2.1. Sampling

A group of 14 "innovative" *alheiras* (Table 2.1) available on the Portuguese market were selected. On each occasion, whenever possible, a traditional *alheira* from the same producer was also selected. This study was conducted between September 2017 and May 2018, during which two *alheiras* from each type were bought from supermarkets on two different occasions (Lot A and B). Samples were transported to the laboratory in portable, insulated cold-bags and stored at 4 °C until analysis, normally between 1 and 2 days after collection. From each lot, two *alheiras* were divided into various pieces and manually mixed.

2.2.2. Physicochemical analysis

The pH value was determined directly with a Crison MicropH 2002 pH-meter (Crison, Barcelona, Spain) equipped with an InLab 427puncture electrode (Mettler Toledo, Columbus, OH, USA). Titratable acidity (TA) was measured by titration from the pH jump in endpoint mode (by default pH value is set to 8.1–8.2) with 0.1 NaOH (Merck, Darmstadt, Germany). Samples were analysed in triplicate and titratable acidity was calculated as a percentage of lactic acid (Zaika, Zell, Smith, Palumbo, & Kissinger, 1976).Water activity was assessed using the AquaLab Series 3 Water Activity Meter (METER Group, Inc. USA) equipment with a probe measuring over the range 0–1 aw with temperature control.

2.2.3. Nitrite and nitrate concentrations

For each sample, 200 g were used for analysis of nitrite and nitrate concentrations following Portuguese standards NP 1846:2006 and NP 1847-1:2009, respectively. A standard curve was established for both analyses. Two readings were performed for each test.

Table 2.1 – Information and main characteristics of analysed *alheiras*

Producer / Sample		Denomination	Information and Main Characteristics							
			Main Ingredient	Casing type	Food Preservatives	Packaging type	Consumption Indications	a_w	pH	TA[a]
A	1	Traditional White Label	Chicken meat; Bread; Bísaro pork meat; Water; Olive oil; Spices	Natural cow gut	E281; E300; E321	Protective atmosphere	No indication	0.981 ± 0.001	6.0 ± 0.02	0.70 ± 0.04
	2	Vegetarian	Bread; Green and black olives; Olive oil; Carrot; Green and red pepper; Spices	100% synthetic	E281; E330	Protective atmosphere	Do not bake in the oven / 75°C Max.	0.991 ± 0.001	4.2 ± 0.01	0.12 ± 0.01
	3	Traditional	Chicken meat; Bread; Bísaro pork meat; Water; Olive oil; Spices	Natural cow gut	E281; E321; E330	Protective atmosphere	Cook until 75°C	0.988 ± 0.001	5.1 ± 0.02	0.34 ± 0.03
	4	Codfish	Bread; Codfish; Green and black olives; Olive oil; Spices	Natural cow gut	E281; E321; E330	Protective atmosphere	Cook until 75°C	0.991 ± 0.002	4.7 ± 0.04	0.17 ± 0.01
B	5	Leek and Mushrooms	Chicken meat; Mushrooms; Bread; Pork loin; Leek; Water; Olive oil; Oregano; Sweet chili	Natural casing	No indication	Protective atmosphere	Cook over 65°C	0.986 ± 0.000	5.3 ± 0.00	0.61 ± 0.03
	6	Traditional	Poultry meat (Chicken, turkey, duck); Bread; Spices	No indication	No indication	Protective atmosphere	Grilled until complete cooking	0.982 ± 0.001	5.7 ± 0.05	0.25 ± 0.01
C	7	Traditional	Pork meat; Wheat bread; Chicken meat; Pork fat; Water; Olive oil; Spices	No indication	E200; E215; E260; E270; E330; E334	Protective atmosphere	Consume after cooking	0.983 ± 0.002	5.5 ± 0.01	0.67 ± 0.02
	8	Soy	Soy; Wheat bread; Olive oil; Spices; Starter cultures	Natural casing	E260; E270; E330; E334; E407; E508; E516;	Protective atmosphere	Consume after cooking	0.982 ± 0.003	4.9 ± 0.03	0.33 ± 0.02
D	9	Vegetables and mushrooms	Shiitake mushrooms; Water; Wheat bread; Vegetables; Olive oil; Spices	Inedible cellulose gut	No indication	No indication	Consume after cooking	0.987 ± 0.001	5.5 ± 0.01	0.34 ± 0.01
E	21	Traditional	Bread; Pork meat and fat; Poultry meat; Spices	Natural pork gut	E250; E252; E262; E301; E331; E450; E451; E452;	Protective atmosphere	Consume after cooking	0.972 ± 0.001	5.6 ± 0.04	0.41 ± 0.01
	10	Shiitake mushrooms	Bread; Pork meat and fat; Poultry meat; Mushrooms; Spices	Natural pork gut	E262; E331; E452	Protective atmosphere	Consume after cooking	0.976 ± 0.001	4.6 ± 0.02	1.00 ± 0.02
	11	Apple	Bread; Pork meat and fat; Apple; Poultry meat; Spices	Natural pork gut	E250; E252; E262; E301; E331; E450; E451; E452;	Protective atmosphere	Consume after cooking	0.970 ± 0.001	4.6 ± 0.05	1.03 ± 0.03
	12	Cheese	Bread; Pork meat and fat; Cheese; Poultry meat; Spices	Natural pork gut	E250; E252; E262; E301; E331; E450; E451; E452	Protective atmosphere	Consume after cooking	0.977 ± 0.001	4.8 ± 0.05	1.16 ± 0.01
F	13	Vegan	Water; Bread; Sunflower oil; Olive oil; Spices; Vinegar	Inedible synthetic gut	No indication	Sterilization heat treatment	Ready to eat	0.979 ± 0.001	6.0 ± 0.08	0.13 ± 0.01

Table 2.1 – Information and main characteristics of analysed *alheiras* (cont.)

Producer / Sample		Denomination	Information and Main Characteristics							
			Main Ingredient	Casing type	Food Preservatives	Packaging type	Consumption Indications	a_w	pH	TA[a]
G	22	Traditional	Bísaro pork meat; Chicken broth; Wheat bread; Olive oil; Spices	Cow gut	No indication	No indication	Heat treatment before consumption	0.981 ± 0.001	4.9 ± 0.01	0.46 ± 0.01
	14	Thyme and marjoram	Wild boar; Pork fat; Water; Wheat bread; Olive oil; Spices natural extracts	Cow gut	E262; E331	No indication	Heat treatment before consumption	0.980 ± 0.001	4.3 ± 0.02	n.d.
H	15	Tofu	Tofu; Whole grain bread; Seitan; Soy sauce; Parsley, Coriander, Cumin, Fennel, Ginger	Inedible cellulose film	No indication	No indication	Grilled, fried or in oven	0.973 ± 0.000	5.2 ± 0.03	0.35 ± 0.00
I	16	Tofu	Tofu; Whole grain bread; Seitan; Soy sauce; Parsley, Coriander, Cumin, Fennel, Ginger	Inedible cellulose film	No indication	No indication	Grilled, fried or in oven	0.983 ± 0.000	5.1 ± 0.03	0.35 ± 0.00
J	17	Vegetarian	Mushrooms; Wheat bread; Olive oil; Spices	Natural pork gut	No indication	No indication	Grilled, fried or in oven	0.973 ± 0.002	4.2 ± 0.01	0.25 ± 0.01
	18	Traditional	Rooster meat; Wheat bread; Pork meat; Spices	Natural pork gut	No indication	No indication	Grilled, fried or in oven	0.965 ± 0.002	5.1 ± 0.02	0.80 ± 0.03
K	19	Vegetarian	Mushrooms; Wheat bread; Green asparagus; Olive oil; Spices	Edible gut	No indication	Vacuum packed	Cook until 72°C	0.961 ± 0.001	4.0 ± 0.01	0.18 ± 0.01
	20	Traditional	Chicken meat; Wheat bread; Bísaro pork meat; Olive oil; Spices	Edible gut	No indication	Vacuum packed	Cook until 72°C	0.959 ± 0.001	4.2 ± 0.04	0.32 ± 0.01

[a] – Titratable Acidity (TA) (lactic acid, %); aw, pH and TA are reported as means ± standard deviations; n.d. – non determined.

2.2.4. Biogenic amines determination

Ten grams of each sample of *alheira* were weighed into 85 ml test tubes and extracted with 20 ml of trichloroacetic acid (5%) (Merck). Extracts were derivatized with o-phthalaldehyde (OPA) (Sigma Diagnostics, St. Louis. MO, USA) and biogenic amines (BA) were determined by High-Performance Liquid Chromatography (HPLC) using a method based on that described by Komprda *et al*. (2004).

2.2.5. Organic acids quantification by High-Performance Liquid Chromatography (HPLC)

Five grams of each sample were added to 50 ml water and the preparation was vigorously shaken and blended with a vortex. Samples were centrifuged at 3000×g for 15 min and the supernatant was filtered initially through filter paper and finally through 0.45 µm HPLC certified disposable syringe filters (Chromafil Pet-45/25; Macherey- Nagel GmbH & Co) before injecting into HPLC. The chromatographic system consisted of a Beckman model 126 pump, a Beckman automatic injector (model 508), a Beckman DAD detector (model 168), and an Aminex HPX-87H cation exchange column at 40 °C. Quantification of each sample was done at 210 nm, after the quantification of several standard compounds at different concentrations. The mobile phase was composed by 2.5 mM of H_2SO_4 with 0.6 ml/min flow. Eleven standard solutions were analysed at different concentrations: between 0.1 and 2 g/L of acetic, citric, lactic, and malic acids and between 0.01 and 0.2 g/L of succinic, butyric, isobutyric and isovaleric acids (all from Merck).

2.2.6. Microbiological analysis

Twenty-five grams of each composite sample (in duplicate) were added to 225 ml of sterile buffered peptone water (Biokar Diagnostics, Beauvais, France), and homogenized in a stomacher (Interscience, Saint Nom la Brèteche, France) for 2 min. Appropriate decimal dilutions were prepared in Ringer's solution (Biokar Diagnostics) for microbial enumeration: colony counts at 30 °C on Plate Count Agar according to ISO 4833-1:2013; lactic acid bacteria (LAB) counts at 30 °C on De Man-Rogosa Sharpe (MRS) medium according to ISO 15214:1998; Enterobacteriaceae on RAPID' *Enterobacteriaceae* medium (Bio-Rad, Hercules, CA, USA; ISO 16140:2016; ISO 21528-2:2017); *Staphylococcus* coagulase positive according to ISO 6888-1:1999; *E. coli* according to ISO 16649-2:2001; enterococci on Bile Esculin Azide Agar (Biokar Diagnostics) incubated at 30 °C for 72 h (Ferreira, Barbosa, Silva, & Vendeiro, 2007); *L. monocytogenes* according to ISO 11290-2:2017 and yeasts and moulds according to ISO 21527-1:2008. Detection of *L. monocytogenes* (ISO

11290-1:2017), *Salmonella* spp. (ISO 6579-1:2017) and sulphite-reducing *Clostridium* spores (NP 2262:1986) were also performed. After appropriate incubation, colonies were counted and/or confirmatory tests performed and the colony forming units (CFU)/g calculated.

2.2.7. Statistical analysis

An analysis of variance was carried out for microbiological analysis to test any significant difference between each lot and formulation of *alheira*. Multiple comparisons were evaluated by Tukey's post-hoc test and all analyses were performed using IBM SPSS Statistics, 24 (IBM Corporation, USA). The mean difference was considered significant at the 0.05 level.

2.3. Results and discussion

2.3.1. Chemical analysis

The results of the physicochemical analyses are shown in Tables 2.1 and 2.2. No differences were observed between traditional and "innovative" *alheiras* with pH values ranging 4.2 to 6.0 and 4.0 to 6.0, respectively (Table 2.1). Also, evident differences between water activity values (Table 2.1) were not observed for traditional (0.959 – 0.988) and "innovative" *alheiras* (0.961 – 0.991). Pathogenic bacteria development is partially inhibited during various sausage production steps, as well as reduction of pH and a_w during this process (Ferreira, Barbosa, Silva, & Felício, 2007). In this kind of products, pH below 4.0 and water activity lower than 0.85 are considered sufficiently to ensure microbiological safety (Marcos *et al*., 2016); in this study, none of the samples showed values of pH and/or water activity below those mentioned, which means that contaminants may be able to grow, specially before product filling or if the product is exposed to temperature abuse (Ferreira, Barbosa, Silva, & Felício, 2007). In food analysis, pH and titratable acidity are two interconnected concepts when dealing with acidity. While pH is important to determine if a microorganism is able to grow in a specific food, titratable acidity can indicate the impact organic acids will have on food flavour (Capita, Llorente-Marigo, Prieto & Alonso-Calleja, 2006). Overall, *alheiras* made with traditional ingredients, such as meat and fat, demonstrated higher titratable acidity values, from 0.25 to 1.16 (Table 2.1).

2.3.2. Nitrite and nitrate concentrations

In all analysed samples (Table 2.2), nitrite and nitrate concentrations were lower than the legal standards, i.e. 150 mg/kg (European Parliament and Council, 2011). Even though in fermented meat products, nitrites and/or nitrates are well-known for their antimicrobial effects against pathogenic bacteria, such as *Clostridium*, *L. monocytogenes* and *Salmonella* spp. (Cammack *et al.*, 1999; Christieans, Picgirard, Parafita, Lebert, & Gregori., 2018; Hammes, 2012; Hospital, Hierro, & Fernández., 2014, 2012). As far as we know (since this information is not specified on all labels), unlike many similar products, *alheiras* are not traditionally prepared using nitrites or nitrates and for that reason it was expected to find very low concentrations of these compounds.

2.3.3. Biogenic amines determination

Considerable variability was observed in the content of biogenic amines in *alheiras* from different producers (Table 2.2). Histamine (0.0 – 18.9 mg/kg), tyramine (0.0 – 2.1 mg/kg), methylamine (0.0 – 59.0 mg/kg) and ethylamine (0.0 – 14.3 mg/kg) were the biogenic amines more often detected in the samples analysed. Histamine and tyramine were detected in 21 samples, while methylamine and ethylamine were found in 15 and 14 analysed products, respectively. Additionally, the highest concentrations of putrescine were detected in samples A2 (39.7 mg/kg; vegetarian) and K20 (27.2 mg/kg; traditional) and cadaverine in samples A2 (29.7 mg/kg) and C7 (40.4 mg/kg; traditional). No correlation between type of *alheira* and/or producer was found. Biogenic amines contents found in this study were similar to those previously reported for fermented sausages (Ekici & Omer, 2018; Gong, Qi, Wang, Lin, & Li, 2014; Hernández-Jover, Izquierdo-Pulido, Veciana-Nogués, Mariné-Font, & Vidal-Carou, 1997; Vidal-Carou, Veciana-Nogués, Latorre-Moratalla, & Bover-Cid, 2014; Singh, Pathak, & Verma, 2012). Histamine, tyramine, putrescine and cadaverine are the most commonly found biogenic amines in dairy products, fish and fish products, meat and meat products, fermented vegetables and soy products being derived by decarboxylation from amino acids (Alvarez & Moreno-Arribas, 2014; Landete, De Las Rivas, Marcobal, & Muñoz, 2011).

2.3.4. Organic acids

In Table 2.3 is presented the quantification of organic acids for all samples analysed. As expected, lactic acid was found in all samples analysed (0.50 – 7.02 mg/g) which corroborates the fact that LAB are the predominant bacteria found in *alheiras* microbiota

(Franciosa, Alessandria, Dolci, Rantsiou, & Cocolin, 2018; Macori & Cotter, 2018). One vegan and two tofu *alheira*-like products showed low lactic acid values 0.50, 0.94 and 0.96 mg/g respectively, which corroborate the low values of LAB counts found. Malic acid, for example, largely found in fruits and vegetables like apple and carrots, was mainly found in "innovative" *alheiras*: sample A2 (vegetarian) with 2.50 mg/g, followed by sample E11 (Apple) with 0.62 mg/g. Succinic acid, another by-product, seems to be predominant in soy, tofu, vegetables and mushrooms "innovative" *alheiras*, except for sample B6 which was the only *alheira* that presents similar values to the same producer of "innovative" *alheira*, 0.58 and 0.62 mg/g, respectively. Acetic (producer C) and citric acid (producer A and C) are often added as food preservatives, however no relation was found since higher values were obtained in samples with no preservatives declared. Butyric acid found in sample B6 (18.11 mg/g) may indicate that the fat present in this *alheira* could be rancid, however no sensorial analysis was performed to support this (Mortera, Zuljan, Magni, Bortolato, & Alarcón, 2018).

Organic acids quantification is important to monitor bacterial growth and activity and their presence has been analysed in several fermented foods and dairy products since acidic conditions minimize pathogen growth and spoilage development (Califano & Bevilacqua, 2000; González de Llano, Rodriguez, & Cuesta, 2008; Mullin & Emmons, 1997; Sirén, Sirén, & Sirén, 2015; Tormo & Izco, 2004; Žulj *et al.*, 2015). Furthermore, they also contribute to the flavour and aroma of fermented products.

Table 2.2 – Nitrite (mg $NaNO_2$/kg), nitrate (mg $NaNO_3$/kg) and biogenic amine (mg/kg) content found in analysed *alheiras*

Producer / Sample		Denomination	Nitrite	Nitrate	Histamine	Methylamine	Ethylamine	Tyramine	Phenylethylamine	Isoamylamine	Putrescine	Cadaverine
A	1	Traditional White Label	6.4	12.7	0.7	59.0	1.2	2.1	1.8	3.1	0.0	0.0
	2	Vegetarian	0.6	37.6	16.7	0.4	2.2	32.1	3.3	0.0	39.7	29.7
	3	Traditional	2.2	4.5	0.5	0.0	1.2	1.0	0.0	0.0	0.0	0.0
	4	Codfish	1.7	13.6	2.2	0.0	2.4	0.9	0.0	0.0	0.0	0.0
B	5	Leek and Mushrooms	2.3	58.1	9.8	0.8	10.0	1.5	0.0	3.1	0.5	0.0
	6	Traditional	11.9	26.0	7.3	1.0	14.3	1.8	0.0	0.0	0.0	0.0
C	7	Traditional	3.5	18.3	18.9	1.0	3.5	7.4	0.0	0.0	5.2	40.4
	8	Soy	2.5	40.9	18.8	1.1	6.0	2.1	0.0	0.0	3.0	0.0
D	9	Vegetables and mushrooms	6.3	31.0	1.3	0.5	0.0	0.0	0.4	0.7	1.0	0.0
E	21	Traditional	1.0	21.0	8.7	0.4	2.7	1.6	2.4	0.0	0.0	0.0
	10	Shiitake mushrooms	0.5	3.1	1.5	0.0	0.0	20.7	0.0	0.0	0.0	0.0
	11	Apple	1.1	2.9	4.2	42.8	1.5	10.7	1.8	0.0	0.0	0.0
	12	Cheese	0.9	34.7	2.7	0.0	0.0	6.2	0.3	0.4	0.0	0.0
F	13	Vegan	0.0	16.2	1.4	10.2	1.1	0.9	0.6	1.1	0.5	0.0
G	22	Traditional	0.5	9.2	0.3	58.2	1.1	1.0	0.0	0.0	0.0	0.0
	14	Thyme and marjoram	0.0	13.0	0.0	7.9	1.1	18.7	1.7	1.6	2.2	0.0
H	15	Tofu	0.0	87.6	1.4	0.0	0.0	0.7	0.6	0.0	0.0	0.0
I	16	Tofu	0.1	87.0	4.6	0.0	0.0	0.9	0.0	0.0	0.0	0.0
J	17	Vegetarian	1.0	53.0	2.3	0.3	0.0	1.0	0.0	0.0	0.0	0.0
	18	Traditional	4.8	73.0	5.8	1.4	0.0	1.8	1.5	0.0	1.5	3.5
K	19	Vegetarian	1.7	27.0	4.4	0.0	1.3	1.9	0.0	0.0	0.0	0.0
	20	Traditional	1.7	1.3	12.4	0.5	0.0	4.8	0.0	0.0	27.2	0.0

Table 2.3 – Quantification of organic acids (mg/g) in analysed *alheiras* (mg/g)

Producer/Sample		Denomination	Organic Acids (mg/g)							
			Acetic	Citric	Lactic	Malic	Succinic	Butyric	Isobutyric	Isovaleric
A	1	Traditional White Label	0.74	< Q.L	4.69	N.D.	N.D.	N.D.	0.70	N.D.
	2	Vegetarian	N.D.	0.16	1.20	2.50	< Q.L	N.D.	0.31	N.D.
	3	Traditional	< Q.L	< Q.L	5.13	N.D.	< Q.L	N.D.	0.81	N.D.
	4	Codfish	N.D.	0.12	1.04	< Q.L	< Q.L	N.D.	0.18	N.D.
B	5	Leek and Mushrooms	N.D.	< Q.L	3.47	N.D.	0.62	N.D.	1.56	N.D.
	6	Traditional	N.D.	< Q.L	1.33	< Q.L	0.58	18.11	N.D.	0.36
C	7	Traditional	< Q.L	N.D.	5.28	N.D.	N.D.	N.D.	0.94	N.D.
	8	Soy	N.D.	1.24	1.60	0.13	0.14	N.D.	< Q.L	N.D.
D	9	Vegetables and mushrooms	N.D.	0.24	3.18	N.D.	1.68	N.D.	0.41	N.D.
E	10	Shiitake mushrooms	1.22	0.35	6.55	< Q.L	0.66	N.D.	1.63	N.D.
	11	Apple	0.55	< Q.L	4.74	0.62	N.D.	N.D.	1.24	N.D.
	12	Cheese	0.52	< Q.L	6.69	0.12	N.D.	N.D.	1.61	N.D.
F	21	Traditional	N.D.	0.50	1.67	N.D.	< Q.L	N.D.	0.23	N.D.
	13	Vegan	N.D.	0.20	0.50	< Q.L	< Q.L	N.D.	< Q.L	N.D.
G	14	Thyme and marjoram	---	---	---	---	---	---	----	---
	22	Traditional	1.84	0.10	4.10	N.D.	N.D.	N.D.	0.54	N.D.
H	15	Tofu	N.D.	0.28	0.96	< Q.L	1.04	N.D.	0.15	N.D.
I	16	Tofu	< Q.L	0.28	0.94	< Q.L	1.34	N.D.	0.20	N.D.
J	17	Vegetarian	N.D.	N.D.	3.23	0.34	0.69	N.D.	0.32	N.D.
	18	Traditional	1.01	N.D.	7.02	N.D.	N.D.	N.D.	0.73	0.35
K	19	Vegetarian	N.D.	N.D.	1.70	< Q.L	< Q.L	N.D.	0.28	N.D.
	20	Traditional	N.D.	< Q.L	2.53	N.D.	N.D.	N.D.	0.28	1.97

N.D. – Non-detected; < Q.L – Below Quantification Limit (0.05 g/L for acetic, lactic and succinic acids; 0.01 g/L for citric, malic, butyric, isobutyric and isovaleric acids).

2.3.5. Microbiological analyses

Regarding the microbiological characterization, in general, variability was observed between producers and even between lots from the same producer (Table 2.4), but no significant differences were observed ($p > 0.05$). Ingredients quality and specific conditions during processing of meat fermented sausages, determines the microbiota developed during fermentation. Some differences found might be related not only with small variations during production, different raw materials used, producer's different geographic locations but also due to the "age" of the sample, since all analyses were performed before the due date, but some may have been packed longer than others.

Significant differences ($p < 0.05$) were observed between traditional and "innovative" *alheiras* and it is possible to observe that any of the biological hazards investigated were found in "innovative" *alheiras* (Table 2.4). A more detailed analysis revealed that total aerobic microorganisms at 30 °C in traditional *alheiras* showed higher values, mainly above 6.1 log CFU/g, when compared to "innovative", except for producer C which showed similar values possibly due to the use of natural casing in both types of *alheiras* (Djordjevic, Pecanac, Todorovic, & Dokmanovic, 2015). Curiously, tofu and vegan *alheiras*-like products showed no microbiological contamination, and this may be due to not being traditionally fermented or because a sterilization step might be applied during production/packaging (Table 2.1).

Numerous authors have described the importance of the natural microbiota throughout the fermentation process, namely LAB, responsible for inhibition of unwanted microbial growth and development of particular colour and flavour (Ferreira *et al.*, 2009). Since traditional *alheiras* are fermented products, it is quite common to find high numbers of LAB and these types of bacteria have been shown to play an important role in the production of numerous fermented sausages (Correia Santos, Fraqueza, Elias, Salvador Barreto, & Semedo-Lemsaddek, 2017; Moretti *et al.*, 2004; Papamanoli, Kotzekidou, Tzanetakis, & Litopoulou-Tzanetaki, 2002; Papamanoli, Tzanetakis, Litopoulou-Tzanetaki, & Kotzekidou, 2003). For this reason, and for most of the *alheiras* analysed, except tofu and vegan *alheiras*-like products as also described, LAB was the dominant microbiota, in some cases higher than 6.0 log CFU/g. These LAB, in particular *Lactobacillus* and *Enterococcus* can be found in relatively high numbers (Albano *et al.*, 2009b; Albano, Henriques, Correia, Hogg, & Teixeira, 2008; Barbosa, Ferreira, & Teixeira, 2009; Correia Santos, Fraqueza, Elias, Barreto & Semedo-Lemsaddek, 2017; Hugas, Garriga, & Aymerich, 2003; Rantsiou & Cocolin, 2006).

Enterococci, found in most of the products from this study in numbers higher than 4.0 log CFU/g, are commonly found in fermented sausages, as they contribute to their aromatization through glycolytic, proteolytic and lipolytic actions (Sarantinopoulos *et al.*, 2001). Nevertheless, presence of enterococci could be a concern as many strains possess virulence factors (Barbosa *et al.*, 2010; Franz, Stiles, Schleifer, & Holzapfel, 2003).

In addition to LAB, yeasts, and moulds play an important role in the development of organoleptic characteristics in fermented sausages. In this study, they were commonly found in most of the samples of both types of *alheiras*. *Alheiras* made with apple (E11), vegetables and mushrooms (B5, D9) presented more yeasts than those mainly constituted by meat, varying from 2.8 to 5.6 log CFU/g. An exception was found in traditional sample J18, which presented yeasts values higher than the similar samples, possibly due to the fact that sampling has been performed closer to the expiration date of the product.

Enterobacteriaceae were found in less than half of the samples (37%), presenting values higher than 3.1 log CFU/g, though they were mainly found in traditional *alheiras* in which meat is the main ingredient. An exception was found in producer A - codfish *alheira*-like product – and one possible explanation is the fact that it contains 1% of cow natural casing (Djordjevic *et al.*, 2015).

Escherichia coli is usually transmitted to humans through food, environmental contact or directly from person to person and some outbreaks have been associated with fermented sausage consumption, but also with vegetables and fruits (Sartz *et al.*, 2008). In this study, *E. coli* was found in six products from five different producers, all of them in traditional *alheiras* samples, presenting values higher than 0.8 log CFU/g. This may be indicative of poor sanitary conditions during manipulation/production or even cross contamination.

Listeria monocytogenes was present in products from three different producers, in levels higher than 2.3 log CFU/g. This was previously described in other studies and may indicate poor facility conditions (Felício *et al.*, 2007; Ferreira *et al.*, 2006; Ferreira, Barbosa, Silva, & Vendeiro, 2007, Ferreira, Barbosa, Silva, & Felício, 2007; Ferreira *et al.*, 2009; Ferreira *et al.*, 2011; Ramalheira, Silva, Hogg, & Teixeira, 2007; Talon & Leroy, 2011; Xavier, Gonzales-Barron, Paula, Estevinho, & Cadavez, 2014). Despite being present in traditional *alheiras*, contamination by this pathogen was not found in "innovative" products which may indicate that contaminations, including cross-contamination, did not occur during common processes. Also, these innovative products are produced in lower numbers which might indicate that better control exists during innovative *alheiras* manufacturing, when compared to traditional *alheiras* production. *Staphylococcus aureus*, *Salmonella* spp. and sulphite

reducing *Clostridium* spores were found neither in the traditional nor in the "innovative" samples.

Alheiras, traditional or "innovative", are generally cooked before consumption, except for vegan *alheira* which is supposed to be Ready To Eat (RTE), either baked in the oven, fried, or grilled. However previous studies suggested that internal temperatures may often not be sufficient to kill all of the pathogens initially present (Felício *et al.*, 2011). Therefore, and in the absence of official microbiological criteria, guidelines for the interpretation of results of microbiological testing of ready-to-eat (RTE) foods placed on the market were followed (Food Safety Authority of Ireland, 2020; revision 3). According to these criteria, all vegetarian *alheira* samples but also vegan and tofu ones presented satisfactory results related to hygiene indicators like *Enterobacteriaceae* and *E. coli* (*Enterobacteriaceae* were found below 10^2 CFU/g and no *E. coli* was found). However, some borderline results were found for *Enterobacteriaceae* in traditional samples like A4, B5 and E21 (results between 10^2 and 10^4 CFU/g) and in samples C7 and E21 for *E. coli* (≥20–10^2 CFU/g). Traditional samples like A1, A3 and J18 were considered unsatisfactory for both *Enterobacteriaceae* and *E. coli* presenting results higher than 10^4 CFU/g and 10^2 CFU/g, respectively. *Alheiras* B6, C7, J18 and K20 were also considered unsatisfactory, but only for *Enterobacteriaceae*. Looking at these values, unsatisfactory results represent unacceptable levels of microbial contamination and this should be investigated in order to detect the cause of the elevated levels. Measures as part of Hazard Analysis and Critical Control Point (HACCP) based procedures and Good Manufacturing Practices (GHP) should be taken into consideration in order to ensure levels in subsequent batches of food are satisfactory. For borderline results, the action carried out should be proportional to the levels detected.

Regarding the presence of pathogens like coagulase-positive staphylococci and *Salmonella* spp., values under 20 CFU/g and absence in 25 g, respectively, are requested in order to consider a product as satisfactory. None of the analysed samples presented *Salmonella* nor coagulase-positive staphylococci. Concerning *L. monocytogenes*, the allowed limit is either '100 CFU/g' or 'absence in 25 g' depending on the category (Food Safety Authority of Ireland, 2020; revision 3). Samples A3, C7 and J18 were considered unsatisfactory, due to the presence of values above the legal limit (100 CFU/g). *Listeria monocytogenes* can be found on a wide range of foods and can persist in food production facilities and contaminate food after processing. Consuming food containing *L. monocytogenes* at levels >100 CFU/g is considered a major food safety risk. It is important to emphasize that *alheiras* are generally cooked before consumption and, if properly cooked, these products may in fact no longer be

considered unsatisfactory. According to the study of (Felício *et al*., 2011), in which internal temperature profiles of *alheiras* were monitored during frying, electric grilling and roasting (in both gas and wood-fired oven), the authors reported that, with exception of roasting, the remaining evaluated cooking methods might not be sufficient to inactivate the foodborne pathogen *L. monocytogenes*. The authors highlighted the fact that some of the internal temperature profiles of *alheiras* were quite low during frying or grilling, which suggest insufficient thawing of frozen *alheiras* (Felício *et al*., 2011). Freezing *alheiras* is a common practice so, in the absence of cooking treatments guidelines, the absence of pathogens in these products becomes important.

Comparatively, the differences between "innovative" and traditional *alheiras* indicate that even being manufactured by the same producer, samples analysed do not present the same biological hazards.

Table 2.4 – Microbiological characterization of *alheiras* (log CFU/g): important microbial parameters in fermented products

Producer /Sample		Type	Lot	Moulds	Yeasts	*Enterococcus*	LAB	Total Counts*	*Enterobacteriaceae*	*E. coli*	*L. monocytogenes*
A	1	Traditional white label	A	0.7 ± 0.0 ^{A}	1.5 ± 0.0 ^{A}	5.8 ± 0.1 ^{ADFGI}	8.5 ± 0.4 ^{ACN}	8.7 ± 0.0 ^{AC}	6.7 ± 0.2 ^{AGM}	3.3 ± 0.1 ^{AB}	<1 ± 0.0 ^{A}
			B	<1 ± 0.0 ^{A}	<1 ± 0.0 ^{A}	5.2 ± 0.0 ^{ADFGI}	6.1 ± 0.0 ^{ACN}	6.1 ± 0.0 ^{AC}	<1 ± 0.0 ^{AGM}	<1 ± 0.0 ^{AB}	<1 ± 0.0 ^{A}
	2	Vegetarian	A	<1 ± 0.0 ^{A}	1.3 ± 0.3 ^{A}	7.1 ± 0.1 ^{AC}	7.5 ± 0.3 ^{ADN}	7.7 ± 0.1 ^{AC}	<1 ± 0.0 ^{BD}	<1 ± 0.0 ^{A}	<1 ± 0.0 ^{A}
			B	<1 ± 0.0 ^{A}	<1 ± 0.0 ^{A}	5.2 ± 0.0 ^{AC}	5.8 ± 0.0 ^{ADN}	6.0 ± 0.0 ^{AC}	<1 ± 0.0 ^{BD}	<1 ± 0.0 ^{A}	<1 ± 0.0 ^{A}
	3	Traditional	A	<1 ± 0.0 ^{A}	<1 ± 0.0 ^{A}	7.5 ± 0.0 ^{ADE}	7.9 ± 0.0 ^{BCEGK}	8.2 ± 0.1 ^{AC}	7.2 ± 0.1 ^{CE}	0.9 ± 0.2 ^{B}	<1 ± 0.0 ^{AD}
			B	<1 ± 0.0 ^{A}	<1 ± 0.0 ^{A}	6.0 ± 0.0 ^{ADE}	8.7 ± 0.1 ^{BCEGK}	8.8 ± 0.0 ^{AC}	7.5 ± 0.0 ^{CE}	3.3 ± 0.0 ^{B}	2.3 ± 0.0 ^{AD}
	4	Codfish	A	1.0 ± 0.0 ^{A}	<1 ± 0.0 ^{A}	5.2 ± 0.2 ^{ADFGI}	5.8 ± 0.0 ^{AN}	6.0 ± 0.0 ^{C}	4.2 ± 0.0 ^{AF}	<1 ± 0.0 ^{A}	<1 ± 0.0 ^{A}
			B	<1 ± 0.0 ^{A}	<1 ± 0.0 ^{A}	4.3 ± 0.0 ^{ADFGI}	6.6 ± 0.0 ^{AN}	6.7 ± 0.0 ^{C}	4.2 ± 0.0 ^{AF}	<1 ± 0.0 ^{A}	<1 ± 0.0 ^{A}
B	5	Traditional with Leek & Mushrooms	A	2.4 ± 0.0 ^{BC}	5.6 ± 0.3 ^{B}	5.4 ± 0.2 ^{ADFGI}	7.3 ± 0.3 ^{AHN}	7.5 ± 0.0 ^{C}	3.9 ± 0.0 ^{AGHJL}	<1 ± 0.0 ^{A}	<1 ± 0.0 ^{A}
			B	2.3 ± 0.0 ^{BC}	5.2 ± 0.0 ^{B}	4.5 ± 0.0 ^{ADFGI}	5.5 ± 0.0 ^{AHN}	5.8 ± 0.1 ^{C}	3.8 ± 0.1 ^{AGHJL}	<1 ± 0.0 ^{A}	<1 ± 0.0 ^{A}
	6	Traditional	A	3.3 ± 0.2 ^{B}	4.5 ± 0.4 ^{BC}	5.8 ± 0.2 ^{ADFGH}	6.0 ± 0.1 ^{AINR}	6.4 ± 0.1 ^{AC}	5.2 ± 0.1 ^{AEG}	<1 ± 0.0 ^{AB}	<1 ± 0.0 ^{AE}
			B	2.5 ± 0.0 ^{B}	4.5 ± 0.0 ^{BC}	6.1 ± 0.1 ^{ADFGH}	7.2 ± 0.0 ^{AINR}	7.3 ± 0.0 ^{AC}	5.3 ± 0.0 ^{AEG}	0.8 ± 0.2 ^{AB}	<1 ± 0.0 ^{AE}
C	7	Traditional	A	1.8 ± 0.2 ^{ACD}	0.7 ± 0.0 ^{A}	8.1 ± 0.1 ^{BCEHK}	8.6 ± 0.1 ^{BCFKQ}	8.8 ± 0.0 ^{AC}	6.3 ± 0.0 ^{CEF}	1.5 ± 0.0 ^{AB}	<1 ± 0.0 ^{A}
			B	<1 ± 0.0 ^{ACD}	<1 ± 0.0 ^{A}	7.3 ± 0.0 ^{BCEHK}	8.7 ± 0.0 ^{BCFKQ}	8.7 ± 0.0 ^{AC}	7.1 ± 0.0 ^{CEF}	<1 ± 0.0 ^{AB}	2.5 ± 0.0 ^{A}
	8	Soy	A	0.8 ± 0.2 ^{A}	0.7 ± 0.0 ^{A}	8.2 ± 0.0 ^{BCEJM}	8.2 ± 0.0 ^{BCFKP}	8.6 ± 0.0 ^{AC}	<1 ± 0.0 ^{BI}	<1 ± 0.0 ^{A}	<1 ± 0.0 ^{A}
			B	<1 ± 0.0 ^{A}	<1 ± 0.0 ^{A}	8.2 ± 0.1 ^{BCEJM}	8.4 ± 0.0 ^{BCFKP}	8.6 ± 0.0 ^{AC}	<1 ± 0.0 ^{BI}	<1 ± 0.0 ^{A}	<1 ± 0.0 ^{A}
D	9	Vegetables & Mushrooms	A	1.3 ± 0.0 ^{A}	3.5 ± 0.1 ^{CDE}	<1 ± 0.0 ^{L}	7.4 ± 0.0 ^{AINR}	7.6 ± 0.0 ^{AC}	<1 ± 0.0 ^{BI}	<1 ± 0.0 ^{A}	<1 ± 0.0 ^{A}
			B	<1 ± 0.0 ^{A}	3.0 ± 0.0 ^{CDE}	1.5 ± 0.1 ^{L}	5.9 ± 0.0 ^{AINR}	5.9 ± 0.0 ^{AC}	<1 ± 0.0 ^{BI}	<1 ± 0.0 ^{A}	<1 ± 0.0 ^{A}
E	10	Traditional with Shiitake Mushrooms	A	2.4 ± 0.0 ^{BDE}	<1 ± 0.0 ^{A}	5.6 ± 0.0 ^{ADFGIK}	7.4 ± 0.0 ^{AEIJPQ}	7.5 ± 0.0 ^{AC}	<1 ± 0.0 ^{BI}	<1 ± 0.0 ^{A}	<1 ± 0.0 ^{A}
			B	2.0 ± 0.1 ^{BDE}	<1 ± 0.0 ^{A}	6.1 ± 0.0 ^{ADFGIK}	8.2 ± 0.0 ^{AEIJPQ}	8.4 ± 0.1 ^{AC}	<1 ± 0.0 ^{BI}	<1 ± 0.0 ^{A}	<1 ± 0.0 ^{A}
	11	Traditional with Apple	A	0.7 ± 0.0 ^{A}	3.1 ± 0.1 ^{CFG}	5.8 ± 0.0 ^{ADFGI}	8.1 ± 0.0 ^{BCDFHJR}	8.1 ± 0.0 ^{AC}	<1 ± 0.0 ^{BN}	<1 ± 0.0 ^{A}	<1 ± 0.0 ^{A}
			B	<1 ± 0.0 ^{A}	2.8 ± 0.0 ^{CFG}	5.1 ± 0.0 ^{ADFGI}	7.9 ± 0.0 ^{BCDFHJR}	7.9 ± 0.0 ^{AC}	<1 ± 0.0 ^{BN}	<1 ± 0.0 ^{A}	<1 ± 0.0 ^{A}
	12	Traditional with Cheese	A	1.2 ± 0.0 ^{A}	1.0 ± 0.0 ^{A}	4.0 ± 0.0 ^{BL}	8.8 ± 0.0 ^{BCFJ}	8.8 ± 0.0 ^{AC}	<1 ± 0.0 ^{BN}	<1 ± 0.0 ^{A}	<1 ± 0.0 ^{A}
			B	<1 ± 0.0 ^{A}	<1 ± 0.0 ^{A}	3.6 ± 0.0 ^{BL}	8.8 ± 0.0 ^{BCFJ}	8.8 ± 0.0 ^{AC}	<1 ± 0.0 ^{BN}	<1 ± 0.0 ^{A}	<1 ± 0.0 ^{A}
	21	Traditional	A	0.7 ± 0.0 ^{A}	3.3 ± 0.0 ^{ADG}	4.7 ± 0.0 ^{ADFGI}	7.7 ± 0.0 ^{BCJL}	7.8 ± 0.0 ^{AC}	<1 ± 0.0 ^{DIJKMN}	<1 ± 0.0 ^{AB}	<1 ± 0.0 ^{A}
			B	<1 ± 0.0 ^{A}	<1 ± 0.0 ^{ADG}	6.7 ± 0.0 ^{ADFGI}	9.2 ± 0.0 ^{BCJL}	9.4 ± 0.0 ^{AC}	3.1 ± 0.0 ^{DIJKMN}	1.4 ± 0.3 ^{AB}	<1 ± 0.0 ^{A}

Table 2.4 – Microbiological characterization of *alheiras* (log CFU/g): important microbial parameters in fermented products (cont.)

Producer /Sample		Type	Lot	Moulds	Yeasts	*Enterococcus*	LAB	Total Counts*	*Enterobacteriaceae*	*E. coli*	*L. monocytogenes*
F	**13**	Vegan	A	<1 ± 0.0 ^{A}	<1 ± 0.0 ^{A}	<1 ± 0.0 ^{LN}	<1 ± 0.0 ^{O}	<1 ± 0.0 ^{B}	<1 ± 0.0 ^{BN}	<1 ± 0.0 ^{A}	<1 ± 0.0 ^{A}
			B	<1 ± 0.0 ^{A}	<1 ± 0.0 ^{A}	<1 ± 0.0 ^{LN}	<1 ± 0.0 ^{O}	<1 ± 0.0 ^{B}	<1 ± 0.0 ^{BN}	<1 ± 0.0 ^{A}	<1 ± 0.0 ^{A}
G	**14**	Wild boar with thyme & marjoram	A	<1 ± 0.0 ^{A}	<1 ± 0.0 ^{A}	<1 ± 0.0 ^{LN}	7.7 ± 0.0 ^{AGIJNPQ}	7.7 ± 0.0 ^{AC}	<1 ± 0.0 ^{BN}	<1 ± 0.0 ^{AB}	<1 ± 0.0 ^{AC}
			B	N.D.	N.D.	N.D.	N.D.	N.D.	N.D ^{BN}	N.D.	N.D.
	22	Traditional	A	<1 ± 0.0 ^{A}	<1 ± 0.0 ^{A}	3.2 ± 0.0 ^{I}	7.1 ± 0.0 ^{BCDHI}	7.7 ± 0.0 ^{AC}	<1 ± 0.0 ^{BN}	<1 ± 0.0 ^{A}	<1 ± 0.0 ^{A}
			B	<1 ± 0.0 ^{A}	1.6 ± 0.0 ^{A}	3.7 ± 0.0 ^{I}	7.6 ± 0.0 ^{BCDHI}	7.9 ± 0.0 ^{AC}	<1 ± 0.0 ^{BN}	<1 ± 0.0 ^{A}	<1 ± 0.0 ^{A}
H	**15**	Tofu	A	<1 ± 0.0 ^{A}	<1 ± 0.0 ^{A}	<1 ± 0.0 ^{LN}	<1 ± 0.0 ^{O}	<1 ± 0.0 ^{B}	<1 ± 0.0 ^{BN}	<1 ± 0.0 ^{A}	<1 ± 0.0 ^{A}
			B	<1 ± 0.0 ^{A}	<1 ± 0.0 ^{A}	<1 ± 0.0 ^{LN}	<1 ± 0.0 ^{O}	<1 ± 0.0 ^{B}	<1 ± 0.0 ^{BN}	<1 ± 0.0 ^{A}	<1 ± 0.0 ^{A}
I	**16**	Tofu	A	<1 ± 0.0 ^{A}	<1 ± 0.0 ^{A}	<1 ± 0.0 ^{LN}	<1 ± 0.0 ^{O}	<1 ± 0.0 ^{B}	<1 ± 0.0 ^{BN}	<1 ± 0.0 ^{A}	<1 ± 0.0 ^{A}
			B	<1 ± 0.0 ^{A}	<1 ± 0.0 ^{A}	<1 ± 0.0 ^{LN}	<1 ± 0.0 ^{O}	<1 ± 0.0 ^{B}	<1 ± 0.0 ^{BN}	<1 ± 0.0 ^{A}	<1 ± 0.0 ^{A}
J	**17**	Vegetarian	A	<1 ± 0.0 ^{A}	<1 ± 0.0 ^{A}	2.8 ± 0.0 ^{BLN}	7.5 ± 0.0 ^{AGIKLN}	7.6 ± 0.0 ^{AC}	<1 ± 0.0 ^{BN}	<1 ± 0.0 ^{A}	<1 ± 0.0 ^{A}
			B	<1 ± 0.0 ^{A}	<1 ± 0.0 ^{A}	2.1 ± 0.1 ^{BLN}	6.8 ± 0.0 ^{AGIKLN}	6.9 ± 0.0 ^{AC}	<1 ± 0.0 ^{BN}	<1 ± 0.0 ^{A}	<1 ± 0.0 ^{A}
	18	Traditional	A	2.4 ± 0.1 ^{ACE}	2.9 ± 0.0 ^{BEF}	5.3 ± 0.1 ^{ADFGIKM}	8.8 ± 0.0 ^{BFMQ}	8.8 ± 0.0 ^{A}	6.4 ± 0.0 ^{AEGH}	<1 ± 0.0 ^{AB}	3.8 ± 0.0 ^{BCDE}
			B	<1 ± 0.0 ^{ACE}	5.3 ± 0.0 ^{BEF}	7.4 ± 0.0 ^{ADFGIKM}	9.9 ± 0.0 ^{BFMQ}	9.9 ± 0.0 ^{A}	4.4 ± 0.0 ^{AEGH}	3.4 ± 0.0 ^{AB}	<1 ± 0.0 ^{BCDE}
K	**19**	Vegetarian	A	<1 ± 0.0 ^{A}	1.6 ± 0.6 ^{A}	5.2 ± 0.0 ^{F}	5.4 ± 0.0 ^{N}	5.7 ± 0.0 ^{C}	<1 ± 0.0 ^{BK}	<1 ± 0.0 ^{A}	<1 ± 0.0 ^{A}
			B	<1 ± 0.0 ^{A}	<1 ± 0.0 ^{A}	3.3 ± 0.0 ^{F}	6.8 ± 0.0 ^{N}	6.8 ± 0.0 ^{C}	<1 ± 0.0 ^{BK}	<1 ± 0.0 ^{A}	<1 ± 0.0 ^{A}
	20	Traditional	A	2.3 ± 0.0 ^{ACE}	2.0 ± 0.4 ^{A}	7.4 ± 0.1 ^{ADFGIKM}	7.8 ± 0.1 ^{AGIJKMQ}	8.0 ± 0.0 ^{AC}	6.5 ± 0.1 ^{AEGHL}	<1 ± 0.0 ^{A}	<1 ± 0.0 ^{A}
			B	<1 ± 0.0 ^{ACE}	<1 ± 0.0 ^{A}	5.2 ± 0.1 ^{ADFGIKM}	7.7 ± 0.1 ^{AGIJKMQ}	7.7 ± 0.0 ^{AC}	5.2 ± 0.0 ^{AEGHL}	<1 ± 0.0 ^{A}	<1 ± 0.0 ^{A}

N.D. – Non-determined; * - Total counts at 30 ºC; Equivalent capital letters, by column, mean no significant differences between each *alheira* (p>0.05).

2.4. Conclusions

In this study, even though pH and water activity levels are insufficient to assure microbiological safety, nitrites, nitrates, biogenic amines and organic acids were found to be within accepted limits for this kind of product. No biological threat was detected in "innovative" *alheiras*, but some of the traditional products analysed have shown concerning microbiological status in terms of food safety. Even though no *S. aureus*, *Salmonella* spp. nor sulphite reducing *Clostridium* spores were detected, *E. coli* and *L. monocytogenes* were found in traditional *alheiras* available in the market.

In conclusion, unlike traditional *alheiras* which often contain pathogenic agents, no harmful organisms nor chemical hazards were found in these new products, even though produced by the same companies. An important aspect to take into consideration in future confirmation studies, is to understand why traditional are more contaminated than "innovative" *alheiras*. Although being produced in the same facilities and eventually under the same conditions, it is undisputed that some ingredients used in their manufacture are different and meats used in traditional *alheiras* might be more contaminated. However, the production of traditional *alheiras* in higher amounts could be one of the answers to diverse types of contamination found and, consequently, to different associated hazards.

CHAPTER 3

Evaluation of the microbial diversity of traditional and non-traditional Portuguese *alheiras*

Abstract

This study represents the first application of next-generation sequencing technology to characterize the microbial communities associated with *alheiras*, traditional Portuguese sausages made from a combination of poultry meat, pork, bread, olive oil and/or fat, salt and spices. The microbial diversity of 14 *alheiras* made with alternative ingredients (innovative *alheiras*) and seven traditional *alheiras* was analysed using high-throughput next-generation sequencing of 16S and ITS rRNA to understand whether this diversity is affected by product composition. The bacterial and fungal communities associated with each *alheira* were obtained by sequencing the 16S rRNA gene V3–V4 and Internal Transcribed Spacer 2 (ITS2) regions of rRNA gene amplicons, respectively. Variations in the composition of the microbiota were found between the samples, reflected in the great differences in the profiles of the dominant species. More than 500 taxa were identified, in particular belonging to the families *Lactobacillaceae* and *Xanthomonadaceae,* which were present in all samples. For the analysis of the bacterial communities, genera belonging to lactic acid bacteria and *Xanthomonas* were predominantly found, while fungal related, the yeast *Pichia* was found in almost all samples followed by the filamentous fungus *Alternaria*. The results indicate that, even when made in the same facilities and possibly under the same conditions, the different ingredients used in traditional and innovative *alheiras* clearly lead to different microbial diversity patterns in most producers. As a first attempt to characterize the microbial diversity of *alheiras*, this study allowed a clearer understanding of the microbial diversity of these innovative products and their homologous counterparts.

3.1. Introduction

The production of *alheiras*, traditional Portuguese meat products made from a combination of poultry meat, pork, bread, olive oil and/or fat, salt, and spices, is a long-established practice in the North of Portugal. Several industries and small artisanal producers in this region keep the tradition of producing this gastronomic delight (Ramalhosa *et al.*, 2012). Even though these products are highly appreciated, in the last ten to fifteen years, different *alheiras* have become available, with ingredients other than pork and/or poultry meat, such as fish, veal, lamb, mushrooms, tofu, soy and vegetables (in this manuscript denominated "innovative" types), as a way to improve competitiveness through innovation, while at the same time meet different consumer expectations (Azevedo *et al.*, 2020; Ferreira *et al.*, 2006, 2007, 2009; Leroy & Degreef, 2015).

In naturally fermented sausages, there is an intrinsic association between the microbiota that develops during transformation and the sensory characteristics of the final product. Even though there is already extensive scientific knowledge on fermentation and pathogen behaviour in traditional *alheiras* (Albano *et al.*, 2009b; Barbosa *et al.*, 2010; Felício *et al.*, 2007; Ferreira *et al.*, 2011), innovative products represent a challenge regarding their safety and microbiota. As the latter are produced by the same companies, it is predictable that problems previously described with traditional products could also be observed with these new products. Considering this, there is a need to evaluate the microbial ecology of innovative *alheiras* in order to understand if these new products are also carriers of microbial and chemical hazards like their traditional counterparts, raising new public health questions (Azevedo *et al.*, 2020).

While traditional microbiological methods easily allow the detection of culturable microorganisms, those that require specific conditions, enrichment, are unculturable or are in an injured state are not usually detected (Rantsiou *et al.*, 2005). To overcome classical limitations, the application of molecular-based methods is paramount to explore and understand the microbial diversity of complex samples (De Filippis *et al.*, 2018; Justé *et al.*, 2008; Rantsiou & Cocolin, 2006; Stefanis *et al.*, 2016). As such, due to its high-throughput data generation, next-generation sequencing (NGS) is currently the key technology used to detect and classify not only dominant but also rare or low-prevalence microorganisms (Hugenholtz & Tyson, 2008; Kergourlay *et al.*, 2015). Moreover, NGS coupled with the targeted amplification is the most common method used to characterize the microbial diversity of a given sample, targeting either the 16S rRNA gene for bacterial communities or internal transcribed spacers (ITS) for fungal communities, (Caporaso *et al.*, 2011; Schoch

et al., 2012). In fact, in the past years, several studies using culture-independent rRNA approaches on fermented sausages have allowed a more detailed understanding of the microbiota of these products. These studies have shown that culture-independent techniques are powerful tools to explore and recognize the microbial dynamics that occur during food fermentation. In particular, they allowed to understand the connection between microbial diversity and how the presence of different strains affects the sensory characteristics of the final product (Barbieri *et al.*, 2021; Cruxen *et al.*, 2019; Franciosa *et al.*, 2021; Macieira *et al.*, 2019; P. Yang *et al.*, 2022).

This study aimed to assess the complex microbial diversity of traditional and innovative *alheiras* using high-throughput 16S and ITS rRNA next-generation sequencing to identify and compare their microbial diversity.

3.2. Material and methods

3.2.1. Sample origin

A group of 14 innovative *alheiras*, individually packed (around 200 g) and available on the Portuguese market were selected for this study. Where possible, a traditional *alheira* from the same producer was also selected (see Table 3.1). In total, 21 *alheiras* from 11 different producers were analysed, each prepared using distinct ingredients and production methods.

3.2.2. Total DNA extraction

For each *alheira* sample, the casing was removed and the paste was homogenized individually in a stomacher for 2 minutes. Subsequently, 2 g were used for total DNA extraction, using the DNeasy mericon Food Kit (Qiagen, Valencia, CA, USA) with modifications to the manufacturer´s protocol. The volume of Proteinase K solution was doubled and traditional and innovative samples were incubated for 60 and 90 minutes, respectively, using constant shaking and vortexing every 15 minutes. These incubation times were adjusted in order to obtain higher DNA yields, especially in the innovative samples.

The concentration and quality of the extracted DNA were checked using a spectrophotometer (Nanodrop Technologies, Rockland, DE, USA) and 0.5% (w/v) agarose gel electrophoresis. All DNA extracts were stored at −20 °C prior to further analysis.

Table 3.1 – Information and main characteristics of analysed *alheiras*

Producer / Sample		Label Indication*	Denomination	Producer location	Main Ingredients
A	1	Traditional White Label	Meat	Mirandela (Bragança)	Chicken meat; Bread; Bísaro pork meat; Water; Olive oil; Spices
	2	Vegetarian	Non-meat		Bread; Green and black olives; Olive oil; Carrot; Green and red pepper; Spices
	3	Traditional	Meat		Chicken meat; Bread; Bísaro pork meat; Water; Olive oil; Spices
	4	Codfish	Non-meat		Bread; Codfish; Green and black olives; Olive oil; Spices
B	5	Leek and Mushrooms	Meat	Chaves (Vila Real)	Chicken meat; Mushrooms; Bread; Pork loin; Leek; Water; Olive oil; Oregano; Sweet chili
	6	Traditional	Meat		Poultry meat (Chicken, turkey, duck); Bread; Spices
C	7	Traditional	Meat	Trancoso (Guarda)	Pork meat; Wheat bread; Chicken meat; Pork fat; Water; Olive oil; Spices
	8	Soy	Non-meat		Soy; Wheat bread; Olive oil; Spices; Starter cultures
D	9	Vegetables and mushrooms	Non-meat	Tondela (Viseu)	Shiitake mushrooms; Water; Wheat bread; Vegetables; Olive oil; Spices
E	21	Traditional	Meat	Vila Nova de Paiva (Viseu)	Bread; Pork meat and fat; Poultry meat; Spices
	10	Shiitake mushrooms	Meat		Bread; Pork meat and fat; Poultry meat; Mushrooms; Spices
	11	Apple	Meat		Bread; Pork meat and fat; Apple; Poultry meat; Spices
	12	Cheese	Meat		Bread; Pork meat and fat; Cheese; Poultry meat; Spices
F	13	Vegan	Non-meat	Sintra (Lisboa)	Water; Bread; Sunflower oil; Olive oil; Spices; Vinegar
G	22	Traditional	Meat	Gimonde (Bragança)	Bísaro pork meat; Chicken broth; Wheat bread; Olive oil; Spices
	14	Thyme and marjoram	Meat		Wild boar; Pork fat; Water; Wheat bread; Olive oil; Spices natural extracts
H	15	Tofu	Non-meat	Covilhã (Castelo Branco)	Tofu; Whole grain bread; Seitan; Soy sauce; Parsley, Coriander, Cumin, Fennel, Ginger
I	16	Tofu	Non-meat	Covilhã (Castelo Branco)	Tofu; Whole grain bread; Seitan; Soy sauce; Parsley, Coriander, Cumin, Fennel, Ginger
J	17	Vegetarian	Non-meat	Ponte de Lima (Viana do Castelo)	Mushrooms; Wheat bread; Olive oil; Spices
	18	Traditional	Meat		Rooster meat; Wheat bread; Pork meat; Spices
K	19	Vegetarian	Non-meat	Mirandela (Bragança)	Mushrooms; Wheat bread; Green asparagus; Olive oil; Spices
	20	Traditional	Meat		Chicken meat; Wheat bread; Bísaro pork meat; Olive oil; Spices

* Samples which were not denominated as traditional were considered *innovative* products

3.2.3. Samples sequencing and data analysis

Amplification and sequencing of the 16S rRNA gene (region V3-V4) and Internal Transcribed Spacer 2 region were performed at Genoinseq (Cantanhede, Portugal). Briefly, amplification was performed using KAPA HiFi HotStart PCR Kit according to manufacturer suggestions and using previously described primers for 16S rRNA (Herlemann *et al.*, 2011; Klindworth *et al.*, 2013) and ITS2 (Tedersoo *et al.*, 2014). PCR products were pair-end sequenced in an Illumina MiSeq apparatus (Illumina, San Diego, CA, USA), according to the manufacturer's instructions (Illumina, 2013).

Raw reads were quality-filtered with PRINSEQ version 0.20.4 (Schmieder & Edwards, 2011) to remove sequencing adapters, reads with less than 150 bases for bacteria and 100 for eukaryotes, and trim bases with an average quality lower than Q25 in a window of five bases. Using default parameters, the forward and reverse reads were merged by overlapping paired-end reads with AdapterRemoval version 2.1.5 (Schubert *et al.*, 2016). 16S rRNA and ITS2 data were analysed with Kraken v2 (Wood and Saltzberg, 2014) using the pre-built Standard-16 database of 10/9/2023 (available at https://benlangmead.github.io/aws-indexes/k2) and the pipITS software (Gweon *et al.*, 2015) using the UNITE 9.0 v27.10.2022 database, respectively. At each taxonomic rank, classification supported by ≤10 reads were excluded. Classification falling below a threshold of 5% across all samples were grouped in an extra group named "Others" for data visualization purposes.

Statistical analysis and alpha diversity metrics, namely Shannon and Simpson indices, were established using *R* software (v1.9.1).

3.3. Results and Discussion

Operational taxonomic unit tags were classified at family, genus, and species levels for both bacteria and fungi metagenomic sequencing to increase knowledge about the microbial communities in these sausages.

In the taxonomic assignment of bacteria, 324 Operational Taxonomic Units (OTUs) were identified from all *alheira* samples, belonging to 116 families and 208 genera. At the family level, for the majority of *alheira* samples, *Lactobacillaceae* (89.1 – 2.8%) was found, followed by *Xanthomonadaceae* (39.1 – 0.4%) (Figure 3.1). In several recent studies, the *Lactobacillaceae* family has proven to be the most representative member found in fermented products (Ferrocino *et al.*, 2018; Fontana *et al.*, 2016; Kavitake *et al.*, 2022; Kergourlay *et al.*, 2015; Macieira *et al.*, 2019; Ucak *et al.*, 2022).

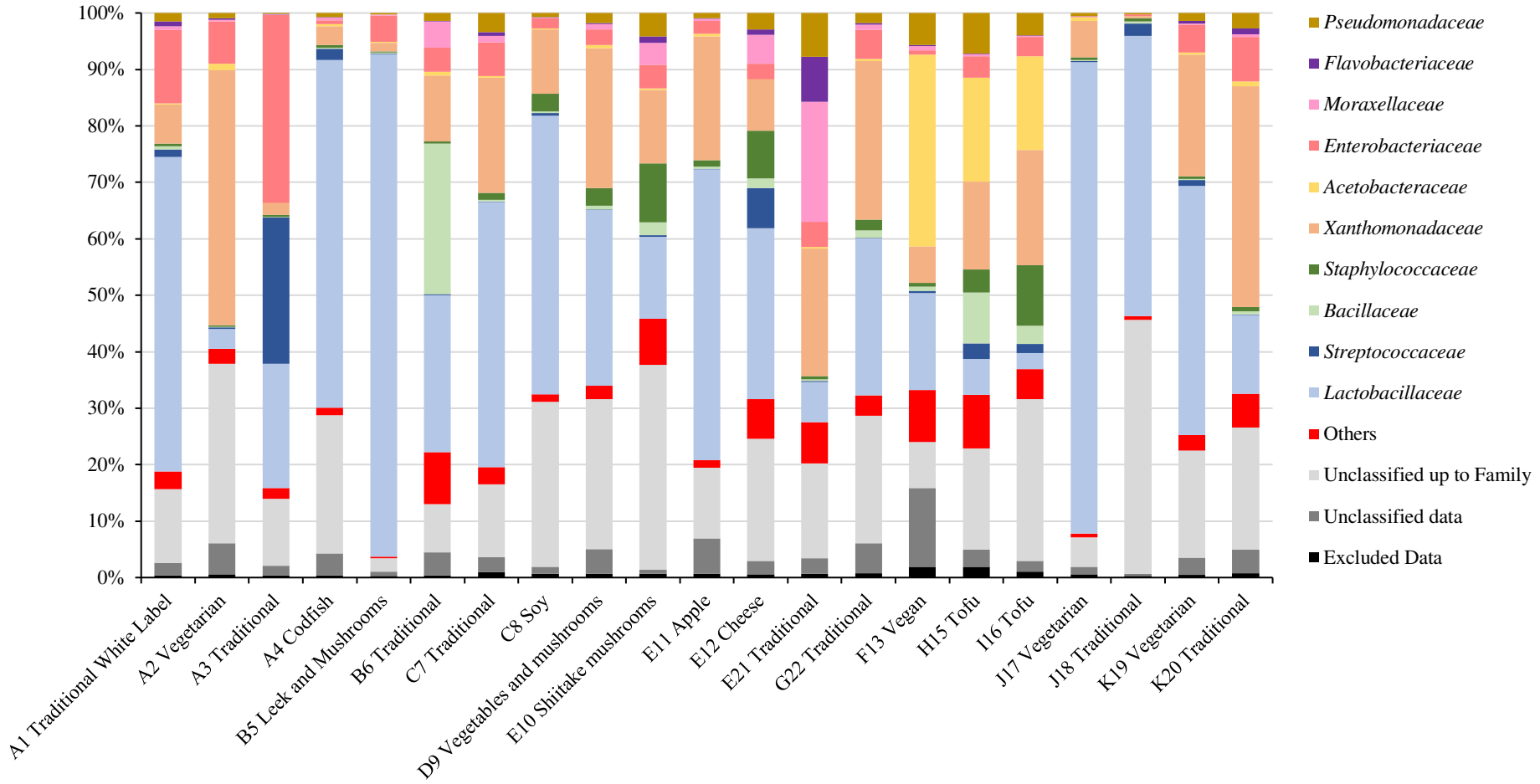


Figure 3.1 – Relative abundance of bacterial communities at the family level determined by 16S metagenomic analysis. OTUs with an incidence above 5% are shown.

Xanthomonadaceae, gram-negative bacteria belonging to the order *Xanthomonodales*, have also been found in previous studies of fermented foods (Einson *et al.*, 2018; Park *et al.*, 2012; Qin *et al.*, 2016; Zang *et al.*, 2018; Zhao *et al.*, 2016). *Acetobacteraceae* were predominantly found in vegan (sample F13 – 34.0%) and tofu *alheiras* (samples H15 – 18.4% and I16 – 16.6%), which is in accordance with the main ingredients used in these *alheiras* manufacturing. Acetic acid bacteria belonging to the *Acetobacteraceae* family, which are widespread and play an important role in food and beverage production, have also been often found in some other fermented foods (Leech *et al.*, 2020; Peng *et al.*, 2015; Pérez-Cataluña *et al.*, 2018; Yegin *et al.*, 2022). Several studies demonstrated the role of acetic acid bacteria in natural fermentation systems, such as vinegar production and soybeans processing (Chen *et al.*, 2022; De Roos and De Vuyst, 2018; Pothakos *et al.*, 2016).

Some rare families were found in at least one sample from all producers, namely *Moraxellaceae* and *Flavobacteriaceae*, which might indicate that these bacteria are intrinsically present in *alheiras* production environments. Another possibility, already reported, includes external sources of contamination, such as DNA extraction kits and contamination originating within the study itself (Cornet & Baurain, 2022; Lou *et al.*, 2022). Similarly, at the genus level, *Xanthomonas* was found in all samples (44.8 to 0.4%) followed by several lactic acid bacteria genera such as *Pediococcus*, *Weissella*, *Latilactobacillus*, *Lactococcus*, *Leuconostoc* and *Companilactobacillus* (up to 33.7, 20.3, 14.4, 13.2, 10.2 and 5.5%, correspondingly) with no distinctions between traditional and innovative *alheiras* (Figure 3.2). Strains of lactic acid bacteria are frequently isolated from fermented foods and beverages (Belleggia *et al.*, 2020; Demirci *et al.*, 2022; Liang *et al.*, 2021; Prathiviraj *et al.*, 2022; Quijada *et al.*, 2018; Sadiq *et al.*, 2014). Xantham gum is a natural heteropolysaccharide produced by *Xanthomonas* species and is frequently used in the food industry as a stabilizer and thickener (Demirci *et al.*, 2019). This additive is commonly used in bread production, which might help explain these genera predominance in *alheira* samples, since this is a common ingredient used in the production of all types of *alheiras*. Some *Enterobacteriaceae* family elements, such as *Salmonella* and *Escherichia*, were found in some samples indicating hygiene failures during production (Alessandra De Cesare, 2018; Al-Mutairi, M.F., 2011; Carrasco *et al.*, 2012).

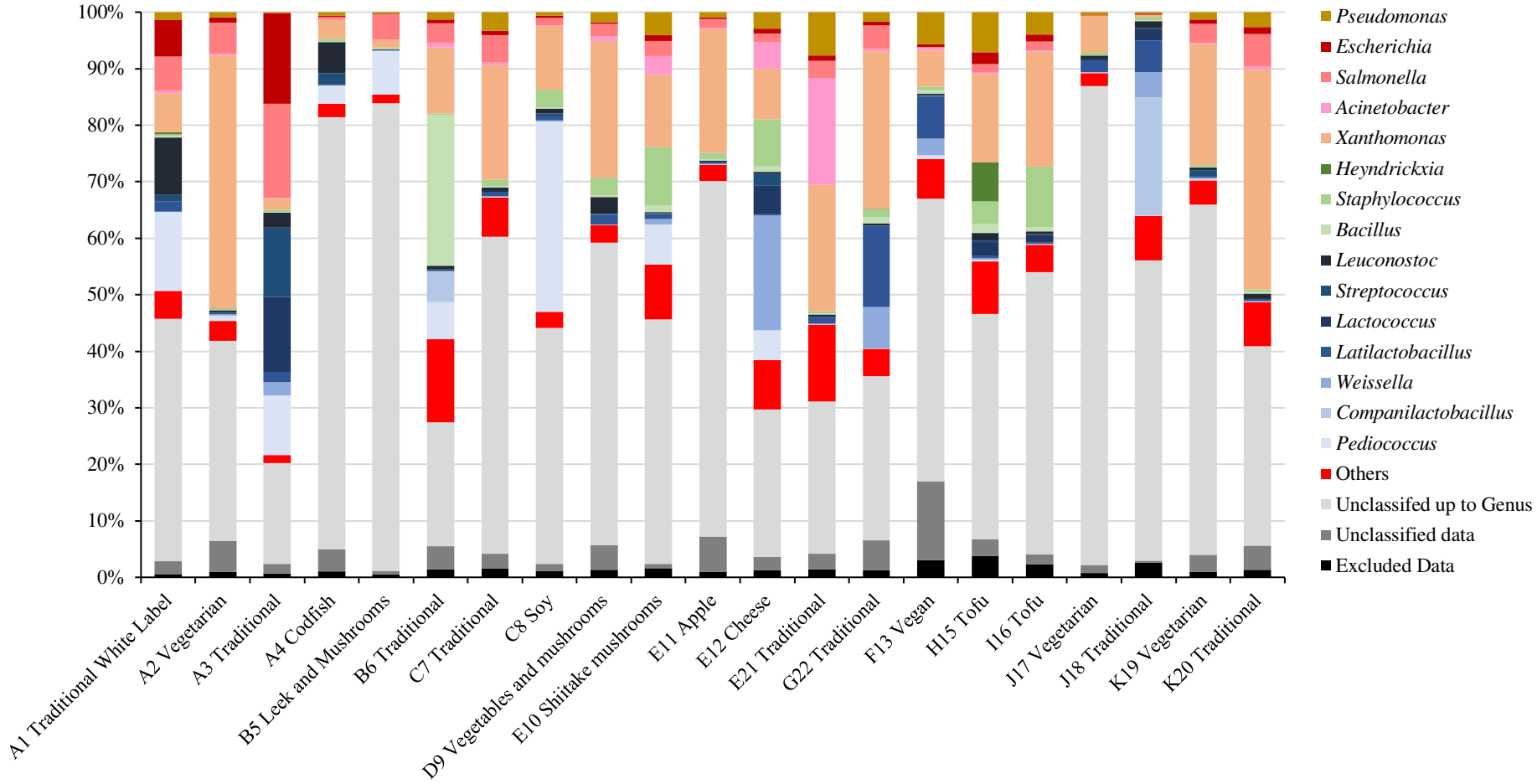


Figure 3.2 – Relative abundance of bacterial communities at the genus level determined by 16S metagenomic analysis. OTUs with an incidence above 5% are shown.

At the species level (Supplementary Figure 3.1), the result that stands out most is the agreement with the results found in the classical analysis performed in our latest study (Azevedo *et al.*, 2020), which indicates the presence of *Enterobacteriaceae* and *Escherichia coli* in several samples, mainly in traditional *alheiras* (Castaño *et al.*, 2002; Chevallier *et al.*, 2006). Despite being present in the NGS analysis, *Staphylococcus aureus* was not found in any samples during classical microbiological analysis.

To summarise, beyond a large number of microbial species, the relative microbiota found was also very variable among all samples, even for those collected from the same producer. Regarding fungal communities, Figure 3.3 summarizes the genera identified and *Pichia,* a yeast belonging to the *Saccharomycetaceae* family, was found in 18 of the 21 samples analysed (up to 16.8%). Numerous *Pichia* species have been identified in fermented meat sausages, leading to the belief that one of its functions is to contribute to the flavour and colour development of fermented meat products (Bhalla & Savitri, 2017). Fermented sausages' ecology is intricate and involves a peculiar microbiota distinctive from the region where they are produced. *Debaryomyces* and *Kazachstania* are other genera commonly found in fermented foods, yet they were found in lower frequency (Copetti, 2019; Franciosa *et al.*, 2018; Punyauppa-path *et al.*, 2022). *Alternaria*, a genus abundant in the environment that has been isolated from a varied selection of food products, was found in 19 out of 21 samples (up to %12.6) in this study. Due to its high prevalence in many food products, this fungus has been the subject of in-depth studies to determine safe limits for toxic metabolites of *Alternaria* in foods and to develop effective control approaches for this pathogen (Patriarca, 2016). *Rhizopus*, *Geotrichum* and *Aspergillus* were found in more than half of the samples, yet this was an expected result since these fungi can inhabit sausages, which often come from the operational environment (Castellari *et al.*, 2010; Coton *et al.*, 2021; Jiang *et al.*, 2023; Parussolo *et al.*, 2019; Q. Yang *et al.*, 2022; Yao *et al.*, 2021). Shiitake, an edible mushroom, is frequently used as a meat substitute in the preparation of vegetarian *alheiras,* and accordingly, samples D9, E10 and J17 have high *Lentinula* values (32.0, 62.6 and 38.4%, respectively) (Ba *et al.*, 2017; Wang *et al.*, 2019). Some other samples, such as B5 and K19, contain evidence of mushrooms in their composition; however, no *Lentinula* was found, leading us to conclude that other mushroom types must be used.

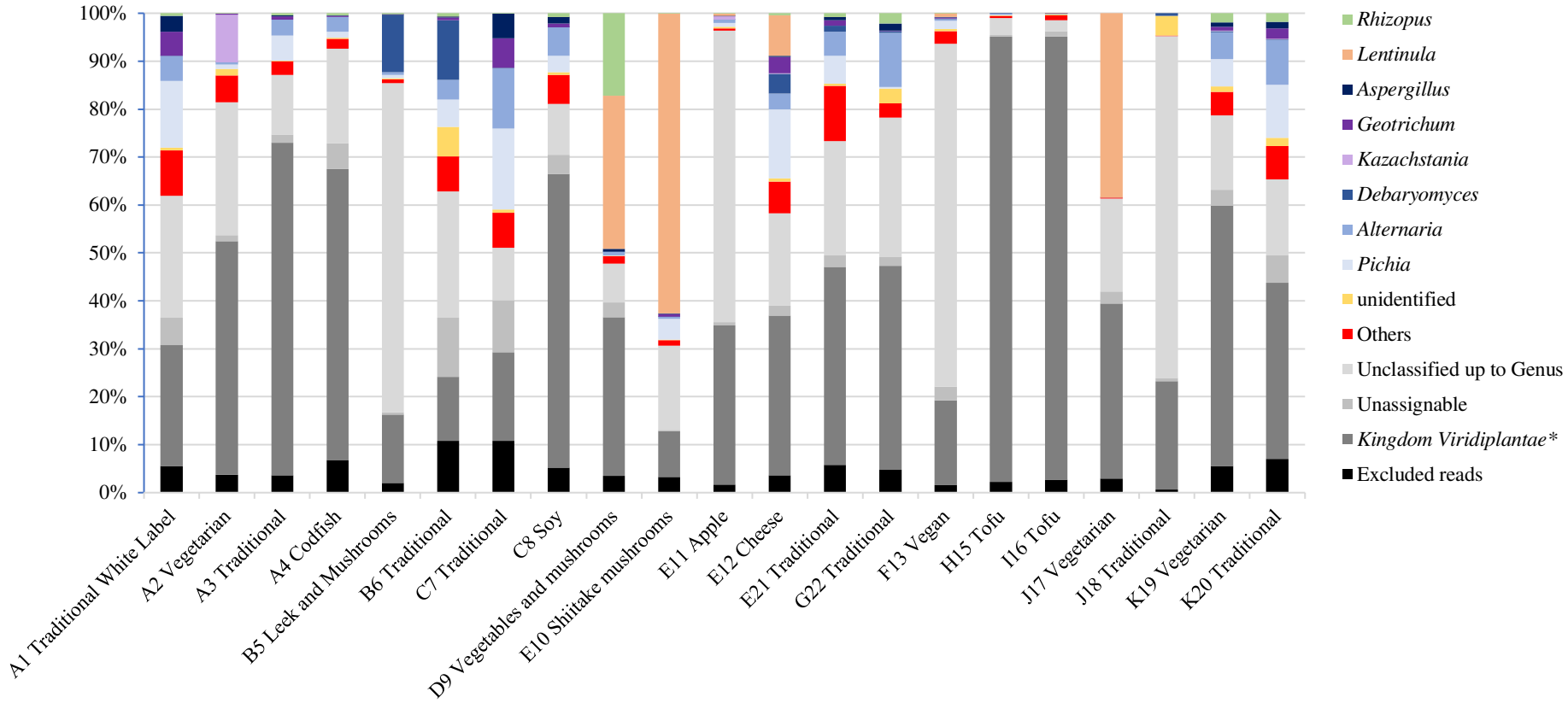


Figure 3.3 – Relative abundance of fungal communities at the genus level determined by ITS2 metagenomic analysis. OTUs with an incidence above 5% are shown.

All of these have been reported as genera found in fermented foods (Ban *et al.*, 2022; Huang *et al.*, 2022; Tamang *et al.*, 2016; Wang *et al.*, 2020; Ye *et al.*, 2022). According to Toldrá *et al.* (2014), yeasts like *Pichia* and moulds such as *Penicillium* and *Aspergillus* are very well studied for their role in fermented sausage flavour, since these microorganisms are responsible for various sugar fermentation, lactic acid reduction, lipolysis, proteolysis, and amino acid degradation. Supplementary Figures 3.2 and 3.3 provide some extra information on the ITS2 analysis, yet even though ITS2 is a well-established marker for phylogenetic analyses in eukaryotes and a reliable resource for reference sequences, its database was last updated in 2011 (Ankenbrand *et al.*, 2015). Despite that, several other species belonging to *Aspergillus* spp., *Penicillium* spp. and *Pichia* spp. are commonly known to be part of the typical coating of some Mediterranean fermented sausages (Garcia *et al.*, 2001; Ludemann *et al.*, 2004; Patrignani *et al.*, 2007; Selgas *et al.*, 2003). These genera are known to grow optimally at pH values between 4.5 and 6.5, and, according to the literature, *alheiras* have pH values between 4.0 and 6.0 (Albano *et al.*, 2009b; Azevedo *et al.*, 2020; Ferreira *et al.*, 2006).

Cluster dendrograms and correlation matrices were created to explain the relationship between all data (Figures 3.4 and 3.5). Also, alpha diversity matrixes like Shannon and Simpson indices were calculated to evaluate and compare bacterial community diversities of all 21 samples (Supplementary Figure 3.4).

In Figure 3.4A, the existence of three distinct clusters is clear, and curiously, the green one encompasses all samples belonging to producers in the Bragança area (Producer A, G and K). The explanation for this situation may lie in the fact that common raw materials are used as ingredients for *alheira* production, namely Bísaro pork, bread wheat and olive oil from Trás-os-Montes. Figure 3.4B presents the relationship between all the samples, and some samples have 16S gene sequences that are family related, with a correlation above 0.95 not only in between samples from the same producer (B5, B6, C7, C8, J17 and J18), but also between producers (B, C, E, J and K). Although it is known that ingredients used and production conditions can influence microbial communities, this is still an unexplored fact (Jung *et al.*, 2011).

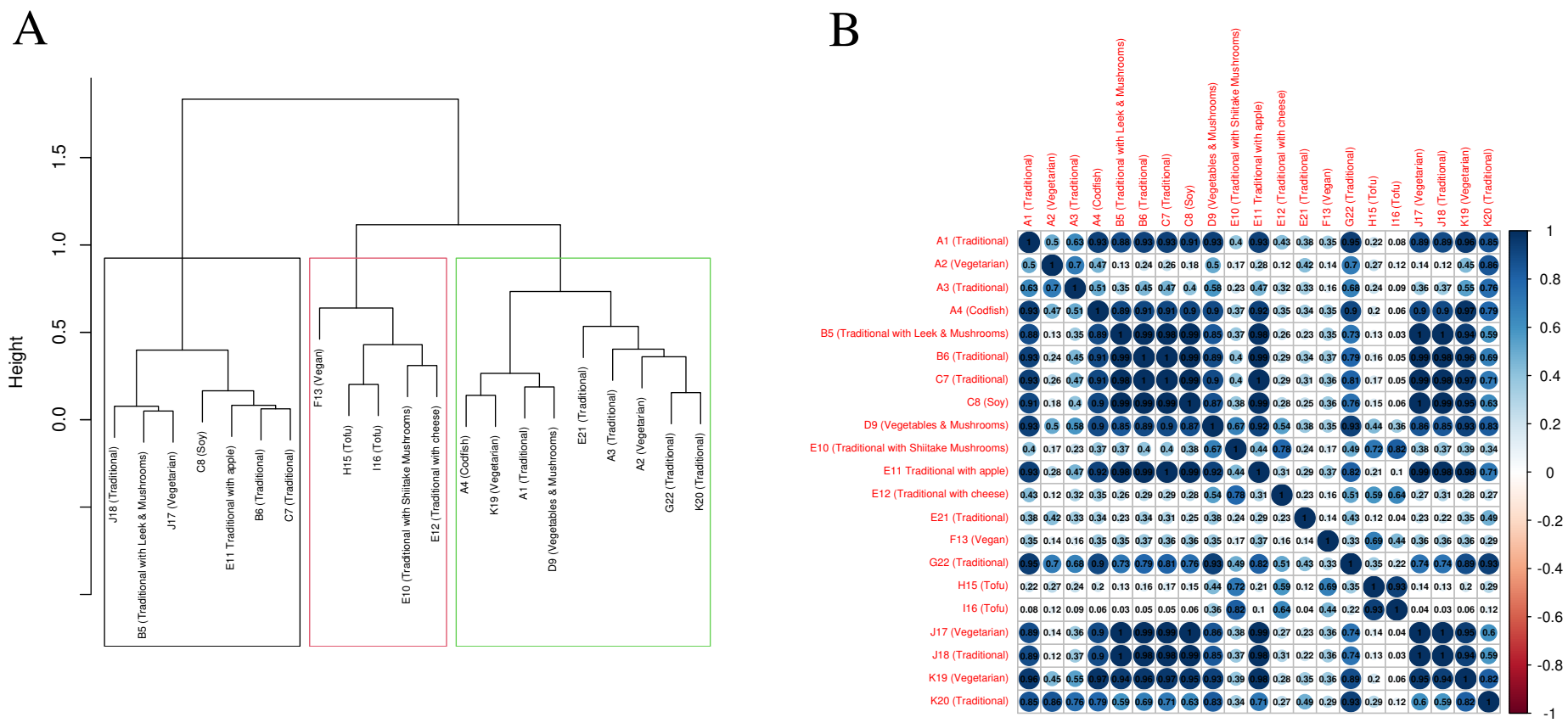


Figure 3.4 – Cluster Dendrogram (**A**) and correlation matrix (**B**) based on *alheira* samples 16S rRNA sequences, at the family level, arranged using *R* software. The colour of the scale bar of the correlation matrix denotes the nature of the correlation, with "1″ indicating a perfect positive correlation (dark blue) and "-1" indicating a perfect negative correlation (dark red).

When related to the ITS2 gene, three distinct clusters might also be observed in Figure 3.5A, where samples B5, I16 and J18 are clearly different from all the others. In Figure 3.5B, only a few samples present a very high correlation (above 0.95) between samples, but producers C, E and K show a similar pattern between traditional and vegetarian samples. In the study by Belleggia *et al.* (2022a), the authors state that, depending on the manufacturing process of the producer, different products may be obtained, although the same raw materials are used.

3.4. Conclusions

Compared to our previous research on microbiological and chemical characterization of non-meat *alheiras*, all dominant bacteria found using classical methods were also dominant by non-culturable analysis. Next-generation sequencing has been demonstrated to be an effective tool to categorize bacterial and fungal communities in food samples where cultural methods are insufficient to accurately determine the diversity of the whole microbiota. To the extent of our knowledge, this study represents the first application of next-generation sequencing to characterize the microbial communities associated with both traditional and innovative Portuguese *alheiras*. It has been demonstrated to be supportive of understanding this fermented product microbiota better and helpful in selecting potential starter strains or their combinations for the food industry. Furthermore, we believe that the data obtained can be a guide for new studies about innovative fermented food products.

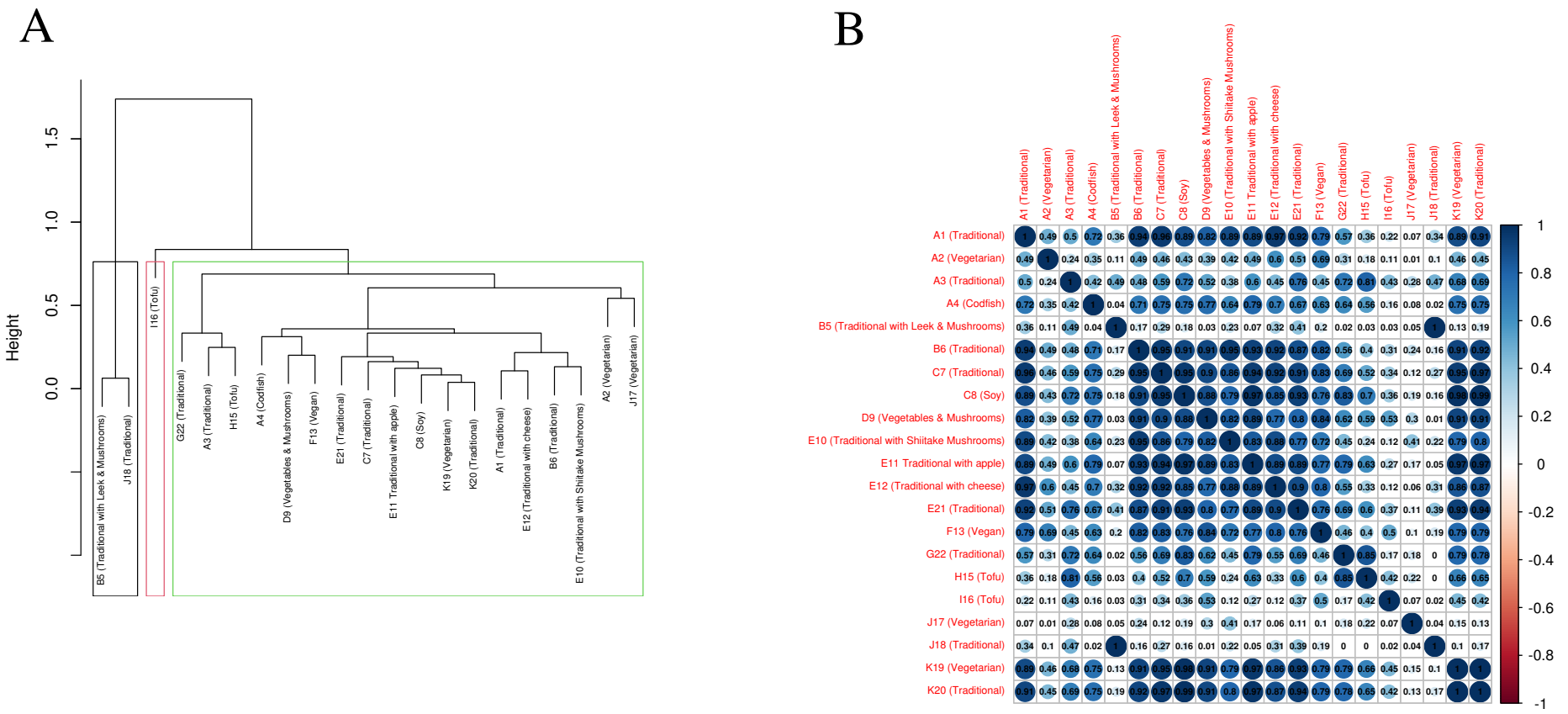


Figure 3.5 – Cluster Dendrogram (**A**) and correlation matrix (**B**) based on *alheira* samples ITS2 sequences, at the family level, arranged using *R* software. The colour of the scale bar of the correlation matrix denotes the nature of the correlation, with "1" indicating a perfect positive correlation (dark blue) and "-1" indicating a perfect negative correlation (dark red).

CHAPTER 4

Behaviour of foodborne pathogens in different *alheira* matrices along their shelf-life

Abstract

Being a well-known delicacy in Portugal, traditional *alheiras* are well-studied either microbiologically or chemically. However, there are very few studies reporting on the characteristics of innovative formulations of these fermented products, whose popularity has increased in recent years. Therefore, this study aimed to investigate the behaviour of foodborne pathogens in the *alheira* matrix once it is difficult to predict it accurately. By this, obtaining information on different formulations of this traditional Portuguese fermented sausage seemed necessary. A challenge test was performed, where traditional and innovative *alheiras* (codfish and vegetarian) from the same producer were inoculated with specific foodborne pathogens to determine their behaviour along the product shelf-life at 4 ºC. Foodborne pathogens selection was carried out considering those more likely to occur as contaminants during manufacturing and posterior handling, consisting of gram-positive and gram-negative bacteria. During storage, pH values of each matrix tended to decrease while water activity (a_w) increased. Lactic acid bacteria were found in higher numbers, especially in traditional *alheira,* but those numbers increased along the shelf-life for all the samples. Each target pathogen showed different behaviours on *alheira* matrices, but interestingly, most pathogens were no longer detected before the expiration date in vegetarian *alheira*. Overall, vegetarian *alheira* has proven to own particular characteristics that conditionate the viability of foodborne pathogens since all tested pathogens were found to have counts below the limit of detection of the technique at the end of shelf-life in this matrix.

4.1. Introduction

The production of fermented meat products, such as *alheiras*, has a long tradition in the north of Portugal. This tradition is carefully maintained by both small artisanal producers and established industries, contributing to the preservation of this gastronomic heritage (Ramalhosa *et al.*, 2012). These products are highly appreciated and, in recent years, different types of *alheiras* have become available to meet the different expectations of modern consumers (Leroy & Degreef, 2015; Yang *et al.*, 2022). These new products (innovative *alheiras*) are mainly produced with ingredients other than pork and/or poultry meats (traditional *alheiras*), such as fish, veal, lamb, mushrooms, tofu, soy, and vegetables (Azevedo *et al.*, 2020).

Although traditional *alheiras* have been very well characterized microbiologically and chemically (Ferreira *et al.*, 2006, 2007; Patarata *et al.*, 2008; Silva *et al.*, 2019), there are very few studies reporting on the characteristics of innovative formulations of these fermented products, whose popularity has increased in recent years.

Alheira is usually grilled, roasted, fried, boiled, or microwaved before consumption and is generally not considered ready to eat. However, it has been shown that some of the traditional cooking methods used may not be effective in reducing any foodborne pathogens that may be present in traditional *alheiras* (Felício *et al.*, 2011).

A previous study (Azevedo *et al.*, 2020) reported that innovative *alheiras* did not exhibit the typical microbiological contamination found in traditional *alheiras* and differences in the organic acids and nitrate and nitrate concentrations, along with differences in organic acids and nitrate/nitrite concentrations. Given that both the physic-chemical characteristics and microbiota of food products influence the survival of foodborne pathogens (Paswan *et al.*, 2020; Romero-Gil *et al.*, 2018), the primary objective of this study was to investigate the behaviour of selected pathogens throughout the shelf life of traditional and innovative (made from cod and vegetables) *alheiras* stored at 4 ºC.

4.2. Materials and methods

4.2.1. Strains used in this study and inocula preparation

Cocktails of *Escherichia coli* (ATCC 25922 and O157:H7), *Listeria monocytogenes* (NCTC 11994, CECT 911, CECT 936 and CEP 104794), *Salmonella* Enteritidis (ATCC 25928, 417536 and 545047) and *Staphylococcus aureus* (ATCC 29213, ESB 18N (MRSA) and ESB 2037 M1 (MSSA)) – all deposited in the culture collection of *Escola Superior de Biotecnologia*, *Universidade Católica Portuguesa* – were used. To prepare each inoculum,

100 µl of each bacteria stock culture (stored at -80 ºC in Brain Hearth Infusion (BHI, Biokar Diagnostics, Allonne, France) broth containing 20% (v/v) glycerol) were inoculated in 10 ml of BHI broth and incubated at 37 ºC overnight. The final inocula were prepared from these tubes, transferring 100 µl to 10 ml of fresh BHI broth and incubating at the same conditions. After this incubation period, about 8.0 log CFU/ml cells were achieved. Through centrifugation (Centrifuge 5427 R, Eppendorf, Hamburg, Germany), cells were washed and resuspended in Buffered Peptone Water (BPW, Biokar). Subsequently, serial decimal dilutions in peptone water were made to achieve a final level of approximately 6.0 log CFU/ml. Equal volumes of each foodborne strain were mixed to prepare the corresponding strain cocktails as inoculum for *alheira* samples. Levels of each pathogen were confirmed by enumeration on Tryptone Bile X-glucuronide (TBX, Biokar) for *E. coli*, Agar Listeria according to Ottaviani & Agosti (ALOA, BioMérieux, Craponne, France) for *L. monocytogenes*, Xylose Lysine Deoxycholate (XLD, VWR, Pennsylvania, USA) for *S.* Enteritidis and Baird Parker Rabbit Plasma Fibrinogen (BPA with RPF, BioMérieux) for *Staph. aureus*.

4.2.2. Preparation of *alheira* samples

Alheiras with different fillings (traditional, cod and vegetarian) but from the same producer, were partially emptied, allowing the ends and centre to be tied together, resulting in two mini *alheiras*, each weighing around 50 g. To these mini *alheiras*, the vacuum was applied into individual bags, and they were then inoculated at several points with 50 µl of individual foodborne pathogens cocktails using a sterile needle. To avoid loss of vacuum, an isolation patch was used to allow inoculation without air entering. Inoculated and uninoculated (control) mini *alheiras* were stored at 4 ºC.

4.2.3. Analysis of *alheira* samples

Counts of each pathogen and lactic acid bacteria (LAB) were performed immediately after inoculation (day 0), and at pre-set time points (3, 7, 14, 21, 28 and 60 days), according to ISO standards (ISO 16649-2:2001 for *E. coli*, ISO 11290-2:2017 for *L. monocytogenes*, ISO 6579-1:2017 for *Salmonella* spp., ISO 6888-1:1999 for *S. aureus* and ISO 15214:1998 for LAB). In addition, and according to ISO standards, detection protocols were performed when counts were below the detection limit of the enumeration technique (ISO 16649-

3:2015 for *E. coli*, ISO 11290-1:2017 for *L. monocytogenes*, ISO 6579-1:2017 for *Salmonella* spp. and ISO 6888-3:2003 for *Staph. aureus*).

pH values were measured directly with a Crison MicropH 2002 pH-meter (Crison, Barcelona, Spain) equipped with an InLab 427 puncture electrode (Mettler Toledo, Columbus, OH, USA). At day 0, water activity (a_w) was assessed using the AquaLab Series 3 Water Activity Meter (METER Group, Inc. Pullman, USA). For each pathogen, three independent experiments were conducted, using two mini *alheiras* at each sampling point.

4.2.4. Statistical analysis

The differences in microbial counts over storage time were analysed for the different type of *alheiras* using the one-way analysis of variance (ANOVA) with Tukey's post hoc test (IBM SPSS software, Version 28.0, Inc., Chicago, IL, USA). Statistical significance was assumed when $p < 0.05$.

4.3. Results and discussion

The viability of each target pathogen was evaluated in three *alheira* matrices from the same producer (codfish, vegetarian and traditional). *Escherichia coli*, *L. monocytogenes*, *S.* Enteritidis, and *Staph. aureus* counts were below the detection limit of the enumeration technique (less than 10 CFU/g) in uninoculated control samples in all experiments (data not shown).

As shown in Figure 4.1, all pathogens exhibited similar behaviour in each *alheira* until the 14th day of storage ($p > 0.05$); however, this pattern changed thereafter. For *E. coli* (Fig. 4.1A), a slight decrease in cell numbers was observed after 21 and 28 days of storage, with no significant differences between *alheiras* ($p > 0.05$). However, after 60 days, although this reduction was maintained for the codfish and traditional matrices, there was a significant reduction ($p < 0.05$) in the vegetarian matrix, with counts below the detection limit of the enumeration technique (lesser than 10 CFU/g).

For *E. coli* (Fig. 4.1A), the cell numbers remained relatively stable over the 28-day storage period, with no statistically significant differences observed between *alheiras* ($p > 0.05$). However, after 60 days, a similar reduction was observed for the cod and traditional *alheiras* (approximately, 1 log) and a significant decrease ($p < 0.05$) occurred in the vegetarian *alheira*, with counts below the detection limit of the enumeration technique (less than 10 CFU/g). A similar pattern was observed for *L. monocytogenes* (Fig. 4.1B), where reductions of more than 5 log cycles were observed by day 60 in the vegetarian matrix. Nevertheless,

it's worth noting that despite being in numbers lower than the detection limit of the enumeration technique, *L. monocytogenes* was still detected.

Salmonella Enteritidis (Fig. 4.1C) and *S. aureus* (Fig. 4.1D) proved to be the most sensitive microorganisms. Over 21 and 28 days of storage, the *Salmonella* spp. and *Staph. aureus* counts, respectively, remained constant in the traditional and codfish *alheiras*. After these periods, there was a reduction in the counts, especially in the codfish sausage. From the 21st day of storage until the end of the storage period, the cell counts had fallen below the detection limit of the enumeration technique ($p < 0.05$) in the vegetarian *alheira*. Between days 21 and 28, the reductions observed in cod and traditional sausages were similar ($p > 0.05$) but more pronounced for *S. Enteritidis*. At the end of storage (60 days), there was a decrease of more than 5 log cycles (counts below the detection limit of the enumeration technique) and 3 log cycles for *S.* Enteriditis and *S. aureus*, respectively, in codfish *alheira*. *Salmonella* spp. was detected in 25 g samples of this matrix on days 21 and 28 but not on day 60.

Several studies demonstrated the presence of foodborne pathogens, such as *E. coli*, *L. monocytogenes*, *Salmonella* spp. and *Staph. aureus*, in fermented sausages, namely *alheiras* (Ferreira *et al.*, 2006, 2007; Silva *et al.*, 2019). In a previous study conducted by Azevedo *et al.* (2020), it has been proven that these innovative products do not own the same microbiota as their traditional homologous. No pathogenic bacteria were found in innovative *alheiras*, which might explain why these innovative matrices are less predisposed to the development of pathogenic bacteria. So far, and to our knowledge, there is still no explanation for this occurrence, yet nutrient availability differences in all three *alheiras* type, is an important bacterial growth conditioning.

The LAB viability and pH values for each matrix on each day of storage are shown in Figures 4.2 and 4.3, respectively. A tendency to decrease over time was observed for the three products, although the traditional *alheira* presented higher pH values when compared to its homologous innovative at all sampling times. This might explain why some foodborne pathogens tend to disappear in the different matrices. Over time, pH decreases in these products mainly due to lactic acid bacteria proliferation (Oshiro *et al.*, 2020; Vera-Peña & Rodriguez-Rodriguez, 2020) and show final values around 4.5 and 3.9 in traditional and innovative products, respectively. Some organic acids tend to increase their effect as the pH drops. In the case of vegetarian sausage, the existence of malic acid in a significant amount (2.50 mg/g) may be a justification for the inactivation of *Salmonella* Enteritidis and *Staphylococcus aureus* in this matrix (Buchanan & Golden, 1998; Marques *et al.*, 2020). All

foodborne pathogens tested possess an optimal pH growth between 6.0 and 7.5 and, by this, might suffer some injury due to acid conditions of the matrix (Culliney & Schmalenberger, 2020; Gautam *et al.*, 2020; Juneja *et al.*, 2014; Oussalah *et al.*, 2007). Water activity was also initially evaluated showing results of 0.981, 0.988 and 0.993 in codfish, traditional and vegetarian *alheiras*, respectively. This parameter does not seem to have any influence on these pathogens' decrease since all of them are comfortable at this a_w range (Schultz, 2016; Sperber, 1983).

Using ComBase Predictive Models (Centre for Food Safety and Innovation, Australia), we were able to verify that when pH reaches very low values (4.03, 4.47 and 3.86 for codfish, traditional and vegetarian *alheiras* at the end of shelf-life, respectively), together with low storage temperature (4 ºC), all foodborne pathogens tend to present values under the detection limit. Although ComBase predicts that these biologic hazards tend to be above the detection limit, in this study it was found that *E. coli* and *L. monocytogenes* are still found in codfish and traditional matrices after 60 days. For that, it seems important that control must be guaranteed at the production level since these pathogens may survive for long periods and may not be destroyed during cooking procedures in the domestic environment.

4.4. Conclusions

From all three matrices tested, vegetarian *alheira* has proven to own particular characteristics that conditionate the viability of foodborne pathogens. All tested pathogens were found to have counts below the detection limit of the technique at the end of shelf-life in this matrix. Moreover, *Salmonella* Enteritidis and *Staph. aureus* were more affected since counts suffered a decrease after 21 days. We believe low temperature and low pH greatly influence bacterial decrease along *alheiras* shelf-life. Further studies should be taken into consideration as a way to clarify the reason why foodborne pathogens possess different behaviour in traditional and innovative *alheiras*.

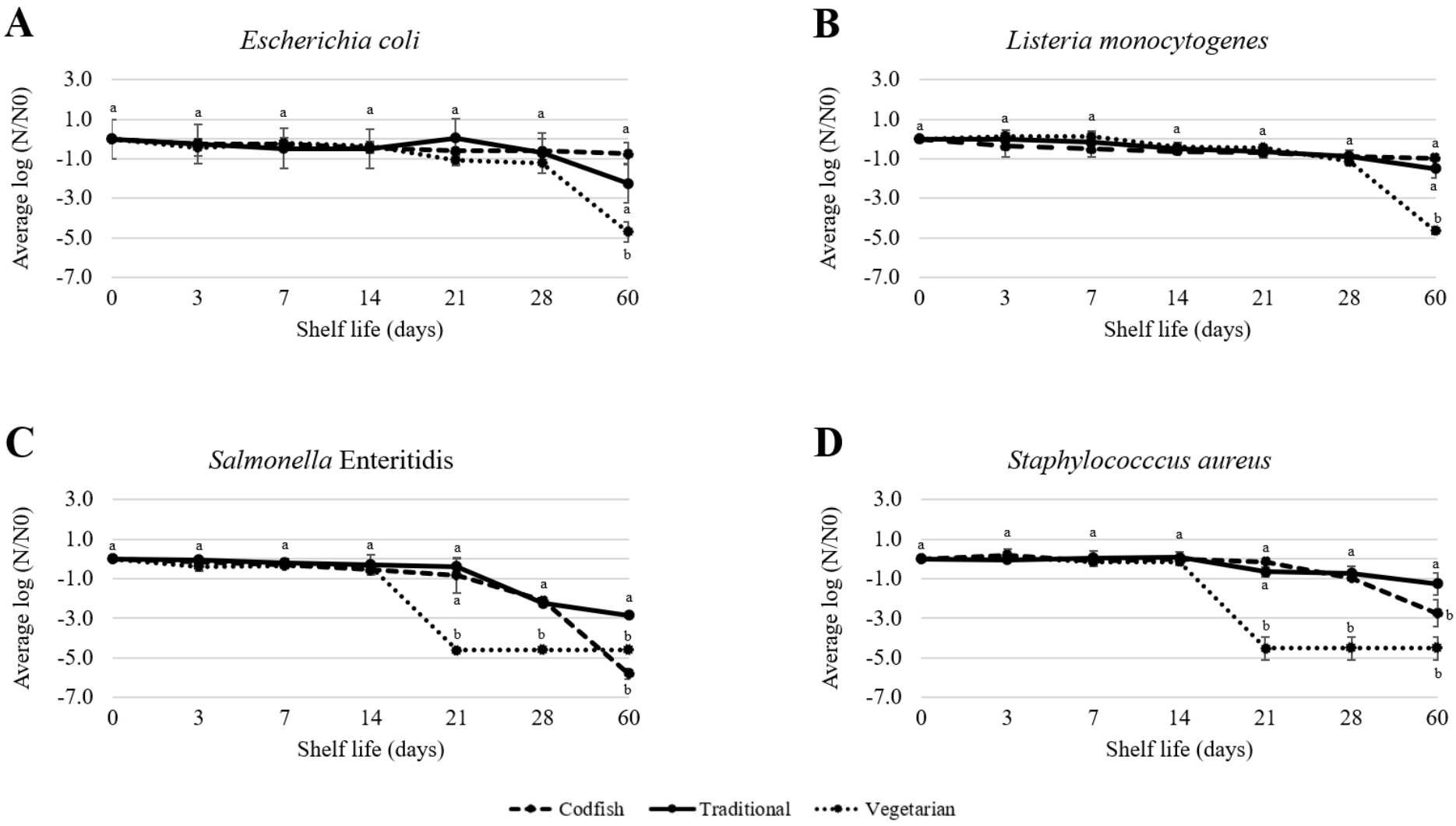


Figure 4.1 – Foodborne pathogens behaviour along shelf-life in all different *alheira* matrices

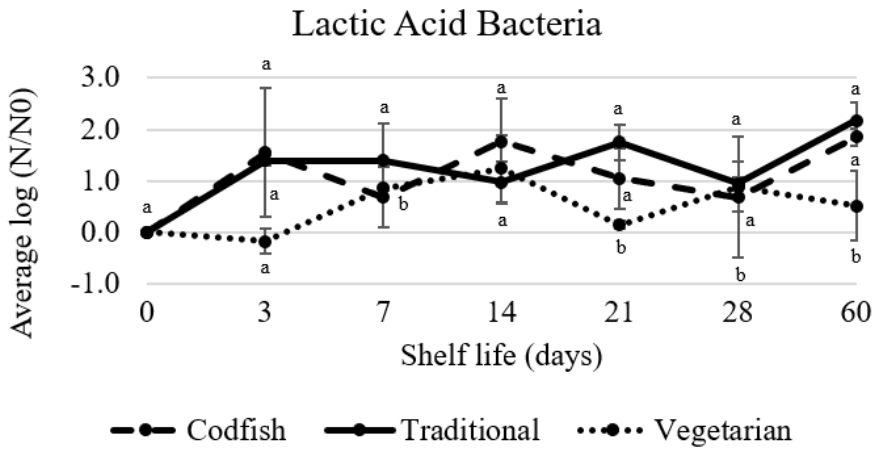


Figure 4.2 – Lactic acid bacteria viability along the shelf-life of each *alheira* matrix. Different letters indicate significant differences between *alheira* samples ($p < 0.05$)

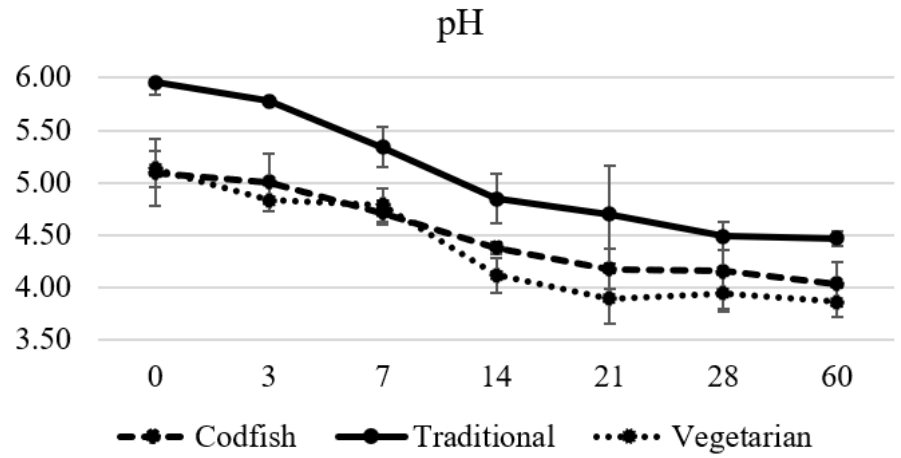


Figure 4.3 – pH value along the shelf-life of each *alheira* matrix

PART III

BIOACTIVE SUBSTANCES

CHAPTER 5

Lactic acid bacteria isolated from traditional and innovative *alheiras* as potential biocontrol agents

Abstract

From a selection of seven traditional and 14 innovative *alheiras*, 491 lactic acid bacteria (LAB) were isolated and tested for their antimicrobial activity against several food-borne pathogens. Among these, six strains revealed antimicrobial activity through potential bacteriocin production against 14 *Listeria monocytogenes* strains, *Enterococcus faecalis* ATCC 29212, *Clostridium sporogenes* ESB050, and *Clostridium perfringens* ESB054. Through whole genome sequencing (WGS), these strains were identified as *Lactiplantibacillus plantarum* (2), *Leuconostoc mesenteroides* (1), and *Pediococcus acidilactici* (3). Furthermore, several orthologues of class II bacteriocins genes were identified, including Plantaricin E, Plantaricin F, Pediocin PA, Enterocin X, Leucocin A, and Coagulin A. No virulence or antibiotic resistance genes' orthologues were detected by WGS analysis. However, the selected LAB strains showed variable phenotypic patterns related to virulence genes and antibiotic resistance when assessed through classical methodologies. None of these strains demonstrated the production of biogenic amines, gelatinase or DNase. Additionally, no hemolytic activity or lipase enzyme production was observed. However, only *Lpb. plantarum* 9A3 was sensitive to all tested antibiotics and was thus chosen for further examination. The bacteriocins produced by *Lpb. plantarum* (9A3) exhibited stability across a broad range of conditions, including temperatures from 4 to 100 ºC, pH values ranging from 2 to 8, exposure to surfactants and detergents (Tween 20 and 80, SDS, EDTA 0.1, 2 and 5 mM, urea, and sodium deoxycholate), and enzymes (papain and catalase). Their maximum activity (AU/mL = 12800) against four *L. monocytogenes* strains was observed between 21 and 36 h of growth of *Lbp. plantarum* 9A3, indicating a bacteriostatic mode of action. Therefore, this strain appears to be a robust candidate for potential application as a protective strain to be used in the food industry. Not only is it safe, but it also produces stable bacteriocins (harbouring genes encoding for the production of three) effectively inhibiting significant pathogens such as *L. monocytogenes* and *C. perfringens*.

5.1. Introduction

Sausages are a valuable part of the gastronomic legacy worldwide and have been a cherished part of gastronomy for centuries. *Alheira*, a traditional smoked and naturally fermented meat sausage originating from northern Portugal, stands as a highly appreciated product (Abrams *et al.*, 2011; Albano *et al.*, 2009a; Macieira *et al.*, 2019). Traditionally, *alheiras* consist mainly of shredded pork and poultry meats, traditional wheat bread, olive oil, pork fat, and spices (Albano *et al.*, 2009b). However, evolving consumer preferences for healthier, tastier, and higher quality products have led to the emergence of new *alheiras* crafted from ingredients such as fish, mushrooms, tofu, soy, and vegetables. These innovative variations, though produced by the same companies, lack harmful organisms often found in traditional *alheiras* (Azevedo *et al.*, 2020). The microbiota of these fermented sausages frequently includes lactic acid bacteria (LAB), pivotal for aroma and flavour alongside the influence of the raw materials and maturation processes. Species like *Lactiplantibacillus plantarum, Leuconostoc mesenteroides* and *Pediococcus acidilactici* are commonly found in fermented meat sausages (Albano *et al.*, 2007; Amaral *et al.*, 2015; Azevedo *et al.*,2020; Campos *et al.*, 2013). Given their usual presence in fermented foods, several LAB are considered "Generally Regarded As Safe" (GRAS) by the American Food and Drug Administration and "Qualified Presumption of Safety" status (QPS) granted by the European Food Safety Authority (EFSA). Extensive research on LAB has been conducted due to their ability to produce bacteriocins - low molecular, thermally stable, antimicrobial, and ribosomal active peptides - that have proven to be active against food-borne pathogens (Barbosa *et al.*, 2021; Martín *et al.*, 2023; Verma *et al.*, 2022). The emerging consumer demand for food products with fewer chemicals and more natural bio-preservation has conducted intense research on bacteriocin studies to discover new antimicrobial compounds that effectively combat pathogens in food products. In addition, the European Commission recently established new limits for nitrites and nitrates (EC, 2023), food additives commonly used in processed meats not only to enhance colour and extend shelf life but also for their antimicrobial properties (e.g. preventing the germination of clostridial spores), prompting food companies to adapt within a two-year period. Given the escalating global concerns about food safety, considerable attention is being directed toward utilizing bacteriocins to control food spoilage and/or growth of food-borne pathogens without compromising the food product itself (O'Connor *et al.*, 2020; Soltani *et al.*, 2021). In the study of Prpich *et al.* (2021), the authors highlighted the significance of indigenous microbiota in defining the uniqueness and distinctive sensory characteristics of fermented sausages. The constant need for innovation

in the artisanal food production makes it essential to explore alternative tools like autochthonous cultures demonstrating technological and/or probiotic traits, or bacteriocin-producer strains, that allow for improving quality and safety. The main objectives of this study were 1) to evaluate the antimicrobial activity of LAB isolated from both traditional and innovative *alheiras*, against several food-borne pathogens; 2) to characterize the bacteriocins produced by selected LAB, considering the absence of antibiotic resistance and virulence factors, using whole genome sequencing (WGS) analysis; and 3) to characterize *in vitro* the mode of action and stability of the produced bacteriocin(s) to ascertain the potential of the selected *Lpb. plantarum* 9A3 as a protective culture in food production.

5.2. Material & Methods

5.2.1. Study of antimicrobial activity potential of isolated LAB

All LAB isolates were collected from both traditional and "innovative" *alheiras*, as detailed in our previous study (Azevedo *et al.*, 2020). Target microorganisms (Supplementary Table 5.1) were grown on Tryptic Soy Agar supplemented with Yeast Extract (6 g/L) (TSA + YE; Biokar) and incubated at 37 ºC for 24 h. Subsequently, a single colony from each isolate was inoculated into 10 mL of Tryptic Soy Broth with 6% Yeast Extract (TSB + YE; Biokar). These overnight cultures, incubated at 37 ºC, were spread-plated onto TSAYE. Antimicrobial activity was assessed by spotting droplets of each LAB culture grown twice in MRS broth (Biokar), onto the bacterial lawns of each target bacterium, following the technique by Van Reenen *et al.* (1998). Inhibition was considered if a translucent halo was detected around the spot after overnight incubation at 37 ºC.

For LAB isolates demonstrating antimicrobial activity, the inhibition nature was determined using a qualitative agar-diffusion technique according to Tomé *et al.* (2006). Briefly, broth cultures of each LAB were centrifuged at 6500×*g* for 10 min at 4 ºC (Hettich 108 Zentrifugen, Rotina 35 R, Germany). The clear supernatants were then sterilized by membrane filtration (CFS) (0.2 µm; Sartorius, Goettingen, Germany) and pH adjustment between 5.0 and 6.0 was performed (CFSn) using a sodium hydroxide solution (1 M NaOH, José M. Vaz Pereira, Lisbon, Portugal). To discriminate inhibition caused by hydrogen peroxide production or by proteinaceous compounds, catalase (500 IU/mL; Sigma-Aldrich) and proteinase K (0.1 mg/mL, sterile; Sigma-Aldrich), respectively, were added to neutralized cell-free supernatant (CFSn) and incubated at 37 ºC for 1 h. Subsequently, the antimicrobial activity of CFS, CFSn, CFSn treated with catalase (CFSnC) and CFSn treated

with proteinase K (CFSnK) was assessed whenever cultures in MRS displayed inhibition against the target organisms. *Pediococcus acidilactici* HA-6111-2 served as *anti*-listerial control strain (Albano *et al.*, 2009a). The presence of a proteinaceous substance, potentially a bacteriocin, was indicated by a clear halo zone surrounding all spots except in the case of CFSnK.

5.2.2. Whole Genome Sequencing and bioinformatic Analyses

The genomic DNA from the six selected LAB strains was extracted following the manufacturer's protocol for total DNA purification from Gram-positive bacteria (Grisp, Porto, Portugal). All samples underwent rigorous analysis to ensure optimal concentration, integrity, and purity for library subsequent preparation. Subsequently, 100 ng of genomic DNA from each sample was run on Illumina DNA Prep (Illumina) and sequencing parameters were obtained using NovaSeq 6000, 2 × 100 bp. For bioinformatic analysis, sequencing reads were demultiplexed using Illumina bcl2fastq 2.20 and adapters were trimmed using Skewer v0.2.2 (Jiang *et al.*, 2014). Quality assessment of FASTQ files was conducted using FastQC v0.11.5-cegat (Andrews, 2010). Both forward and reverse fastq files were subjected to de novo assembly using SPAdes v3.13.1 (Prjibelski *et al.*, 2020) to generate fasta DNA files, which were annotated using Prokka 1.14.6 (Seemann, 2014). Mash 2.3 software was employed to estimate the closest bacterial genome from the refseq genome database (accessed March 15, 2023 at https://gembox.cbcb.umd.edu/mash/refseq.genomes.k21s1000.msh). The closest genome for each LAB genome was downloaded from the Refseq database (accessed on May 22, 2023 at https://ftp.ncbi.nlm.nih.gov/genomes/refseq/bacteria) and utilized as a reference while the genome assemblies were evaluated using QUAST v5.2.0 (Mikheenko *et al.*, 2018) and circus 0.69–8 (Lui *et al.*, 2021) for visual inspection of the assemblies. All programs were run in a Linux operating system environment.

To predict resistance phenotypes from the assembled genomes, the ResFinder-EFSA server was used accessed on March 22, 2022 (samples 4–8 and 10A2), November 17, 2022 (samples 9A3, 18–8 and 21–2/2) and March 23, 2023 (sample 1A5), at https://cge.food.dtu.dk/servic es/ResFinder-EFSA/) (Bortolaia *et al.*, 2020). Similarly, the presence of virulence factor determinants was assessed through a blastn alignment (accessed on March 23, 2022 (samples 4–8 and 10A2), November 17, 2022 (samples 9A3, 18–8 and 21–2/2) and March 24, 2023 (sample 1A5)), at http://www.mgc.ac.cn/cgi-bin/VFs/v5/main.cgi?func=V Fanalyzer) (Liu *et al.*, 2019), using the default parameters.

To identify bacteriocins produced by these LAB strains, the fasta files containing the generated contigs were searched for bacteriocins on the BAGEL4 web server (accessed on March 22, 2022 (samples 4–8 and 10A2), November 17, 2022 (samples 9A3, 18–8 and 21–2/2) and March 23, 2023 (sample 1A5), at http://bagel4.mongenrug.nl) (Van Heel *et al.*, 2018).

5.2.3. Bacteriocin-producing LAB selection and characterization

5.2.3.1. Selected strains

Six strains were chosen based on their demonstrated ability to produce proteinaceous compounds, potential bacteriocins, with activity against various pathogens. These stains comprised two *Lpb. plantarum* (1A5 and 9A3), one *Ln. mesenteroides* (4–8), and three *P. acidilactici* (10A2, 18–8 and 21/2-2), each isolated from distinct sources. *Lactiplantibacillus plantarum* 1A5, *P. acidilactici* 18–8 and 21/2-2 were isolated from traditional *alheiras* while the remaining strains were isolated from *alheiras* made with "innovative" ingredients such as codfish (*Ln. mesenteroides* 4–8), vegetables & mushrooms (*Lpb. plantarum* 9A3), and shiitake mushrooms (*P. acidilactici* 10A2).

5.2.3.2. Antibiotic susceptibility testing

The minimum inhibitory concentrations (MIC's, μg/mL) of antibiotics ampicillin, chloramphenicol, clindamycin, erythromycin, gentamicin, kanamycin, streptomycin, and tetracycline (all from Sigma - Aldrich) were determined using the broth microdilution method according to Clinical and Laboratory Standards Institute (CLSI, 2012; CLSI, 2017). Antibiotics were chosen based on the recommendations of the European Food Safety Authority, ensuring that concentrations covered the defined breakpoints (EFSA, 2012) for the selected LAB. To prepare each LAB inoculum, overnight cultures grown on MRS agar were used to prepare a suspension in Ringer's solution (Biokar), adjusted to a turbidity equivalent to 0.5 McFarland standards. Broth dilutions of each antibiotic were prepared in 96-well microtiter plates (Sarstedt, Sintra, Portugal) using cation-adjusted Mueller Hinton Broth (CAMHB) (Sigma-Aldrich) supplemented with 2.5% (v/v) of Lysed Horse Blood (LHB) (Thermo Fisher Scientific, Massachusetts, USA). Following 24 h incubation at 37 ºC, the presence or absence of turbidity was observed in each well. The minimum inhibitory concentration was considered as the first concentration with no observed growth. Positive controls included isolates grown on CAMHB with and without lysed horse blood with no

antibiotic. *Escherichia coli* ATCC 25922 and *Enterococcus faecalis* ATCC 29212 strains were used to monitor the accuracy of MIC values. The susceptibility of isolates was categorised as sensitive, intermediate, or resistant based on the criteria established by EFSA (2012). Two independent replicates were carried out for each test.

5.2.3.3. Virulence factors

5.2.3.3.1. Determination of biogenic amine-forming capacity

The screening plate method developed by Bover-Cid and Holzapfel (1999) was used to assess the potential of the six selected LAB strains in producing biogenic amines (BA) histamine, tyramine, putrescine, and cadaverine. Plates without amino acid were used as controls. A positive reaction was identified by the presence of a purple colour, or the disappearance of the tyrosine precipitate surrounding the colonies. Two replicates were performed for each isolate.

5.2.3.3.2. Production of hydrolytic enzymes: gelatinase, lipase and DNase

The production of gelatinase and DNase by the six selected isolates was evaluated according to Tiago *et al.* (2004) and Omar *et al.* (2004). Lipase production was assayed in MLB broth supplemented with 2.0 g/L of CaCl2 (Sigma-Aldrich) and 10 g/L of Tween® 80 (Sigma-Aldrich). A positive reaction was denoted by the presence of a clear halo around the colonies after 7 days of incubation at 37 ºC. All the experiments were conducted in duplicate, and *Staphylococcus aureus* ATCC 29213 served as a positive control.

5.2.3.3.3. Hemolytic activity

Hemolysis activity was determined by streaking isolates onto Columbia agar plates (Oxoid, Hampshire, United Kingdom) and incubated for 24 h at 37 ºC according to the method of Semedo *et al.* (2003). Alfa(α)-hemolysis and beta(β)-hemolysis were denoted by greenish and translucent zones around the colonies, respectively. Gamma(γ)-hemolysis indicated the absence of hemolytic activity, characterized by the absence of clear zones around the colonies. *Enterococcus faecalis* F2 and *E. faecalis* DS16 were used as β- and α-hemolytic control strains, respectively (Oliveira *et al.*, 2020).

5.2.3.3.4. Presence of virulence genes

The six LAB isolates were examined for the presence of fifteen virulence genes encoding for the various virulence factors and amino acid decarboxylating enzymes: *ace* (collagen

adhesion), *hyl* (hyaluronidase gene), *asa1* (aggregation substance precursor), *agg* (aggregation substance), *esp* (enterococcal surface protein), *gelE* (gelatinase), *efaAfs* and *efaAfm* (cell wall adhesins), *cylA*, *cylB*, *cylM, cylLL* and *cylLS* (cytolytic activity), and *hdc1*, *tdc* and *odc* (histidine, tyrosine and ornithine decarboxylase activity, respectively).

PCR amplifications were conducted in 0.2 mL reaction tubes using a ThermoCycler (Bio-Rad, Hercules, California, USA), each containing 25 µL of mixtures. Details regarding PCR target genes and primers used, respective sequence, and product size of each virulence gene tested are listed in Supplementary Table 5.2. Electrophoretic separation and controls were carried out according to Oliveira *et al.* (2020).

5.2.4. Characterization of bacteriocin(s) produced by *Lpb. plantarum* 9A3

As a result of the preliminary studies, *Lpb. plantarum* 9A3 was selected for further testing.

5.2.4.1. Maximum bacteriocin production (AU/mL) during *Lpb. plantarum* growth

To ascertain the maximum bacteriocin production during its growth, 1% (v/v) of an overnight *Lpb. plantarum* 9A3 culture was inoculated into 100 mL of MRS broth and incubated at 37 ºC. At regular intervals over 36 h of incubation, aliquots were taken. Changes in pH, viable counts (Colony Forming Units (CFU)/mL) and bacteriocin activity (AU/mL) against four *L. monocytogenes* serovars (NCTC 11994 (1/4 b), CECT 911 (1/2c), CECT 936 (1/2 b), CEP 104794 (1/2a)) were recorded every hour until 24 h, as described by Van Reenen *et al.* (1998). Three independent replicates were performed.

Bacteriocin activity was determined by successive dilutions of 9A3 CFSn in phosphate buffer (pH 6.5), and 10 µL aliquots from each dilution were spotted onto soft agar plates (BHI with 0.7% w/v agar) inoculated with approximately 10^6 CFU/mL of each target *L. monocytogenes* strain. Plates were then incubated at 37 ºC for 24 h. *Pediococcus acidilactici* HA 6111–2 was used as a control. Antimicrobial activity was expressed as Arbitrary Units per mL (AU/mL), calculated as ab x 100, where "a" represents the dilution factor and "b" is the last dilution that resulted in an inhibition zone of at least 2 mm in diameter. Activity was expressed per mL by multiplying by 100.

5.2.4.2. Effect of enzymes, detergents/surfactants/proteases, pH, and temperature on bacteriocin activity

Lactiplantibacillus plantarum 9A3 was grown in MRS broth overnight at 37 ºC followed by cell harvesting through centrifugation (8000×*g* for 10 min at 4 ºC). The resulting supernatant (CFS) was pH-adjusted to the range of 5–6 using 1 M NaOH and incubated at 80 ºC for 10 min (CFSn). Subsequently, 1 mL of this CFSn was incubated at 37 ºC (or at the specific temperature to be studied) under various conditions of pH and studied compound, as outlined in Albano *et al.* (2007). Antimicrobial activity determination against all four *L. monocytogenes* strains, was carried out according to Van Reenen *et al.* (1998).

5.2.4.3. Cell lysis

The mode of action of the bacteriocin(s) produced by *Lpb. plantarum* 9A3 was assessed according to Van Reenen *et al.* (1998). A volume of 20 mL of filter-sterilized supernatant containing bacteriocin (12800 AU/mL, pH ≈ 6.0) was added to 100 mL of early exponential phase cultures (6 h old) of each target *L. monocytogenes* serovars. Samples were taken every hour for 12 h and at 24 h and 30 h for enumeration of *L. monocytogenes*. Each *L. monocytogenes* culture without added bacteriocin was used as control and two independent replicates were accomplished.

5.2.4.4. Adsorption studies, partial purification, and determination of *Lpb. plantarum* bacteriocin molecular size

Adsorption of *Lpb.* p*lantarum* 9A3 bacteriocin was conducted according to Yang *et al.* (1992). Succinctly, bacteriocin-producing cells, cultured for 24 h at 37 ºC and adjusted to pH 6.0, were harvested by centrifugation (8000×*g*, 10 min, 4 ºC) and washed using sterile 0.1 M phosphate buffer (pH 6.5). Pellets were resuspended in 10 mL of 100 mM NaCl (pH 2.0) for 12 h at 4 ºC to facilitate bacteriocin detachment from cells. After cell collection, the cell-free supernatant was neutralized and assessed for bacteriocin activity following Van Reenen *et al.* (1998). For partial purification, the initial supernatant was refrigerated at 4 ºC after which ammonium sulphate was gradually added to reach 60% of saturation and kept at low stirring for 4 h at 4 ºC.

The precipitated proteins, in the pellet and floating on the surface, were dissolved in 25 mM ammonium acetate buffer (pH 6.5), after collection by centrifugation (18000×*g*, 20 min, 4 ºC), according to the method of Sambrook *et al.* (1989). All samples were stored at - 20 ºC. To determine the molecular size of *Lpb.* p*lantarum* 9A3 bacteriocin(s), saturated samples

were separated via tricine-SDS-PAGE as previously described by Schägger and von Jagow (1987). A low molecular weight marker ranging from 6.5 kDa to 270 kDa (GRS Protein Marker PLUs; Grisp) was used. Samples were loaded in duplicate onto the acrylamide gel, followed by gel division after run completion. One portion was fixed with 20% isopropanol and 10% acetic acid. Coomassie Brilliant Blue R250 (Bio-Rad) was used to stain the other half to visualise the peptide band position. After unstained and extensively pre-washed with the sterile distilled water, the other half was overlaid with *L. monocytogenes* CECT 911 and CECT 936 cells (10^6 CFU/mL), initially incorporated in BHI soft agar (0.7% agar w/v; Biokar), to determine the position of the active bacteriocin.

5.3. Results and Discussion

A total of 491 isolates, comprising 299 from vegetarian and 192 from traditional *alheiras*, previously isolated and classified as LAB, were selected for assessment regarding potential bacteriocinogenic activity against food-borne pathogens.

5.3.1. Study of antimicrobial activity potential of isolated LAB

A first screening against 49 target microorganisms (Supplementary Table 5.1) was performed with 491 LAB isolates, of which 98 were selected due to antimicrobial activity against *E. faecalis* ATCC 29212, various strains of *L. monocytogenes*, *C. sporogenes,* and *C. perfringens*. Furthermore, one among the 98 LAB isolates exhibited inhibition of *S. aureus* ATCC 29213. Subsequently, antimicrobial activity due to bacteriocinogenic activity was investigated using cell-free extracts (CFS) subjected to neutralization (CFSn), addition of catalase (CFSnC), and digestion with proteinase K (CFSnK). Ten LAB strains were selected based on CFS activity, and eight of these maintained their antimicrobial activity even after CFSn and CFSnC treatments against the aforementioned food-borne pathogens. Ultimately, only six isolates demonstrated potential bacteriocin production, demonstrating antimicrobial activity against all strains of *L. monocytogenes*, *E. faecalis* ATCC 29212, one strain of *C. sporogenes* and one strain of *C. perfringens*. Lactic acid bacteria have been widely recognized and described by several authors for its effectiveness in inhibiting Gram-positive bacteria (Abrams *et al.*, 2011; Albano *et al.*, 2007, 2009a; Peng *et al.*, 2017).

5.3.2. Characterization of lactic acid bacteria bacteriocin producers by whole-genome sequencing

No orthologues corresponding to antibiotic resistance genes were identified using the ResFinder EFSA software. Similarly, no significant blastn alignments were detected between the assembled contigs and databases containing virulence pathogens of pathogenic bacteria, such as proteins involved in secretion systems and their effectors, toxins, and iron acquisition, adhesion, and invasion by the bacterial cell. Previous studies have also found LAB isolates lacking virulence factors (Behera *et al.*, 2018; Muñoz-Atienza *et al.*, 2013). On the other hand, the BAGEL4 software showed that these LAB encode multiple bacteriocins, which may explain their producer phenotype. Supplementary Figs. 5.1 – 5.6 show the genetic organization maps of the bacteriocin-encoding regions. Supplementary Fig. 5.1A display a contig in the assembled bacterial genome that encodes a two-peptide protein. This protein, exhibiting 100% identity with class II bacteriocins, Plantaricin E and Plantaricin F from *Lpb. plantarum*, is adjacent to two *lanT* gene homologues involved in synthesizing lantibiotic compounds, which are synthesised antimicrobial peptides (Singh and Sareen, 2014), and genes encoding the bacteriocin ABC transporter, the ATP-binding protein, and the permease protein PlnG. Another sequence contig in Supplementary Fig. 5.1B showed 100% identity with pediocin proteins from *P. acidilactici*. Downstream is a *lanT* gene encoding the pediocin PA-1 transport/processing ATP-binding protein PedD and a gene encoding pediocin PA-1 biosynthetic protein PedC. A contig in strain 4-8 encoding Enterocin X, a 100% identical class II bacteriocin chain beta identical to that found in *Ln. mesenteroides* was revealed in Supplementary Fig. 5.2. Originally discovered in *E. faecium*, Enterocin X is a two-peptide bacteriocin (Xα and Xβ) with a narrow spectrum of weak to moderate antibacterial activity (Hu *et al.*, 2010). This bacteriocin was later identified in *Leuconostoc* species isolated from a traditional Korean fermented vegetable - kimchi (Mun *et al.*, 2021). Supplementary Fig. 5.3A shows a contig encoding a two-peptide protein that is 100% identical to the class II bacteriocins, Plantaricin E and Plantaricin F from *Lpb. plantarum*. Upstream of these plantaricin-encoding genes and in the same operon, two *lanT* gene homologues encode the bacteriocin ABC transporter, the ATP-binding and permease protein PlnG. Similarly to what is shown in Suppl. Fig. 5.1B, Suppl. Fig. 5.3B displays a sequence contig 100% identical with pediocin proteins, as well as a LanT gene and a gene encoding the pediocin PA-1 biosynthetic protein. Moreover, this operon also contains a gene encoding a leucocin A homologue. These class II bacteriocins have been reported in several other studies (Barbosa *et al.*, 2021; Holo *et al.*, 2001; Loessner *et al.*, 2003; Wang *et al.*,

2018). In Supplementary Fig. 5.4, the genetic organisation map of *P. acidilactici* strain 10A2 shows the coagulin A and pediocin gene clusters. Coagulin A, a pediocin-like inhibitory substance produced by *Bacillus coagulans*, was first reported in a *Pediococcus* strain by Zommiti *et al.* (2018), but several other authors have found this bacteriocin in *Pediococcus* strains (Jiang *et al.*, 2021; Rodrigues Blanco *et al.*, 2022; Todorov *et al.*, 2023). Downstream of this gene encoding Coagulin A, and in the same operon, is a *LanT* gene encoding the Pediocin PA-1 transport/processing ATP-binding protein PedD and a gene encoding the pediocin PA-1 biosynthetic protein PedC. Supplementary Figs. 5.5 and 5.6 for *P. acidilactici* 18–8 and 21/2-2, respectively, show a similar genetic organisation map. In both, a gene encoding Enterolysin A can be identified, which has a high prevalence in *Enterococcus* and *Pediococcus* strains, but also in *Lactococcus lactis* (Mileriene *et al.*, 2023; Ormaasen *et al.*, 2023).

5.3.3. Bacteriocin-producing LAB selection and characterisation

5.3.3.1. Antibiotic susceptibility testing

Table 5.1 presents the antibiotic susceptibility results for all six LAB isolates. According to EFSA guidelines, evaluating vancomycin susceptibility is not required for obligate and facultative heterofermentative lactobacilli, *Pediococcus* spp. and *Leuconostoc* spp. As these strains are inherently resistant (Keter *et al.*, 2022; Swenson *et al.*, 1990).

In a recent study, Colautti *et al.* (2022) emphasized lactobacilli as a reservoir of antibiotic resistance (AR) genes. While lactobacilli are generally considered more resistant to vancomycin and aminoglycosides like gentamicin, kanamycin, and streptomycin, and susceptible to erythromycin, β-lactam antibiotics (ampicillin), chloramphenicol, and tetracycline. Despite that, in our study, all isolates demonstrated sensitivity to all aminoglycoside antibiotics tested. Despite the general susceptibility to erythromycin, *Lpb. plantarum* (1A5) and both *P. acidilactici* (10A2 and 18–8) exhibited resistance to this antibiotic, consistent with prior reports (Čanžek Majhenič *et al.*, 2007; Holmes *et al.*, 2016). *Pediococcus acidilactici* (10A2) showed resistance to four out of eight antibiotics tested, namely ampicillin, chloramphenicol, erythromycin, and tetracycline. While some studies found *Pediococcus* to be susceptible to ampicillin (Barathikannan *et al.*, 2022; Silva *et al.*, 2019; Singla *et al.*, 2018), all pediococci tested in the study of Federici *et al.* (2014) were resistant to ampicillin which corroborates our findings related to *P. acidilactici* 10A2. Similarly, while most *Pediococcus* are considered sensitive to chloramphenicol and

erythromycin, exceptions have been observed (Basbülbül *et al.*, 2015; Shi *et al.*, 2019; Temmerman *et al.*, 2003) consistent with our study. Some studies reported intrinsic resistance of pediococci to tetracyclines (Danielsen *et al.*, 2007; Federici *et al.*, 2014; Lüdin *et al.*, 2018; Rojo-Bezares *et al.*, 2006), while others found no resistance in isolates from food products (Abbasiliasi *et al.*, 2012; de Sant'Anna *et al.*, 2017; Fguiri *et al.*, 2016; Morandi *et al.*, 2015). Contrary to vancomycin resistance, there is evidence that the *tet (M)* gene can be conjugatively transferred among microorganisms *in vitro* and that resistance to tetracycline is acquired (Gevers *et al.*, 2003), which is a matter of concern.

Regarding *Ln. mesenteroides* (4–8), this LAB exhibited resistance to the same antibiotics as *P. acidilactici* 10A2, plus clindamycin. Regarding ampicillin resistance, *Leuconostoc* species are typically sensitive, but some strains show resistance to other β-lactams (Morandi *et al.*, 2013; Rodríguez-Alonso *et al.*, 2009). While *Leuconostoc* strains are usually susceptible to erythromycin, chloramphenicol, clindamycin, and tetracyclines, cases of resistance have been reported by several authors (Akpinar and Yerlikaya, 2021; Basbülbül *et al.*, 2015; Flórez *et al.*, 2005; Morandi *et al.*, 2013) consistent with our findings. As previously stated by others (Basbülbül *et al.*, 2015; Flórez *et al.*, 2016; Morandi *et al.*, 2013), *Leuconostoc* strains are intrinsically resistant to vancomycin.

5.3.3.2. Virulence factors

5.3.3.2.1. Determination of biogenic amine-forming capacity

Lactic acid bacteria are recognized for their decarboxylase activity, potentially leading to the generation of biogenic amines from available amino acids (Alfaia *et al.*, 2018; Özogul and Hamed, 2018). Notably, none of the tested LAB isolates produced cadaverine, histamine, putrescine, or tyramine. Several studies suggest the use of "amine-negative" starter cultures to prevent amine formation in fermented foods (Grujović *et al.*, 2022; Héquet *et al.*, 2007; Lim, 2022). Certain bacteriocin-producing LAB strains possess the ability to reduce biogenic amines levels in fermented foods by producing amine oxidase production or catalysing the oxidative deamination of amines. Consequently, the application of particular strains capable of degrading and/or inhibiting biogenic amines formation has been suggested as a safe approach in the preservation of fermented foods and beverages within the food industry (Joosten and Nunez, 1996; Lim and Choi, 2018).

5.3.3.2.2. Production of hydrolytic enzymes: gelatinase, lipase and DNase

Gelatinase activity is regarded as harmful as it has the potential to break down collagen, initiating an inflammatory response (Leonardo and Pennypacker, 2009). While lipases are vital enzymes with wide industrial applications, growing evidence suggests their role as significant microbial virulence factors (Dinçer and Kıvanç, 2018; Stehr *et al.*, 2003). DNase, capable of degrading DNA by cleaving phosphodiester linkages in the DNA backbone, may be involved in bacterial growth, biofilm maturation, and evasion of the immune system, thereby acting as a virulence factor (Varela-Ramirez *et al.*, 2017). Interestingly, none of the studied LAB strains produced gelatinase, lipase or DNAse. Similar findings have been reported in several studies indicating the absence of gelatinase and DNase-producing LAB strains (Javed *et al.*, 2022; Keter *et al.*, 2022; Pinto *et al.*, 2020; Silva *et al.*, 2019). However, it is noteworthy that lipase-producing strains are commonly found among lactobacilli, *Pediococcus* and *Leuconostoc* strains (García-Cano *et al.*, 2019).

5.3.3.2.3. Hemolytic activity

Hemolysis is a virulence factor that supports microorganisms in accessing iron and can lead to host anemia (Keter *et al.*, 2022). In this study, all studied isolates exhibited no hemolytic activity. Lactobacilli are commonly recognized as non-hemolytic (Chen *et al.*, 2022; Cizeikiene and Jagelaviciute, 2021; Halder *et al.*, 2017). Similar observations were made in the studies of Jeong and Lee (2015) and Wang *et al.* (2018), where *Leuconostoc* strains also showed an absence of hemolytic activity. Recent studies have further confirmed the lack of hemolytic activity in *Pediococcus* species (Barathikannan *et al.*, 2022; Shazadi and Arshad, 2022).

Table 5.1 – Distribution of MICs of tested antibiotics for all six LAB selected

Origin	Sample	Strain	Antibiotics Minimum Inhibitory Concentration (MICs) (mg/L)							
			AMP	**CHL**	**CLI**	**ERY**	**GEN**	**KAN**	**STR**	**TET**
			Microbiological cut-off values (mg/L) proposed by EFSA for *Lactiplantibacillus plantarum/pentosus*							
			2	8	2	1	16	64	n.r.	32
Traditional *Alheira*	1A5	*Lactiplantibacillus plantarum*	0.125	4	0.06	**4**	0.5	8	1	8
Vegetables & Mushrooms *Alheira*	9A3		0.125	2	0.125	1	2	32	4	2
			Microbiological cut-off values (mg/L) proposed by EFSA for *Leuconostoc*							
			2	4	1	1	16	16	64	8
Codfish *Alheira*	4-8	*Leuconostoc mesenteroides*	**16**	**128**	**2**	**64**	0.25	16	1	**128**
			Microbiological cut-off values (mg/L) proposed by EFSA for *Pediococcus*							
			4	4	1	1	16	64	64	8
Shiitake mushrooms *Alheira*	10A2		**128**	**128**	0.5	**64**	4	64	64	**128**
Traditional *Alheira*	18-8	*Pediococcus acidilactici*	0.125	2	0.25	**8**	4	8	0.5	4
Traditional *Alheira*	21/2-2		0.06	2	0.06	0.5	0.125	1	0.5	8

MICs higher than EFSA cut-off values are in bold and indicate resistance to the corresponding antibiotic
AMP – Ampicillin; CHL – Chloramphenicol; CLI – Clindamycin; ERY – Erythromycin; GEN – Gentamycin; KAN – Kanamycin; STR – Streptomycin; TET – Tetracycline
n.r. – not required

Table 5.2 – Presence of virulence genes

Origin	Sample	Strain	Virulence Genes															
			agg	*esp*	*gelE*	*efaAfm*	*efaAfs*	*cylA*	*cylB*	*cylM*	*cylLL*	*cylLS*	*ace*	*hyl*	*asa*1	*hdc*1	*tcd*	*odc*
Traditional *Alheira*	1A5	*Lactiplantibacillus plantarum*	–	–	+	–	+	–	–	–	+	+	–	–	–	–	–	–
Codfish *Alheira*	4-8	*Leuconostoc mesenteroides*	+	–	+	+	+	–	–	+	+	–	+	–	+	–	–	–
Vegetables & Mushrooms *Alheira*	9A3	*Lactiplantibacillus plantarum*	+	–	+	+	+	–	–	–	–	–	+	–	+	–	–	–
Shiitake mushrooms *Alheira*	10A2	*Pediococcus acidilactici*	+	–	+	–	+	–	–	+	+	–	–	–	+	–	–	–
Traditional *Alheira*	18-8	*Pediococcus acidilactici*	+	–	+	+	+	–	–	–	+	–	+	–	+	–	–	–
Traditional *Alheira*	21/2-2	*Pediococcus acidilactici*	+	–	+	+	+	–	–	+	+	–	+	–	+	–	+	–

5.3.3.2.4. Presence of virulence genes

The absence of biogenic amine production as well as hemolytic, gelatinase and DNase activities by our isolates does not necessarily mean that they are not potentially pathogenic or express virulence. In the present study, all isolates lacked *esp*, *cylA*, *cylB*, *hyl*, *hdc1* and *odc* genes (Table 5.2). Virulence factors have been reported in LAB isolates although they are more commonly associated with enterococci strains. Gelatinase gene (*gelE*) (Suppl. Fig. 5.7), for example, is commonly found in *Enterococcus* strains and was previously described by Eaton and Gasson (2001) as highly influenced by culture conditions and laboratory manipulation. This might lead to the loss of the structural genes and can explain why, although all tested isolates harboured the *gelE* gene, none presented gelatinase activity during *in vitro* testing.

Aggregation substance, encoded by *agg* and *asa1* genes (Suppl. Fig. 5.7), was detected in all isolates except *Lpb. plantarum* 1A5. This is a plasmid-carried gene that facilitates the conjugative transfer of sex pheromone gene-containing plasmids through the clattering of one bacterium to another (Galli *et al.*, 1990). *Leuconostoc* and lactobacilli strains tested in the study of Jeronymo-Ceneviva *et al.* (2014) harboured *asa1* gene yet, from what we know this far, this gene has never been reported in *P. acidilactici* strains.

5.3.4. Characterization of bacteriocin produced by *Lpb. plantarum* 9A3

Following the phenotypic characterization, *Lpb. plantarum* 9A3 was selected due to its susceptibility to all tested antibiotics and absence of virulence factors. The crude supernatant served as the primary material for all experiments conducted to characterize 9A3 bacteriocin(s). WGS confirmed that strain 9A3 has the capability to produce more than one bacteriocin, and therefore, all reported outcomes can be attributed to one or more bacteriocins.

5.3.4.1. Growth of *Lpb. plantarum* 9A3 and bacteriocin production

Fig. 5.1 illustrates the viable counts and antimicrobial activity (AU/ mL) of *Lpb. plantarum* 9A3 cell-free supernatant against *L. monocytogenes* serovars NCTC 11994 (1/4 b), CECT 911 (1/2c), CECT 936 (1/2 b), CEP 104794 (1/2a) over time. The maximum bacteriocin activity (12800 AU/mL) against all serovars was achieved after 24 h except for *L. monocytogenes* CEP 104794 (1/2a), where it was observed at 21 h. Viable cell counts increased by approximately 3 log CFU/mL after 12 h of incubation, reaching the maximum

cell growth around 21 h of incubation. Considering the characterization of bacteriocin-producing *Lpb. plantarum*-producing strains, various authors have highlighted their robust bacteriocin production, particularly showing potent activity against diverse *L. monocytogenes* strains (Barbosa *et al.*, 2021; García-Reyes *et al.*, 2023; Zareie *et al.*, 2023). Based on the observed maximum cell growth and achievement of maximum bacteriocin activity (12800 AU/mL), 24 h was selected for all subsequent assays.

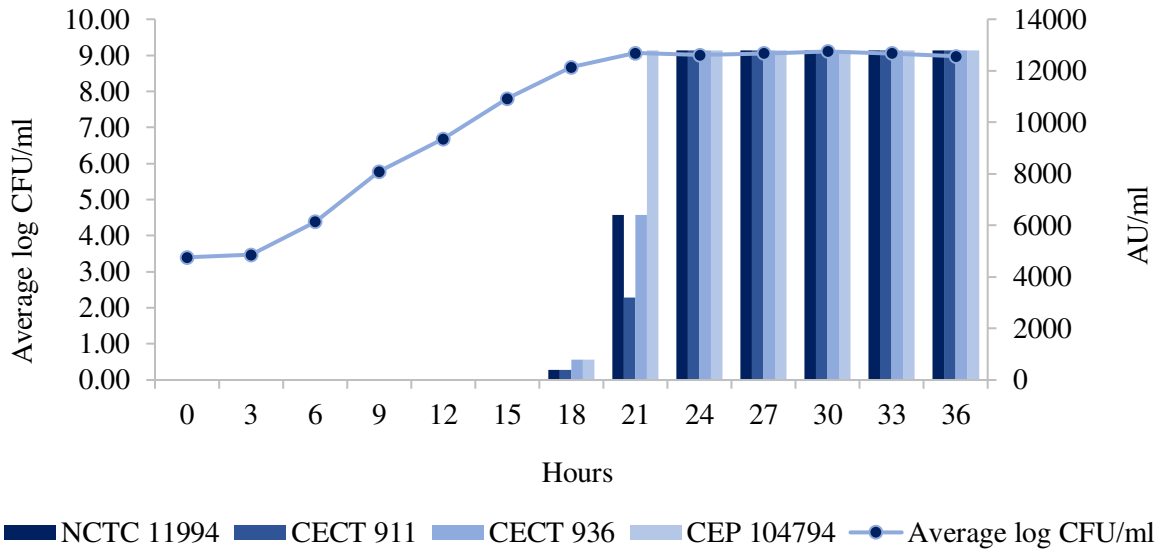


Figure 5.1 – Bacteriocin production by *Lpb. plantarum* 9A3 in MRS broth. The antimicrobial activity of cell-free supernatant is presented as AU/mL (bars) for *L. monocytogenes* serovars NCTC 11994 (1/4 b), CECT 911 (1/2c), CECT 936 (1/2 b), CEP 104794 (1/2a). Viable cell counts are also presented as log CFU/mL (line).

5.3.4.2. Effects of enzymes, temperature, pH, and surfactants on bacteriocin activity

A variety of detergents, enzymes, pH, and temperature that have an impact on bacteriocin(s) activity against *L. monocytogenes* serovars NCTC 11994 (1/4 b), CECT 911 (1/2c), CECT 936 (1/2 b), CEP 104794 (1/2a) are presented in Tables 5.3 and 5.4. The bacteriocin activity of *Lpb. plantarum* 9A3 remained stable within a temperature range of 4 ºC to 60 ºC against most *L. monocytogenes* serovars (Table 5.3). However, for serovar 1/2c, residual activity was observed below 30 ºC, while activity persisted above 80 ºC for all serovars tested. Similar thermal stability has been noted in bacteriocins produced by other lactobacilli strains (Barbosa *et al.*, 2021; Zhao *et al.*, 2022). It was also observed that bacteriocin(s) from *Lpb. plantarum* 9A3 were quite resistant to a wide pH range (from 2 to 10), indicating sensitivity

to alkaline conditions (Table 5.4). Similar decreases in antimicrobial activity have been reported for other bacteriocins such as plantaricins and pediocins under altered pH conditions (Barbosa *et al.*, 2021; Heredia-Castro *et al.*, 2015; Ramos *et al.*, 2016). Regarding surfactants commonly used in the food industry, *Lpb. plantarum* 9A3 bacteriocin(s) showed resistance (Table 5.4), with exceptions noted to treatments with sodium carbonate, NaCl and Triton X-100, which promoted the loss of bacteriocin (s) activity. Among the enzymes tested, only papain and catalase treatments did not reduce bacteriocin(s) activity, while proteinase K and pepsin drastically reduced bacteriocinogenic activity by 100%. Furthermore, a lower reduction in activity was noted following treatment with papain and the antioxidant enzyme (catalase). This has been reported in several other studies not only when bacteriocins were treated with proteinase K (Barbosa *et al.*, 2021; Oliveira *et al.*, 2020) but also when exposed to pepsin, papain, and catalase (Ramos *et al.*, 2016; Todorov *et al.*, 2013).

Table 5.3 – Temperature effect on antimicrobial activity reduction of *Lactiplantibacillus plantarum* 9A3 bacteriocin, expressed in percentage values, against four different *Listeria monocytogenes* serovars

Listeria monocytogenes		**NCTC 11994**		**CECT 911**		**CECT 936**		**CEP 104794**	
		1h	2h	1h	2h	1h	2h	1h	2h
T (ºC)	4	0.00%	0.00%	50.00%	50.00%	0.00%	0.00%	0.00%	0.00%
	25	0.00%	0.00%	50.00%	50.00%	0.00%	0.00%	0.00%	0.00%
	30	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%
	37	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%
	60	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%
	80	0.00%	0.00%	50.00%	50.00%	0.00%	0.00%	0.00%	50.00%
	100	75.00%	87.50%	87.50%	87.50%	50.00%	75.00%	75.00%	87.50%
	121	98.44%		98.44%		100.00%		98.44%	

Note: With no treatment, maximum activity of 9A3 bacteriocin (s) for each *L. monocytogenes* strain was 12.800 AU/mL. Percentage values of inhibition are represented according to this maximum activity.

Table 5.4 – Antimicrobial activity reduction of *Lactiplantibacillus plantarum* 9A3 bacteriocin, expressed in percentage values, against four different *Listeria monocytogenes* serovars, under pH, detergents, surfactants, and protease inhibitors effect

Listeria monocytogenes		**NCTC 11994**	**CECT 911**	**CECT 936**	**CEP 104794**
pH	2	0.0%	0.0%	0.0%	0.0%
	4	0.0%	0.0%	0.0%	0.0%
	6	0.0%	0.0%	0.0%	0.0%
	8	0.0%	0.0%	0.0%	0.0%
	10	50.0%	50.0%	75.0%	50.0%
	12	99.2%	99.2%	100.0%	99.2%
Detergents	Tween 20	0.0%	0.0%	75.0%	0.0%
	Tween 80	0.0%	0.0%	75.0%	0.0%
	Triton X-100	50.0%	50.0%	87.5%	0.0%
	SDS	0.0%	0.0%	50.0%	0.0%
	EDTA 0.1mM	0.0%	0.0%	75.0%	0.0%
	EDTA 2mM	0.0%	0.0%	75.0%	0.0%
	EDTA 5mM	0.0%	0.0%	75.0%	0.0%
	Urea	0.0%	0.0%	75.0%	0.0%
	NaCl	0.0%	50.0%	87.5%	50.0%
	Sodium carbonate	87.5%	87.5%	96.9%	87.5%
	Sodium deoxycholate	0.0%	0.0%	75.0%	0.0%
Enzymes (mg/ml)	Proteinase K 1.0	100.0%	100.0%	100.0%	100.0%
	Proteinase K 0.1	100.0%	100.0%	100.0%	100.0%
	Papain 1.0	0.0%	50.00%	87.50%	50.00%
	Papain 0.1	0.0%	50.00%	75.00%	0.0%
	Pepsin 1.0	100.0%	100.0%	100.0%	100.0%
	Pepsin 0.1	100.0%	100.0%	100.0%	100.0%
	Catalase 1.0	0.0%	75.0%	87.5%	50.0%
	Catalase 0.1	0.0%	0.0%	75.0%	0.0%

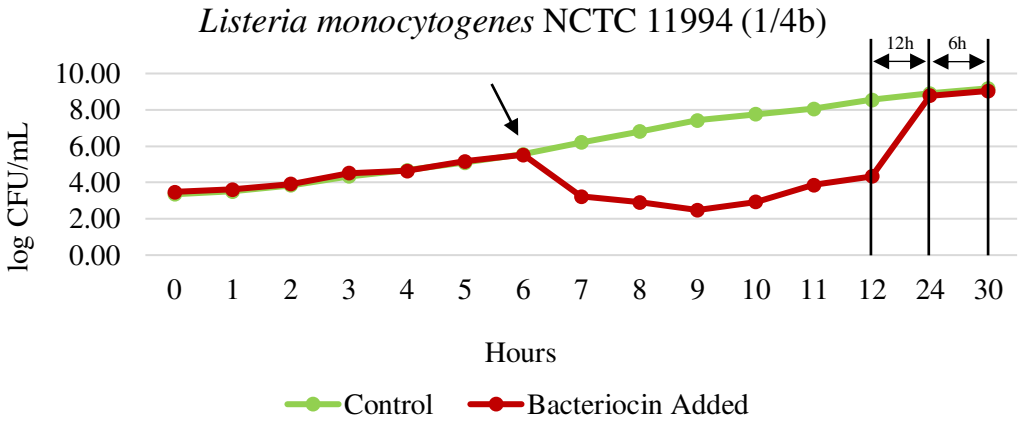
Note: With no treatment, maximum activity of 9A3 bacteriocin (s) for each *L. monocytogenes* strain was 12800 AU/mL. Percentage values of inhibition are represented according to this maximum activity.

5.3.4.3. Cell lysis

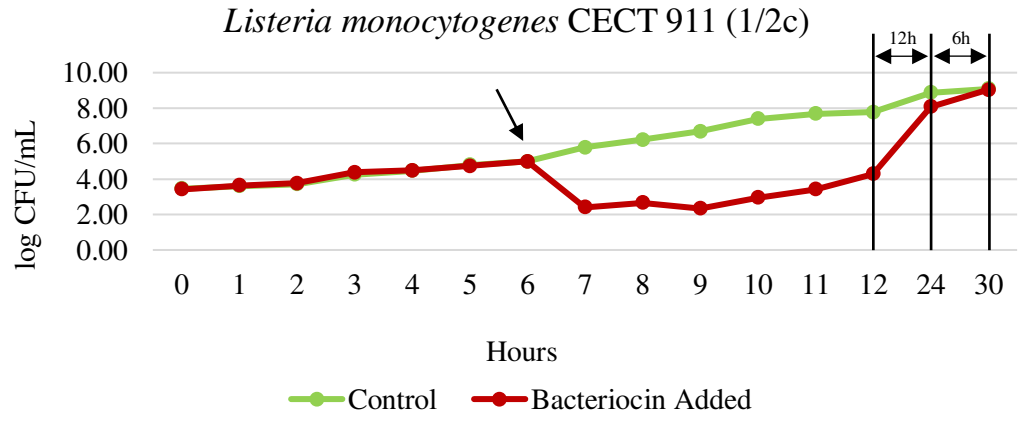
The impact of *Lpb. plantarum* 9A3 bacteriocin(s) on the growth of *L. monocytogenes* serovars NCTC 11994 (1/4 b), CECT 911 (1/2c), CECT 936 (1/2 b) and CEP 104794 (1/2a) is exhibited in Fig. 5.2. Upon the addition of *Lpb. plantarum* 9A3 bacteriocin(s) to mid-log *L. monocytogenes* cultures (6 h-old), a suppression of cell growth was evident, particularly notable for *L. monocytogenes* CEP 104794 (serovar 1/2a) with a reduction of 3.57 log

CFU/mL (Fig. 2D). Conversely, no noticeable change in cell counts was observed for untreated samples (control). Across all studied *L. monocytogenes* serovars, the treated cell-free supernatant of *Lpb. plantarum* 9A3 significantly impacted their viability, effectively controlling their growth for at least 12 h. These findings parallel those reported by Martín *et al.* (2023), where the numbers of *L. monocytogenes* and *E. faecalis* decreased following the addition of a bacteriocin produced by *Latilactobacillus sakei* 205 (3200 and 6400 AU/mL, respectively). Similarly, Barbosa *et al.* (2021) documented a decrease of approximately 2 log cycles in *L. monocytogenes* 7947 upon the addition of the cell-free supernatant containing *Lpb. plantarum* R23 bacteriocin (12800 AU/mL). Notably, observations at 24 and 30 h indicated that despite growth suppression, *L. monocytogenes* were able to recover after 24 h across all cases. Previous studies typically assessed the behaviour of *L. monocytogenes* over a 12-h growth period (Barbosa *et al.*, 2021; Martín *et al.*, 2023).

A



B



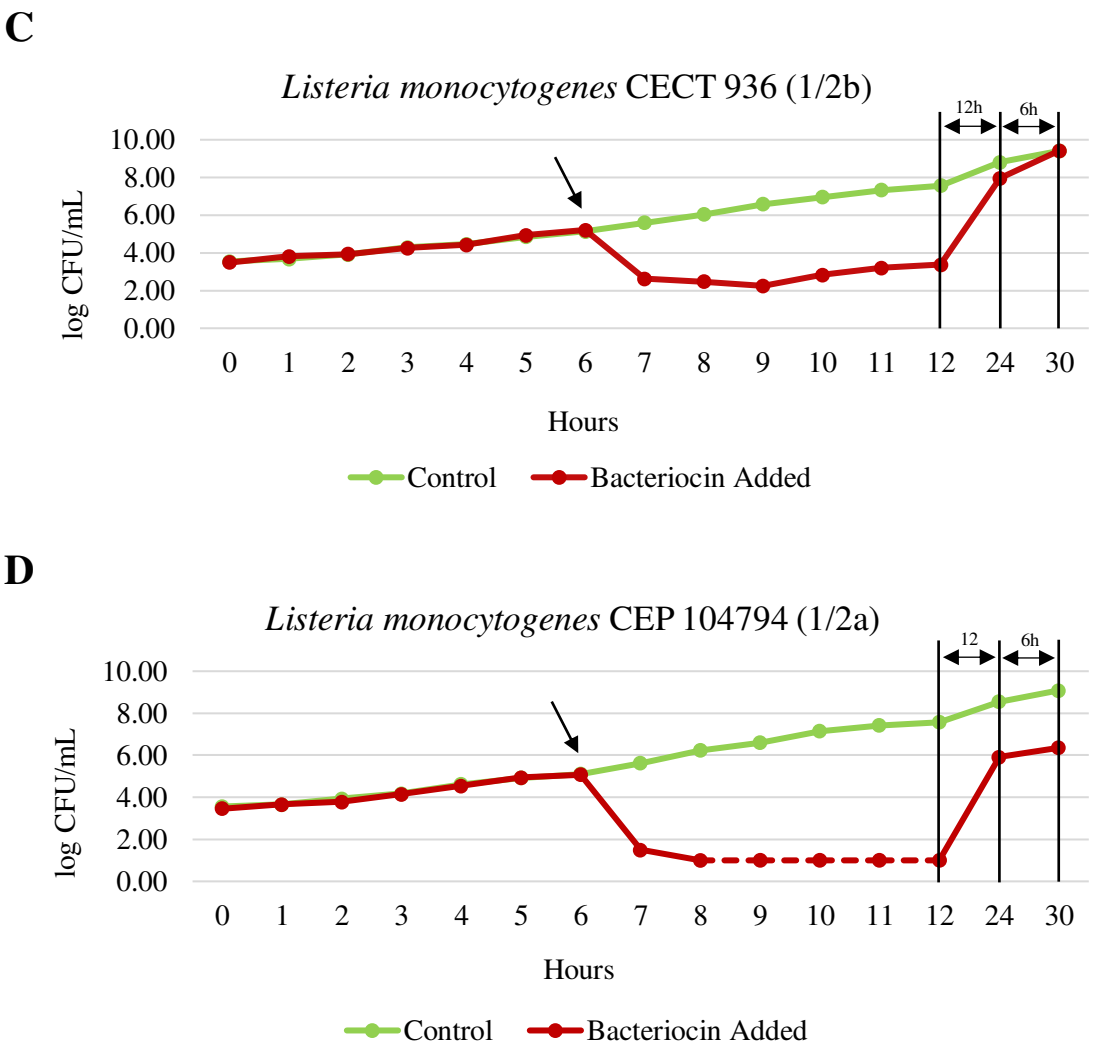


Figure 5.2 – Effect of *Lpb. plantarum* 9A3 bacteriocin (s) on the growth of *L. monocytogenes* NCTC 11994 (**A**), CECT 911 (**B**), CECT 936 (**C**) and CEP 104794 (**D**) presented as log CFU/mL. Green lines represent target cultures without added bacteriocin. The arrow indicates the point at which the bacteriocin was added.

5.3.4.4. Adsorption studies and molecular size determination

Bacteriocin adherence to the surface of producer cells was not observed upon treating *Lpb. plantarum* 9A3 with 100 mM NaCl at pH 2.0, aligning with findings reported for other bacteriocins produced by different lactobacilli (Barbosa *et al.*, 2021; Martín *et al.*, 2023; Martinez *et al.*, 2013).

Tricine/SDS-PAGE analysis estimated the molecular size of *Lpb. plantarum* 9A3 bacteriocin(s) to range between 37 and 52 kDa, deduced from the correlation between the

position of the peptide band and the growth inhibition clear zone of *L. monocytogenes* CECT 911 (Fig. 5.3). A comparable outcome for *L. monocytogenes* CECT 936 is presented in Supplementary Fig. 5.8. Surprisingly, the molecular weight estimated by SDS-PAGE exceeded the anticipated range, considering the bacteriocins suggested by WGS. Class II bacteriocins like Plantaricin E, Plantaricin F, Pediocin PA, Enterocin X, Leucocin A and Coagulin A generally exhibit a molecular weight of less than 10 kDa (Drider *et al.*, 2006), consistent with most bacteriocins produced by *Lpb. plantarum* strains (Han *et al.*, 2023; Sidhu and Nehra, 2021; Wang *et al.*, 2023). However, without further isolation and characterization studies of the purified bacteriocins, any explanation would remain speculative. This leaves us with hypotheses requiring validation: the observed halo on the gel could result from the activity of multiple bacteriocins, and therefore, the value determined may be an artefact of the technique; the molecular weight standards may not be sufficiently accurate (Neris *et al.*, 2020); or we may even be in the presence of new bacteriocin(s). Other authors have reported high molecular weights for bacteriocins produced by other lactobacilli: Noroozi *et al.*, (2019) reported a novel large molecular weight bacteriocin (68 kDa) produced by a *Lacticaseibacillus casei* strain and Islam *et al.* (2020) identified lactobacilli producing 30 and 40 kDa bacteriocins. Moreover, the SwissProt database lists only three bacteriocins from *Lpb. plantarum*: plantaricin ASM1 (https://www.uniprot.org/uniprotkb/C7G1H4, accessed on October 19, 2023), plantaricin-A (https://www.uniprot.org/uniprotkb/P80214, accessed on October 19, 2023) and plantaricin KL-1Y (https://www.uniprot. org/uniprotkb/C0HJC0, accessed on October 19, 2023) with molecular weights of 6891 kDa, 5458 kDa and 3498 kDa respectively. The bacteriocin Plantaricin ASM1 produced by *Lpb. plantarum* A-1 has been isolated and characterized, and its molecular mass of 5045.72 Da was determined by MALDI mass spectrometry (Hata *et al.*, 2010), suggesting that the protein is processed into a mature form. Bacteriocin KL-1Y from *Lpb. plantarum* was also successfully purified and its molecular mass, determined by electrospray mass spectrometry, was 3498 Da (Rumjuankiat *et al.*, 2015), which is consistent with the prediction. Concerning Leucocin A, identified as a homologous sequence in the genome of *Lpb. plantarum* 9A3, a putative Leucocin A (https://www.uniprot.org/uniprotkb/A0A2S3U2A4, accessed on October 19, 2023) with a predictive molecular weight of 13,375 kDa, have been deposited in the UniProtKB unreviewed TrEMBL database. These data suggest that the bacteriocins from *Lpb. plantarum* remains inadequately understood, posing challenges in confirming their molecular weight via SDS-PAGE.

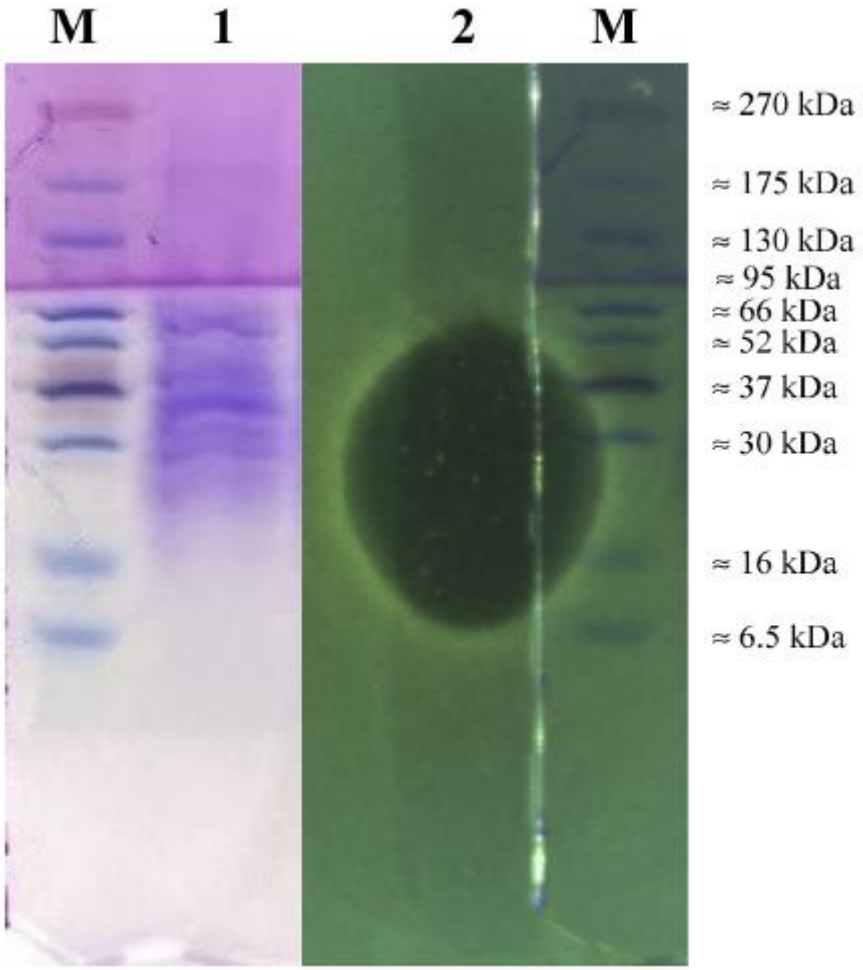


Figure 5.3 – Tricine/SDS-PAGE of *Lpb. plantarum* 9A3 bacteriocin: Lanes 1 and 4: Molecular mass marker (M); Lane 2: peptide bands in the stained gel with Coomassie Blue R250; Lane 3: growth inhibition zone, corresponding to the position of the peptide band in lane 2 (the gel was covered with viable cells of *L. monocytogenes* CECT 911 (10^6 CFU/mL), embedded in BHI soft agar and incubated at 37 ◦C for 24 h).

5.4. Conclusions

Lactiplantibacillus plantarum 9A3, isolated from vegetables & mushrooms *alheira*, was chosen due to its high bacteriocin activity spectrum against not only several *L. monocytogenes* strains but also against *E. faecalis* ATCC 29212, *C. sporogenes* and *C. perfringens*. Both phenotypic and WGS analysis confirm *Lpb. plantarum* 9A3 as a safe culture for potential use in the food industry, as it lacks virulence and antibiotic resistance genes. Additionally, this bacterium produces safe and stable bacteriocin(s), suggesting its potential as a bio-preservative culture. Notably, it not only inhibits *L. monocytogenes* but also *Clostridium* spp. commonly present in fermented meat products. This capacity might

extend food shelf life and mitigate microbiological risk associated with *alheiras* and similar processed meats. With a two-year window approaching for companies to comply with new regulations requiring a reduction in the use of nitrites, exploring viable alternatives for this preservative, especially those effective against clostridial species, becomes crucial. The presence of genes encoding Plantaricin E, Plantaricin F, Pediocin and Leucocin A in *Lpb. plantarum* 9A3 prompts the need to establish conditions conducive to the production of one or more bacteriocins. Understanding these conditions is crucial to enhance our understanding of about its applicability. This strain possesses promising characteristics that warrant further investigation to assess its effect on the flavour and quality of fermented sausages. This exploration could pave the way for its use as a protective culture in the food industry.

PART IV

CONCLUSIONS AND FINAL REMARKS

CHAPTER 6

Conclusions

Due to the increase in demand for vegetarian, vegan, and flexitarian diets over the past years, consumers' search for products with high functional and nutritional values increased substantially. Looking for products not derived from animals (i.e., vegetarian and/or vegan) has increased globally. Additionally, the current abundance of products based on beans, seeds, and nuts is mainly due to consumers' demands for plant-based alternatives not only for dietary reasons, lifestyle, or health concerns but also due to the concerns associated with environmental sustainability. This dynamic process and change in consumer preferences have made the overall population more health-conscious and have demanded beneficial health-targeted value of food and its sustainability in the food chain. As a result, the food industry is compelled to advance functional foods, providing innovative foods that possess higher quality while adding value based on their functional properties. Despite the known advantages, the biggest hurdle associated with these innovative products lies in the fact that meat alternatives are currently much more expensive when compared to their meat counterparts, representing an economic challenge to vegetarian and vegan consumers. Despite that, meat analogues present a more sustainable method of production when compared to traditional meat production systems requiring smaller natural source volumes. Several studies have been done incorporating a broad range of ingredients to improve the physicochemical, nutritive, textural, and sensory properties of these meatless products.

This study demonstrated that even though pH and water activity levels are insufficient to assure microbiological safety, nitrites, nitrates, biogenic amines, and organic acids were within accepted limits for this kind of product. No biological threat was detected in innovative *alheiras*, but some of the traditional products analysed have shown a concerning microbiological state in terms of food safety. Even though no *Staph. aureus*, *Salmonella* spp. nor sulphite-reducing *Clostridium* spores were detected, *E. coli* and *L. monocytogenes* were found in traditional *alheiras* available in the market. Therefore, unlike traditional *alheiras*, which often contain pathogenic agents, no harmful organisms nor chemical hazards were found in these new products, even though they were produced by the same companies.

Next-generation sequencing demonstrated to be an effective tool for categorizing bacterial and fungal communities in food samples where cultural methods are insufficient to accurately determine the diversity of the whole microbiota. Nevertheless, all dominant bacteria found using classical methods were also dominant by non-culturable analysis. This study allowed to understand this fermented products microbiota better, and it was helpful to select potential starter strains or their combinations for the food industry.

During the challenge test, from all three matrices tested, vegetarian *alheira* has proven to own characteristics that conditionate the viability of foodborne pathogens. All tested pathogens were found to have counts below the detection limit of the technique at the end of shelf-life in this matrix. Moreover, *S.* Enteritidis and *Staph. aureus* were more affected since counts suffered a decrease after 21 days. We believe low temperature and low pH greatly influence bacterial decrease along *alheiras* shelf-life.

Due to its high bacteriocin activity spectrum against not several *L. monocytogenes* strains, *E. faecalis* ATCC 29212, *C. sporogenes* and *C. perfringens*, *Lpb. plantarum* 9A3, isolated from vegetables and mushrooms *alheira*, was chosen. Both phenotypic and WGS analysis confirm *Lpb. plantarum* 9A3 as a safe culture for potential use in the food industry, as it lacks virulence and antibiotic resistance genes. Additionally, this bacterium produces safe and stable bacteriocin(s), suggesting its potential as a bio-preservative culture. Notably, it not only inhibits *L. monocytogenes* but also *Clostridium* spp. commonly present in fermented meat products. This capacity might extend food shelf life and mitigate microbiological risks associated with *alheiras* and similar processed meats. With a two-year window approaching for companies to comply with new regulations requiring a reduction in the use of nitrites, exploring viable alternatives for this preservative, especially those effective against clostridial species, becomes crucial. The presence of genes encoding Plantaricin E, Plantaricin F, Pediocin and Leucocin A in *Lpb. plantarum* 9A3 prompts the need to establish conditions conducive to producing one or more bacteriocins. Understanding these conditions is crucial to enhancing our understanding of their applicability. This strain possesses promising characteristics that warrant further investigation to assess its effect on the flavour and quality of fermented sausages. This exploration could pave the way for its use as a protective culture in the food industry.

CHAPTER 7

Future research and development

Initially, this research aimed to characterize innovative *alheiras* regarding their microbial and chemical hazards and develop biocontrol solutions to mitigate any identified risks. After extensive analysis, no pathogenic microorganisms or chemical hazards were detected in these new products, despite being produced by the same companies as traditional *alheiras* known to have these challenges. Nevertheless, some questions remain unanswered, which leads to some suggestions for future work:

- To investigate the reasons behind the higher contamination levels in traditional *alheiras* compared to innovative *alheiras*. Despite the differences in ingredients and the possibility that the meat used in traditional *alheiras* may be more contaminated, both products undergo similar heat treatments and are produced in the same facilities.
- Conduct further studies using both culture-based techniques and next-generation sequencing to gain a deeper understanding of the microbiota present in both traditional and innovative *alheiras* and confirm that, in fact, these innovative products do not have pathogen contaminants.
- Develop further experiments to clarify why foodborne pathogens behave differently in traditional and innovative *alheiras*.
- Conduct further characterization of *Lpb. plantarum* 9A3 regarding its potential use as a protective culture, including: identification of optimal growth conditions that contribute to the highest production of one or more bacteriocins; assessment of strain stability during storage under various conditions (e.g., dried or frozen states); characterization of the bacteriocin(s) produced, including their potential toxicity.

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ANNEX

Table 1.1 – Origin and ingredients of available worldwide fermented non-meat sausages

Origin	Brand	Product	Ingredients	Reference
Australia	Made with Plants	Vegan Bangers And Mash	Vegan mash (45%) (potato, soy milk powder (soybean), salt, white pepper), plant-based sausages (35%) (plant-based mince (33%) (water, vegetable protein (soy), vegetable oil, cocoa butter, thickeners (461, 412), vegetable protein powder, vegetable protein extract (soy), smoke flavour, yeast extract, beetroot powder, salt, natural colour (150a)), garlic, parsley, black pepper), gravy (15%) (water, gravy powder (thickener (1442), maltodextrin, salt, natural flavours, sugar, vegetable powder, tomato powder, vegetable gums (415, 412), natural colours (150a, 160c), herbs)), peas (5%)	https://madewithplants.com.au/
	Quorn	Quorn Sausage Rolls	Wheat flour (calcium carbonate, iron, niacin, thiamin), mycoprotein (23%), water, vegetable oil, seasoning {textured wheat protein [wheat gluten, firming agent (516), preservative (223)], salt, free range egg white powder, whey powder (from milk), wheat flour (calcium carbonate, iron, niacin, thiamin), yeast extract, dextrose, thickener (pectins), dried onion, herbs, sunflower oil, barley malt extract, yeast, acidity regulator (503), white pepper extract, herb extract, colour (172)}, onion, canola oil	https://www.quorn.com.au/
	V2 Food	V2 Plant Based Sausages	Water, soy protein, vegetable oils, thickeners (methylcellulose, modified cornstarch, carrageenan), flavours (contains glutamic acid), colours (caramelised sugar, beetroot powder), onion, dehydrated garlic, salt, yeast extract, herbs (parsley, thyme), minerals (zinc, iron), vitamins (niacin (B3), pyridoxine (B6), cobalamin (B12)), antioxidants (tocopherol, ascorbic acid)	https://www.v2food.com/
Belgium	The Vegan Butcher's Choice	Vegan Garlic Salami Vegan Italian Sausage	Vital wheat gluten, water, sunflower oil, organic nutritional yeast, miso paste, brown sugar, apple cider vinegar, spices, salt Vital wheat gluten, water, extra virgin olive oil, onion, garlic, silken tofu, tomato paste, white miso paste, nutritional yeast, spices, liquid smoke, sugar, salt	https://shop.veganbutcher.be/

Origin	Brand	Product	Ingredients	Reference
Brazil	SuperBom	Pea-based Smoked Sausage	Water, sodium caseinate, powdered egg white, isolated pea protein, pea starch, sunflower oil, pea fiber, casein, salt, hyposodium salt, yeast extract, sugar, calcium chloride firming agent, stabilizer -zantes (carrageenan, xanthan gum and locust bean gum), thickeners (sodium alginate and methylcellulose), maltodextrin, hydrolyzed corn protein, spices, aromatic herbs, aroma similar to natural sausage, radish, carrot and bell pepper, natural smoke aroma, iron and zinc minerals, vitamins A, B9 and B12	https://superbom.com.br
		Pea Sausage	Water, sodium caseinate, powdered egg white, isolated pea protein, pea starch, sunflower oil, pea fiber, salt, hyposodium salt, yeast extract, sugar, carrageenan and xanthan gum stabilizers, maltodextrin, hydrolyzed protein corn, spices, aromatic herbs, aroma identical to natural sausage, radish, carrot and pepper concentrates, iron and zinc minerals, vitamins A, B9 and B12	
		Traditional Pea Sausage	Water, sodium caseinate, powdered egg white, isolated pea protein, pea starch, sunflower oil, pea fiber, casein, salt, hyposodium salt, yeast extract, sugar, calcium chloride firming agent, stabilizer -zantes (carrageenan, xanthan gum and locust bean gum), thickeners (sodium alginate and methylcellulose), maltodextrin, hydrolyzed corn protein, spices, aromatic herbs, aroma similar to natural sausage, radish, carrot and bell pepper, natural smoke aroma, iron and zinc minerals, vitamins A, B9 and B12	
Canada	Sol Cuisine	Veggie Breakfast Sausages	Filtered water, soy protein, expeller pressed sunflower oil, yeast extract, cellulose gum, evaporated cane syrup, signature superfoods blend (fava bean powder, maca powder*, lucuma powder*, mesquite powder*), spices, sea salt, onion, vitamin blend (potassium chloride, maltodextrin, magnesium oxide, ferric orthophosphate, niacinamide, zinc oxide, cyanocobalamin, D-calcium pantothenate, copper gluconate, pyridoxine hydrochloride, thiamine mononitrate, riboflavin) (*organic)	https://solcuisine.com/
	Yves Veggie Cuisine®	Jumbo Veggie Dogs	Water, soy protein isolate, vital wheat gluten, tofu (water, soybeans, magnesium chloride). Contains 2% or less of: spices, natural flavour, vitamin & mineral blend (dipotassium phosphate, dimagnesium phosphate, ferric orthophosphate, zinc oxide, niacinamide, vitamin B12, calcium pantothenate, thiamine hydrochloride, pyridoxine hydrochloride, riboflavin, iron oxide), extractives of spices, wheat starch, yeast extract, red beet powder (colour), carrageenan	http://yvesveggie.com/

Origin	Brand	Product	Ingredients	Reference
Canada (cont.)	Yves Veggie Cuisine® (Cont.)	The Good Dog	Water, soy protein isolate, vital wheat gluten, tofu (water, soybeans, magnesium chloride). Contains 2% or less of: cane sugar, wheat starch, yeast extract, salt, ground mustard seed, carrageenan, natural flavour, tomato lycopene extract (colour), rice flour, ferric orthophosphate (iron), vitamin B12, extractives of spices	http://yvesveggie.com/
		Veggie Dogs	Water, soy protein isolate, vital wheat gluten. Contains 2% or less of: spices, natural flavour, dehydrated onion, vitamin and mineral blend (dipotassium phosphate, dimagnesium phosphate, ferric orthophosphate, zinc oxide, niacinamide, vitamin B12, calcium pantothenate, thiamine hydrochloride, pyridoxine hydrochloride, riboflavin, iron oxide), extractives of spices, beet powder and paprika (colour), wheat starch, maltodextrin, yeast extract, carrageenan, sugar, salt	
		Veggie Tofu Dogs	Water, soy protein isolate, vital wheat gluten, tofu (water, soybeans, magnesium chloride). Contains 2% or less of: wheat starch, yeast extract, salt, sugar, canola oil, ground mustard seed, carrageenan, seasoning (maltodextrin, salt, spice, dehydrated onion), natural flavour, vitamin and mineral blend (dimagnesium phosphate, ferric orthophosphate, zinc oxide, niacinamide, vitamin B12, calcium pantothenate, thiamine hydrochloride, pyridoxine hydrochloride, riboflavin, iron oxide), paprika, extractives of spices, beet powder (colour), salt blend (sea salt, potassium chloride, magnesium chlorides, magnesium sulfates)	
	Zoglos	Incredible HotDogs	Water, wheat gluten, isolated soy protein, soybean or, salt, methylcellulose, carrageenan, spices, onion, vitamin mix (potassium phosphate, magnesium oxide, niacinamide, ferric phosphate, zinc oxide, calcium pantothenate, thiamine mononitrate, pyridoxine hydrochloride, riboflavin, copper sulphate, folic acid, cyanocobalamin), garlic, paprika, flavours, locust bean gum, yeast extract, maltodextrin, xanthan gum, potassium chloride, smoke flavour, iron oxide, flavours, calcium phosphate, citric acid, disodium guanylate and inosinate	https://zoglos.com/

Origin	Brand	Product	Ingredients	Reference
Canada (cont.)	Zoglos (cont.)	Meatless Franks	Water, wheat gluten, isolated soy protein, soybean or, salt, methylcellulose, carrageenan, spices, onion, vitamin mix (potassium phosphate, magnesium oxide, niacinamide, ferric phosphate, zinc oxide, calcium pantothenate, thiamine mononitrate, pyridoxine hydrochloride, riboflavin, copper sulphate, folic acid, cyanocobalamin), garlic, paprika, flavours, locust bean gum, yeast extract, maltodextrin, xanthan gum, potassium chloride, smoke flavour, iron oxide, flavours, calcium phosphate, citric acid, disodium guanylate and inosinate	https://zoglos.com/
Germany	Gut Bio	Tofu Sausages	Potable water, wheat protein * (27%), tofu (soy bean *, potable water, solidifying agents: magnesium chloride (E-511) and calcium sulfate (E-516) (2%), vegetable no. hydrogenated *, onion *, sea salt, condiment *, raw sugar *, yeast *, thickening agent: carob seed flour * (E-410), spices *, garlic *, celery * (* Ingredients from ecological agriculture)	https://www.aldi.pt
	Taifun	Grill Cracker	Tofu * 80% (soybeans * 55%, water, coagulant: magnesium chloride, calcium sulfate), cold-pressed sunflower oil *, oats *, vegetable broth * (sea salt, yeast extract *, sunflower oil *, leek *, carrots *, celery *, mace *, nutmeg *, parsley *), white pepper *, coriander *, black pepper *, garlic *, ginger *, nutmeg *, oregano *, parsley *, mace * (* from controlled organic cultivation)	https://www.taifun-tofu.de/
		Grilled Roast	Tofu * 62% (soybeans * 55%, water, coagulant: magnesium chloride, calcium sulfate), brown rice *, wheat protein *, cold-pressed sunflower oil *, vegetable broth * (sea salt, yeast extract *, sunflower oil *, leek *, carrots *, celery *, mace *, nutmeg *, parsley *), onions *, marjoram *, caraway *, black pepper *, mace *, nutmeg *, ginger *, turmeric * (* from controlled organic cultivation)	
	Vantastic Foods	Sim Sala Mi Vegan smoked salami in one piece	Seitan (76%) (water, wheat protein), yeast extract, flavour, onions, soy sauce (water, soybeans , sea salt), spices (contains mustard), salt, thickener: agar agar, locust bean gum, wheat starch , colouring: iron oxides	https://www.vantastic-foods.com/

Origin	Brand	Product	Ingredients	Reference
Germany (cont.)	Vantastic Foods	Vegan Bockwurst sausages	Seitan (water, wheat protein) 80%, rapeseed oil, yeast extract, salt, onions, flavouring, thickener: locust bean gum, agar-agar, xanthan; spices, wheat starch, colouring: iron oxide; beechwood smoke	https://www.vantastic-foods.com/
		Vegan Frying Sausages	Seitan* (water, wheat protein*) 80%, sunflower oil*, onion*, yeast extract*, salt, wheat starch*, spices*, thickener: locust bean gum*. (* = certified organic)	
	Veggyness	Garlic Sujuk	Seitan* (water, wheat protein*) (70%), textured wheat protein* (wheat protein*, wheat flour*), high oleic sunflower oil*, yeast extract*, paprika*, garlic* (2%), spices*, onion*, rock salt, thickening agent: guar gum and locust bean gum, acidifying agent: vegan lactic acid, paprika extract*. *organic ingredient	https://www.veggyness.com/
		Spacebar Chorizo vegan	Seitan* (67%): (water, wheat protein*), red pepper*, coconut fat*, onion*, yeast extract*, rock salt, spices* (contains celery* and mustard*) thickening agent: locust bean gum*, beech wood smoke. (* certified organic)	
		Spacebar Hem	Seitan* (86%): (water, wheat protein*), coconut oil*, hemp seed* (2.00%), rock salt, yeast extract*, spices*, onion*, thickening agent: locust bean gum* and guar gum*, beech wood smoke. (* certified organic)	
		Vegan Merguez	Seitan* (72.00%): (water, wheat protein*), onions*, high oleic (high oleic) sunflower oil*, spices*, red peppers*, yeast extract*, rock salt, thickening agent: agar-agar*, guar gum* and locust bean gum*, smoke**. (*certified organic, **natural beech wood smoke)	
		Vegane Bockwurst	Seitan (water, wheat protein) (80%), rapeseed oil, yeast extract, salt, onions, flavour, thickeners: locust bean gum, agar-agar and xanthan; spices, wheat starch, colouring agent: iron oxide; beechwood smoke	
		Vegane Bratwurst	Seitan (water, wheat protein) (72%), rapeseed oil, onions, yeast extract, rock salt, spices, flavourings, thickeners: agar-agar, locust bean gum	

Origin	Brand	Product	Ingredients	Reference
Germany (cont.)	Veggyness (cont.)	Vegane Bratwurst Chorizo	Seitan* (64.00%): (water, wheat protein*), red peppers*, coconut fat*, onion*, yeast extract, spices* (contains celery and mustard), rock salt, thickening agent: locust bean gum*, smoke**. (*certified organic, **natural beech wood smoke)	https://www.veggyness.com/
		Vegane KaLeWu	Seitan (water, wheat protein) (80%), rapeseed oil, yeast extract, salt, onions, flavour, thickeners: locust bean gum, agar-agar and xanthan; spices, wheat starch, colouring agent: iron oxide; beechwood smoke	
		Vegane Sausage Rostbratwurst	Seitan* (81.00%): (water, wheat protein*), high oleic (high oleic) sunflower oil*, yeast extract*, rock salt, wheat starch*, onion*, spices*, thickening agent: carob seed flour (* certified organic)	
		Vegane Winzi-Weenies	Seitan* (82%): (water, wheat protein*), high oleic (high oleic) sunflower oil*, yeast extract*, stone salt, spices* (contains mustard*), onions* thickening agent: locust bean gum*, paprika extract*, smoke** (* certified organic, ** natural beech wood smoke)	
	Viana	Original Currywurst - Tofu Queen	Sauce (60%): (tomato paste *, drinking water, raw cane sugar *, sunflower oil *, apple cider vinegar *, sea salt, curry *, thickener: locust bean gum *, yeast *), vegetarian sausage (40%): tofu (soy beans *, drinking water , Coagulant magnesium chloride), drinking water, wheat protein *, sunflower oil *, sea salt, spices *, raw cane sugar *, thickener: locust bean gum *, celery * (* controlled organic cultivation)	http://www.viana.de/
	Vitaquell	Tofu-Dings Feingewürzt	Tofu 41% (water, soybeans , coagulant nigari (magnesium chloride), calcium sulfate), carrot juice, egg white , rapeseed oil, onions, herbs, potato starch, sea salt, yeast extract, natural flavour, brandy vinegar, spices (contain mustard seeds)	https://www.vitaquell.de/
		Tofu-Wurst Gartenkräute	Tofu 42% (water, soybeans , coagulant nigari (magnesium chloride), calcium sulfate), egg white , rapeseed oil, onions, oil seed mixture (linseed, sesame , pumpkin seeds, sunflower seeds, almonds , mustard seeds), herbs, potato starch, sea salt, cane sugar, yeast extract	

Origin	Brand	Product	Ingredients	Reference
Germany (cont.)	Vitaquell (cont.)	Vegetarische Brat-Dings	Tofu (water, soybeans , coagulant nigari (magnesium chloride), calcium sulfate), rapeseed oil, water, egg white , herbs, spices, sea salt, yeast extract, mustard seed , brandy vinegar, raw cane sugar, natural flavour, smoke flavour	https://www.vitaquell.de/
		Vegetarische Curry-Brat-Ding	Water, egg white, rapeseed oil, sweet lupine flour, corn starch, onions, potato starch, herbs, spices, barley malt , mustard seeds , curry (0.4%), brandy vinegar, sunflower oil, carrots, parsnips, rice flour, yeast extract, sea salt, natural flavour, freshly developed smoke made of natural woods	
		Vegetarische Vier Weiße	Tofu 32% (water, soybeans , nigari coagulant (magnesium chloride, calcium sulfate), rapeseed oil, water, palm oil, egg white , onions, stabilizer gum arabic, sea salt, yeast extract, potato starch, spices, herbs, smoke flavour, cane sugar, lemon oil.	
	Wheaty	Bavarian Style	Seitan* (water, wheat protein*) 82%, high oleic sunflower oil*, rock salt, yeast extract*, onion*, spices*, thickening agent guar gum*, parsley*, lemon* (* certified organic)	https://www.wheaty.com/
		Chorizo	Seitan* (water, wheat protein*) 78%, coconut fat*, onion*, yeast extract*, soy sauce* (water, soybeans*, sea salt), red bell pepper*, garlic*, rock salt, spices*, paprika extract*, acidifying agent: vegan lactic acid. (* certified organic)	
		Country Style	Seitan* (water, wheat protein*) 86%, high oleic sunflower oil*, coconut fat*, rock salt, spices* (contains celery* and mustard*), yeast extract*, thickening agent locust bean gum* and guar gum*, onion*, red bell pepper*, paprika extract*, natural beech wood smoke (* certified organic)	
		Vegan Merguez	Seitan* (water, wheat protein*) 72%, onion*, high oleic sunflower oil*, spices*, red bell pepper*, yeast extract*, rock salt, thickening agent: agar-agar*, guar gum* and locust bean gum*, natural beech wood smoke (* certified organic)	

Origin	Brand	Product	Ingredients	Reference
Germany (cont.)	Wheaty (cont.)	Hot Dog / Tiny Hot Dog	Seitan* (water, wheat protein*) 82%, high oleic sunflower oil*, yeast extract*, rock salt, spices* (contains mustard*), onion*, thickening agent locust bean gum*, paprika extract*, natural beech wood smoke (* certified organic)	https://www.wheaty.com/
		Herbalicious	Seitan* (water, wheat protein*) 81%, high oleic sunflower oil*, yeast extract*, rock salt, wheat starch*, onion*, spices*, thickening agent locust bean gum* (* certified organic)	
		Mini-Herby	Seitan* (water, wheat protein*) 81%, high oleic sunflower oil*, yeast extract*, rock salt, wheat starch*, onion*, spices*, thickening agent locust bean gum* (* certified organic)	
		Red Hot Chili Peppers	Seitan* (water, wheat protein*) 82%, coconut fat*, spices* (contains celery* and mustard*), yeast extract*, rock salt, onion*, thickening agent locust bean gum*, chili* (0,3%), paprika extract*, natural beech wood smoke (* certified organic)	
		Red Sausages	Seitan* (water, wheat protein*) 73%, onion*, high oleic sunflower oil*, yeast extract*, rock salt, paprika*, spices*, thickening agent: agar-agar*, guar gum* and locust bean gum*, natural beech wood smoke (* certified organic)	
		Vegan BBQ Mix	Seitan* (water, wheat protein*) 74%, coconut fat*, red bell pepper*, high oleic sunflower oil*, rock salt, yeast extract*, spices* (contains celery* and mustard*), onion*, oat fiber*, wheat starch*, potato starch*, thickening agent xanthan* and locust bean gum, natural beech wood smoke. (*certified organic)	
		Vegan farmer crackers	Seitan * (water, wheat protein *) 86%, high oleic sunflower oil *, coconut fat *, rock salt, spices * (contains celery * and mustard *), yeast extract *, thickener: locust bean gum * and guar gum *, onions *, Red pepper *, pepper extract *, beech wood smoke (* from controlled organic cultivation)	
		Vegan Sucuk	Seitan* (water, wheat protein*) (70%), textured wheat protein* (wheat protein*, wheat flour*), high oleic sunflower oil*, yeast extract*, paprika*, garlic* (2%), spices*, onion*, rock salt, thickening agent:(guar gum* and locust bean gum*), acidifying agent: vegan lactic acid, paprika extract* (* certified organic)	

Origin	Brand	Product	Ingredients	Reference
Ireland	Denny	Meat Free Sausages	Water rehydrated textured **soya** and **wheat** protein(16%), **soya** protein, **wheat** pro tein, salt, **soya** bean oil, natural flavouring), rusk (**wheat**), coconut oil, **soya** protein concentrate (4%), **wheat** starch, chicory root fiber, natural flavourings, stabilizer: methyl cellulose; salt, yeast extract, sodium alginate, colouring foods: beetroot, safflower; spice extracts: black pepper, ginger, nutmeg; herbs, spices, flavouring	https://www.denny.ie/
	Eden	Eden Vegan Sausages	Water, soya, sunflower oil, potato starch, maize semolina, rice semolina, cellulose, tomato puree, spices, salt, rusk [wheat flour (wheat flour, calcium carbonate, iron, niacin, thiamin), salt], maltodextrin, tapioca starch, stabilisers: sodium triphosphate, sodium diphosphate, yeast extract, barley malt extract, spice extracts, rape oil, preservative: sodium sulphite, beetroot juice powder, natural flavourings, antioxidant: sodium ascorbate, thickener: guar gum, smoke flavouring	https://store.edenfoods.com/
	Glas	Perfectly Plant-based Sausages	Water, soya flour, potato starch, textured soya, sunflower oil, maize semolina, rice semolina, soya protein, spices, wheat gluten, salt, cellulose, tomatoes, tapioca starch, yeast extract, maltodextrin, wheat flour, soybean oil, rapeseed oil, barley malt extract, spice extract, natural flavours, beetroot juice powder, thickener: guar gum, smoke flavouring, antioxidant: sodium ascorbate, calcium carbonate, iron, niacin, thiamin, filled into sodium alginate casing	https://glasfoods.ie/
Israel	Tivall Food Industries Ltd.	Cocktail Sausage	Rehydrated wheat and soya proteins (46%), water, vegetable oil (sunflower, rapeseed in varying proportions), egg white powder, potato starch, dextrose, hydrolyzed vegetable protein (soya, maize), garlic powder, salt, yeast extract, stabilisers (guar gum, xanthan gum), flavouring, spices, maltodextrin, colour (iron oxide), vitamins & minerals (tricalcium phosphate, vitamin C, vitamin B3, zinc oxide, ferric diphosphate, vitamin E, vitamin B5, vitamin B6, vitamin B2, vitamin B1, folic acid, vitamin B12)	http://www.trialiafoods.com.au/

Origin	Brand	Product	Ingredients	Reference
Israel (cont.)	Tivall Food Industries Ltd. (cont.)	Smoked Frankfurters	Rehydrated wheat and soya proteins (46%), water, vegetable oil (sunflower, rapeseed in varying proportions), egg white powder, potato starch, dextrose, hydrolysed vegetable protein (soya, maize), garlic powder, salt, yeast extract, stabilizers (guar gum, xanthan gum), flavouring, spices, maltodextrin, colour (iron oxide), vitamins & minerals (tricalcium phosphate, vitamin C, vitamin B3, zinc oxide, ferric diphosphate, vitamin E, vitamin B5, vitamin B6, vitamin B2, vitamin B1, folic acid, vitamin B12)	http://www.trialiafoods.com.au/
Italy	Veghiamo – Deli Slices	Black Pepper	Water, gluten from wheat* 32%, durum wheat flour* 8%, chickpea flour*, extra virgin olive oil*, red beetroot powder*, natural flavouring, tamari sauce* (water, soybean seeds*, salt, shochu* (water, rice*, salt), toasted soybean seeds*), black pepper*1.5%, soft wheat flour*, sourdough, coconut oil*, salt, mixture of spices and aromatic herbs* (*organic ingredients)	https://www.felsineoveg.com/
		Carpaccio	Water, gluten from wheat *34%, durum wheat flour * 8%, sunflower oil *, natural flavourings, colouring: betanin, citric acid, salt, soft wheat flour *, pea protein, sourdough, white pepper powder*, garlic powder* (*organic ingredients)	
		Classic	Water, gluten from wheat* 31%, durum wheat flour* 8%, chickpea flour*, extra virgin olive oil*, natural flavourings, red beetroot powder*, soft wheat flour*, salt, sourdough, sunflower oil*, pepper powder*, garlic powder* (*organic ingredients)	
		Cured Ham Flavoured	Water, gluten from wheat 31%, natural flavourings, durum wheat flour 7%, sunflower oil, colouring: beetroot red, pea protein, salt, soft wheat flour, sourdough, white pepper powder, garlic powder	

Origin	Brand	Product	Ingredients	Reference
Italy (cont.)	Veghiamo – Deli Slices (cont.)	Lupins	Water, lupin flour* 27%, gluten from wheat* 23%, sunflower oil*, wheat starch*, natural flavouring, salt, apple vinegar*, tamari sauce* (water, soybean seeds*, salt, shochu* (water, rice*, salt), toasted soybean seeds*), coconut oil*, chickpea flour*, durum wheat flour*, dehydrated onion powder*, sourdough, mixture of spices and aromatic herbs*, acidity regulator: sodium bicarbonate (*organic ingredients)	https://www.felsineoveg.com/
		Pepperoni Flavoured	Water, gluten from wheat 35%, natural flavourings, durum wheat flour 7%, chickpea flour, extra virgin olive oil, colouring: beetroot red, salt, soft wheat flour, chili pepper powder, sourdough, sunflower oil, garlic powder	
		Salmon Flavoured	Water, gluten from wheat 31%, colouring concentrate from paprika, durum wheat flour 6%, natural flavourings, chickpea flour, extra virgin olive oil, soft wheat flour, salt, sourdough, sunflower oil, white pepper powder, garlic powder	
		Smoked Flavoured	Water, gluten from wheat* 34%, durum wheat flour* 8%, chickpea flour*, natural flavourings, extra virgin olive oil*, tamari sauce* (water, soybean seeds*, salt, shochu* (water, rice*, salt), toasted soybean seeds*), coconut oil*, soft wheat flour*, sourdough, salt, mixture of spices and aromatic herbs* (*organic ingredients)	
		Smoked Ham Flavoured	Water, gluten from wheat 30%, durum wheat flour 7%, chickpea flour, natural flavourings, extra virgin olive oil, colouring: beetroot red, flavour, smoke flavourings, soft wheat flour, salt, sourdough, sunflower oil, pepper powder, garlic powder	
		Spicy	Water, gluten from wheat* 32%, durum wheat flour* 8%, chickpea flour*, extra virgin olive oil*, natural flavouring, tamari sauce* (water, soybean seeds*, salt, shochu* (water, rice*, salt), toasted soybean seeds*), mixture of spices and aromatic herbs*, coconut oil*, soft wheat flour*, sourdough, salt (*organic ingredients)	

Origin	Brand	Product	Ingredients	Reference
Italy (cont.)	Veghiamo – Deli Slices (cont.)	Truffle	Water, gluten from wheat* 32%, durum wheat flour* 8%, chickpea flour*, extra virgin olive oil*, natural flavourings, truffle dices (*Tuber aestivum*)* 2%, tamari sauce* (water, soybean seeds*, salt, shochu* (water, rice*, salt), toasted soybean seeds*), coconut oil*, soft wheat flour*, sourdough, salt, mixture of spices and aromatic herbs* (*organic ingredients)	https://www.felsineoveg.com/
		Turmeric & Ginger	Water, gluten from wheat* 32%, durum wheat flour* 9%, chickpea flour*, sunflower oil*, natural flavouring, salt, ginger powder*0,4%, turmeric powder* 0,3%, chili pepper powder*, sourdough, pepper powder*, garlic powder* (*organic ingredients)	
Japan	Terra Foods	Vegan Marude Sausage	Soy protein, okara, devil's root powder, salt, beet sugar, yeast extract, spices, vegetable powder (onion, celery), maltodextrin / calcium hydroxide, trehalose	https://www.terrafoods.shop/
New Zeland	Tonzu	Organic Vegan Sausages – Curry Spice	Certified organic activated whole soybeans, organic sunflower oil, organic buckwheat, organic tapioca flour, curry spices, organic onion flakes, organic quinoa, organic rice flour, psyllium husks, sea salt, vegan yeast extract, tomato powder, cocoa, ground nutmeg, pure nigari	https://tonzu.co.nz/
		Organic Vegan Sausages – Garlic & Chilli	Certified organic activated whole soybeans, filtered water, organic sunflower oil, organic tapioca flour, organic garlic granules (2.8%), organic rice flour, New Zealand sea salt, tomato powder, yeast extract, mixed herbs, organic cocoa, chilli powder (0.2%), ground nutmeg, pure nigari	
		Organic Vegan Sausages – Italian Herb	Certified organic activated whole soybeans, filtered water, organic sunflower oil, organic tapioca flour, organic onion flakes, organic rice flour, New Zealand Sea salt, yeast extract, pure nigari, mixed herbs (0.4%), organic cocoa, organic garlic granules, ground nutmeg	

Origin	Brand	Product	Ingredients	Reference
New Zeland (cont.)	Tonzu (cont.)	Organic Vegan Sausages – Sage & Onion	Certified organic activated whole soybeans, filtered water, organic sunflower oil, organic tapioca flour, organic onion flakes (2.7%), organic rice flour, sage (0.9%), New Zealand Sea salt, yeast extract, tomato powder, organic cocoa, ground nutmeg, pure nigari	https://tonzu.co.nz/
Poland	Meatless Meat / Bezmiesnymiesny	Meatless Classic Kabanos	Water, vegetable proteins (**wheat** , pea), rapeseed oil, coconut oil, inactive yeast flakes, **soybeans** , soy **sauce** , Kłodawa salt, black pepper, natural spices, spice extracts, rice flour, dextrose, natural flavours, fiber (bamboo, pea, plantain), maltodextrin	https://www.bezmiesnymiesny.pl/
		Meatless Chilli Kabanos	Water, vegetable proteins (**wheat** , pea), rapeseed oil, coconut oil, inactive yeast flakes, **soybeans** , soy sauce, salt, natural spices (including chili flakes), spice extracts, rice flour, dextrose, natural flavours, fiber (bamboo, pea, plantain), maltodextrin	
		Meatless Country Sausages	Water, vegetable proteins (**wheat** , pea), rapeseed oil, tofu, inactive yeast flakes, **soy** flour , **soybeans** , Kłodawa salt, soy **sauce** , natural spices (including dried onion, dried garlic), smoke flavour, fibers (bamboo, pea, plantain)	
		Meatless Italian Sausages	Water, vegetable proteins (pea, **wheat**), rapeseed oil, inactive yeast flakes, **soy** flour , **soybeans** , Kłodawa salt, soy **sauce** , natural spices (including dried tomatoes, smoked paprika, oregano), smoke flavour, fibers (bamboo, pea, plantain), spice extracts	
		Meatless Onion Kabanos	Water, vegetable proteins (**wheat** , pea), rapeseed oil, coconut oil, inactive yeast flakes, **soya** , soy **sauce** , Kłodawa salt, natural spices (including dried garlic, dried onion), spice extracts, rice flour, dextrose, natural flavours, fibers (bamboo, pea, plantain), maltodextrin	
		Meatless Pepperoni	Water, **wheat** protein , tomato concentrate, rapeseed oil, inactive yeast flakes, **soybean** , potato starch, fiber (bamboo, pea, plantain), natural spices (including sweet pepper, hot pepper, smoked pepper), **mustard** , Kłodawa salt	

Origin	Brand	Product	Ingredients	Reference
Poland (cont.)	Meatless Meat / Bezmiesnymiesny (cont.)	Meatless sausages	Water, **wheat** protein , rapeseed oil, inactive yeast flakes, **soybean** , Kłodawa salt, natural spices, fibers (bamboo, pea, plantain), glucose, flavours (including smoke flavour), spice extracts, carboxymethylcellulose, starch (rice, potato)	https://www.bezmiesnymiesny.pl/
		Meatless Sausages Curry	Water, vegetable proteins (pea, **wheat**), rapeseed oil, apple juice, inactive yeast flakes, **soy** flour , **soy** , Kłodawa salt, soy **sauce** , glucose, sugar, natural spices (including turmeric, sweet pepper, coriander), fiber (bamboo, pea, plantain), spice extracts	
		Meatless Spanish Sausages	Water, vegetable proteins (pea, **wheat**), rapeseed oil, inactive yeast flakes, **soy** flour , **soybeans** , Kłodawa salt, soy sauce, natural spices (including cumin, chili pepper), smoke flavour, fibers (bamboo, pea) , plantain), spice extracts	
		Meatless White Sausages	water, vegetable proteins (**wheat** , pea), rapeseed oil, inactive yeast flakes, **soybean** , soy **sauce** , Kłodawa salt, black pepper, natural spices (including marjoram), modified corn starch, **soy** flour , glucose, aromas, sugar , fibers (bamboo, pea, plantain)	
	Polsoja	Breakfast Sausages	Water, soy protein, vegetal oil, wheat protein (gluten),methyl-cellulose (binder), corn starch (e1422), spice aromas and extracts, salt, yeast extract, vegetal proteins	http://www.polsoja.com.pl/
Portugal	Biodharma	*Salsichão*	Tofu (soybeans, nigari), cornmeal, arrowroot flour, beer yeast flakes, seaweed agar, olives, onion, garlic, sweet pepper, curry, mustard, extra virgin olive oil, tamari, water	https://biodharma.pt/
	Casa da Prisca	Soy *Alheira*	Soybean (70%), wheat bread (wheat flour type 65, water, salt, yeast), olive oil, salt, spices, garlic, starter crops, thickener (E407), stabilizer (E508; E516), water, water regulator acidity (E260; E270), dextrose, antioxidant (E330, E334)	https://www.casadaprisca.pt/
	Irmãos Oliveira	Vegetables and Mushrooms *Alheira*	Mushrooms 36% (Shiitake *), water, wheat bread * (wheat flour, water, natural yeast, salt) vegetables 16% (onion *, pepper *, tomato *, garlic *, parsley *), olive oil * salt and spices. Non-edible cellulose casing (* biological ingredients)	https://www.irmaosoliveiras.pt/

Origin	Brand	Product	Ingredients	Reference
Portugal (cont.)	Nobre	Vegalia Soy Sausages	Water, sunflower oil, soy protein (7%), egg albumin, starch, salt, flavourings, smoke aroma, yeast extract, gelling agents (guar gum, xanthan gum), spices	https://www.nobre.pt/
		Vegalia Tofu Sausages	Water, sunflower oil, tofu (7%) (water, soya, coagulants: nigari (water, magnesium chloride), calcium chloride), egg albumin, soya protein, potato starch, salt, flavourings, smoke flavour, yeast extract, gelling agents (guar gum, xanthan gum), spices, fine herbs (0.2%)	
	Próvida	Tofu *Alheira*	Tofu * (38%) (soy *, water, natural magnesium nigari-chloride], whole wheat bread * (whole and white wheat flour *, water, sea salt, natural yeast (wheat flour * and water)), seitan * (wheat flour *, wheat gluten *, water), pepper *, garlic powder *, soybean sauce * (water, soybean *, whole wheat, sea salt and koji-aspergillus oryzae), parsley *, coriander * , cumin *, fennel * and ginger * Non-edible casing (* organic production)	https://www.provida.pt/
	Topitéu	Wild *Alheira*	Mushrooms (45%), wheat bread (wheat flour, water, salt, yeast) (31%), cooking syrup (11%), green asparagus (6%), extra virgin olive oil (5%), spices (1%), salt (1%). Edible casing	https://topiteu.pt/
	Veg In	Vegan *Alheira*	Water, bread baked in a wood oven (wheat flour, water, yeast, salt), protein and vegetable fibers (wheat gluten, soy flour, isolated soy protein, wheat flour, rice bran), sunflower oil, olive oil, red pepper mass (red pepper, salt), spices, vinegar, onion, salt, garlic paste (garlic, salt) and natural smoke	http://www.vegin.pt/
Spain	Bio Veggie Sausages (Ahimsa)	Cheese	Water, sunflower oil*, wheat gluten*, oat flakes*, Gouda cheese*(5.8%), textured soya*, onion*, wheat flour*, egg albumin*, sea salt, spices*, brewer's yeast* (contains wheat*), vegetable stock* (contains celery*) (*Organically grown).	https://www.ecologicosahimsa.com/

Origin	Brand	Product	Ingredients	Reference
Spain (cont.)	Bio Veggie Sausages (Ahimsa) (cont.)	Chorizo Tofu and Seitan	Water, wheat gluten* (seitan) (22.3%), sunflower oil*, tofu* (11.07%) (soybeans*, water, food grade coagulant) calcium sulphate)), spices* (sweet paprika*, sea salt, cane sugar*, onion*, thyme*, garlic*, corn starch*, rosemary*), textured soya*, tomato*, brewer's yeast*(contains wheat*), spices*, cornstarch*, thickeners (xanthan gum and carrageen), hummus (common salt, natural hummus aromas), emulsifier (soy lecithin) (*Organically grown)	https://www.ecologicosahimsa.com/
		Fine herbs	Water, sunflower oil*, wheat gluten*, oat flakes*, textured soya*, onion*, tomato*, wheat flour*, egg albumin*, sea salt, brewer's yeast* (contains wheat*), spices*, parsley*(0.5%), sweet paprika* (*Organically grown)	
		German style	Water, wheat gluten*, Tofu* (water, soybeans*, food grade coagulant [calcium sulphate]), sunflower oil*, brewer's yeast* (contains wheat*), sea salt, corn starch*, spices*, soy sauce*(contains wheat*), sweet cayenne pepper*, thickener (xantana gum), garlic*, thickener (carrageen), apple vinegar*, hummus (common salt, natural hummus aromas), emulsifier (soy lecithin) (*Organically grown)	
		Smoked	Water, sunflower oil*, wheat gluten*, soya protein*, tomato*, yeast* (contains wheat*), soy sauce* (contains wheat*), hummus aroma (salt, hummus aroma) (0.5%), sea salt, thickener (carrageen), yeast extract* (contains wheat*), emulsifier (soy lecithin) (*Organically grown)	
		Tofu and Seitan	Wheat gluten*(seitan) (25.6%), sunflower oil*, tofu*(12.8%) (soybeans*, water, nigari), brewer's yeast*, spices*(contains celery*), sea salt, corn starch*, emulsifier /soya lecithin), thickener (xanthan gum), hummus (common salt, natural hummus aromas), thickener (carrageen) (*Organically grown)	

Origin	Brand	Product	Ingredients	Reference
Spain (cont.)	Biosurya	Salsicha vegetal de tofu y quinoa	Water, tofu * (23.6%) (water, soybeans *, stabilizer: calcium sulfate), wheat gluten *, cooked quinoa * (11.3%) (water, quinoa *, sea salt) , sunflower oil *, carrot *, toasted onion *, vegetable broth * (sea salt *, rice flour *, yeast extract *, onion *, garlic *, sunflower oil *), onion *, sea salt, starch corn *, garlic *, thickeners: xanthan gum and carrageenan, sweet paprika *, emulsifier: soy lecithin * * From organic farming	https://www.biosurya.com/
	Hijas del Sol	Alemana	Water, wheat gluten *, sunflower oil *, tofu * (water, soy beans *, coagulant [calcium sulfate]), beer yeast * (wheat *), carrots *, sea salt, soy sauce * (soy *, whole wheat (gluten) *, *Aspergillus oryzae*, water), onion *, corn starch *, spices * (bay leaf *, apple blossom *, garlic *, onion *, corn starch *, sweet pepper * pepper black *), thickener (xanthan gum), smoke (common salt, natural smoke flavours), garlic *, thickener (carrageenan), apple cider vinegar *, emulsifier (soy lecithin). * From organic agriculture	https://www.elcorteingles.es/
		Chipolata	Water, wheat gluten *, sunflower oil *, tofu * (water, soybeans *, coagulant [calcium sulfate]), beer yeast * (contains wheat *), sea salt, soy sauce * (soy beans) *, whole wheat *, *Aspergillus oryzae*, water), corn starch *, thickener (xanthan gum), parsley *, thickener (carrageenan), white pepper *, black pepper *, emulsifier (soy lecithin). Ecological cultivation	
		Seitan	Water, wheat gluten * (seitan *) (26%), sunflower oil *, tofu (water, soy *, stabilizer [calcium sulfate]), yeast * (contains wheat * [gluten]), sea salt, starch corn *, garlic *, thickener (xanthan gum), white pepper *, gelling agent (carrageenan), emulsifier (soy lecithin *). * from organic farming	
Sweden	Hänsans Kök	Plant-Based Chorizo	Water, wheat protein (10.1%), vegetable oil (rapeseed, sunflower), onion, stabilizer (methylcellulose, carrageenan, guar gum, xanthan gum), soy protein (3.5%), starch (wheat, potato), potato protein, yeast extract, dried red pepper, salt, spices (chili, dried garlic), garlic, vinegar, flavourings (lycopene), pea fiber, spice extract (jalapeno, paprika / pepper)	https://www.halsanskok.se/

Origin	Brand	Product	Ingredients	Reference
Sweden (cont.)	Hänsans Kök (cont.)	Plant-Based Sausage	Water, wheat protein (10.3%), soy protein (5.3%), spices (dried onion, dried garlic, black pepper, mustard, pepper, nutmeg / muscat), onion, stabilizers (methylcellulose, xanthan gum, guar gum, carrageenan), starch (wheat, potato), potato protein, vegetable oil (rapeseed, sunflower), salt, yeast extract, beetroot powder, rice flour, hydrolyzed soy protein, aromas, vinegar, smoked maltodextrin, lycopene, pea protein	https://www.halsanskok.se/
	Peas of Heaven	Chorizo	Water, rapeseed oil, pea protein, Stabilizer (carrageenan, methylcellulose), corn starch, spices, salt, paprika, chili, garlic, vegetable broth, citrus fibers, dextrose, aroma, yeast extract, acidity regulator (malic acid), sugar, vitamin B12, iron, riboflavin	https://peasofheaven.com/
		Hot Dog	Water, rapeseed oil, pea protein, Stabilizer (carrageenan, methylcellulose), corn starch, salt, spices, onion, paprika, vegetable broth, citrus fibers, dextrose, aroma, yeast extract, acidity regulator (malic acid), sugar, vitamin B12, iron, riboflavin	
		Perfect Bratwurst	Water, rapeseed oil, pea protein, pea flour, starch (pea, potato), salt, thickening agents (methylcellulose, processed eucheuma seaweed), spices, garlic, citrus fibers, caramel powder, acidity regulator (tartaric acid), yeast extract, grape sugar, aroma, stabilizer (E450)	
		Perfect Chorizo	Water, rapeseed oil, pea protein, pea flour, starches (pea, potato), salt, thickener (methylcellulose, processed *Eucheuma* seaweed), spices, chili, garlic, paprika, citrus fibers, acidity regulator (tartaric acid), yeast extract, dextrose, aroma, stabilizer (E450), Sugar	
		Prinskorv	Water, rapeseed oil, pea protein, Stabilizer (carrageenan, methylcellulose), corn starch, salt, spices, onion, paprika, vegetable broth, citrus fibers, dextrose, aroma, yeast extract, acidity regulator (malic acid), sugar, vitamin B12, iron, riboflavin	
Switzerland	Migros	V-Love Plant-Based Bratwurst	Water, rapeseed oil, potato protein (contains **sulfites**), pea protein, thickeners: carrageenan, methyl cellulose, processed *Eucheuma* algae, locust bean gum, konjac, spice extract, iodized table salt, seasoning, spices, starch, flaxseed flour, enzymes	https://www.migros.ch/

Origin	Brand	Product	Ingredients	Reference
Switzerland (cont.)	Migros (cont.)	V-Love Plant-Based Grilled Sausage	Water, rapeseed oil, pea protein, thickeners: carrageenan, methyl cellulose, processed *Eucheuma* algae, locust bean gum and konjac, potato protein (contains sulfites), table salt, spices, glucose, colouring: paprika extract, starch, yeast extract, seasoning, flavour, flaxseed flour, enzymes	https://www.migros.ch/
	Vegusto	No Moo, Vegi-sausage Wiener	Water, wheat protein (gluten), coconut oil, sunflower oil, spices (contains celery), rock salt, rice starch, yeast, potato starch, rice flour, binder (guar gum, xanthan), almond butter, Colourants (radish and carrot concentrate), binder (carrageen), flavouring (vegetable), natural smoke flavour, lactic acid (vegetable)	https://www.vegusto.ch/
		Vegi-sausage, Bianca	Water, wheat protein (gluten), coconut oil, sunflower oil, spices (contains mustard), breadcrumbs (wheat, table salt (not iodized), yeast), rock salt, rice starch, natural smoke flavour, lemon juice, binder (guar gum, xanthan), vegetable fibres	
		Vegi-sausage, Country-style	Water, wheat protein (gluten), sunflower oil, spices, yeast, rock salt, wheat flour, pear syrup, rice starch, natural smoke flavour, lactic acid (vegetable), paprika oil, binder (guar gum, xanthan)	
		Vegi-sausage, Curry	Water, wheat protein (gluten), sunflower oil, spices (contains celery), rock salt, breadcrumbs (wheat, table salt (not iodized), yeast), rice starch, curry 0.7%, carrot and apple concentrate	
		Vegi-sausage, Farmhouse-style	Water, wheat protein (gluten), sunflower oil, coconut oil, spices (contains celery), rock salt, breadcrumbs (wheat, table salt (not iodized), yeast), rice starch, Colourants (radish and carrot concentrate), antioxidant (ascorbic acid), binder (guar gum, xanthan), natural smoke flavour	
		Vegi-Sausage with No-Muh	Water, rice flour, pea protein, corn flour, coconut oil, sunflower oil, yeast, plant fibres, spices, potato starch, rock salt, flavouring (vegetable), rice starch, almond butter, fruit juice, binder (carrageen), natural smoke flavour, Colourants (radish and carrot concentrate, beta-carotene), antioxidant (ascorbic acid)	

Origin	Brand	Product	Ingredients	Reference
Switzerland (cont.)	Vegusto (cont.)	Vegi-sausage, Onion	Water, wheat protein (gluten), sunflower oil, spices (contains celery), rock salt, breadcrumbs (wheat, table salt (not iodized), yeast), rice starch, onions 1.6%	https://www.vegusto.ch/
		Vegi-sausage, Rosso	Water, wheat protein (gluten), coconut oil, sunflower oil, spices, breadcrumbs (wheat, table salt (not iodized), yeast), rice starch, dried tomatoes, rock salt, natural smoke flavour, Colourants (radish and carrot concentrate), stabilizer (ascorbic acid)	
The Netherlands	Schoutenfood	Frankfurter	Water, 14% vegetable proteins (**soy, wheat gluten**), rapeseed oil, onion, **free range egg white**, vinegar, natural flavouring, rice bran, yeast extract, dextrose, salt, **wheat flour**, smoked water, extracts (onion, garlic, spice), sugar, mineral (ferrous fumarate), vitamin B12, colour (E172)	https://www.schoutenfood.com/
		Smoked Sausage	Water, 14% vegetable proteins (**soy, wheat gluten**), rapeseed oil, onion, **free range egg white**, vinegar, natural flavouring, rice bran, yeast extract, dextrose, salt, **wheat flour**, smoked water, extracts (onion, garlic, spice), sugar, mineral (ferrous fumarate), vitamin B12, colour (E172)	
	Unox	Vegetarian Smoked Sausage	Water, vegetable oils 15.9% (sunflower, coconut), thickeners (guar gum, sodium alginate, xanthan gum, cellulose, carrageenan, methylcellulose), protein powder 4.1%, pea fiber, pea protein 1.5%, aroma, spices, salt , hydrolyzed corn protein, food acid: citric acid, yeast extract, mushroom extract. aroma, dextrose, glucose syrup, smoke flavour, sugar, caramel powder, antioxidants (ascorbic acid, alpha-tocopherol), colours (iron oxide, anthocyanin), stabilizer: E450	https://www.unox.nl/
United Kingdom	BonSan	Bonsan Organic Kofu Grill Sausages	Tofu*80% (water, soya beans*, coagulant: kombucha*[black tea* with sugar* fermented into vinegar] and nigari [magnesium chloride]), sunflower oil*, sea salt, tamari* (soya sauce)*, curry spices* 1.3% (contains celery*, mustard*, garlic*), sultanas*, yeast flakes*, thickening agent: locust bean gum*, corn starch*. *certified organic ingredients	https://www.bonsan.co.uk/

Origin	Brand	Product	Ingredients	Reference
United Kingdom (cont.)	Cauldron Foods	Vegetarian Cumberland Sausages	Rehydrated textured vegetable protein (45%) (water, soya protein, potato starch, wheat gluten, stabilizer: dicalcium phosphate), onion, rapeseed oil, cumberland seasoning (5%) (yeast extract, salt, black pepper, sugar, rosemary, barley malt extract, bay, carrot, leek, rapeseed oil), dried free range egg white, soya protein, stabilizer: methylcellulose	https://www.cauldronfoods.co.uk/
		Vegetarian Lincolnshire Sausages	Rehydrated textured vegetable protein (45%) (water, soya protein, potato starch, wheat gluten, stabilizer: dicalcium phosphate), water, onion, rapeseed oil, lincolnshire seasoning (5%) (yeast extract, salt, potassium chloride, herbs, fructose, white pepper, rusk (wheat flour, salt, raising agent: ammonium bicarbonate) barley malt extract, carrot powder, leek powder, sage extract, nutmeg extract), dried free range egg white, soya protein, stabilizer: methylcellulose	
	GoodLife	Good Life Mighty Non-Meaty Sausages	Water, mushrooms (15%), red onions (13%), lentils (12%), soy protein, spinach (4.5%), fried onions (onions, sunflower oil), fortified wheat flour (wheat flour, calcium carbonate, iron, niacin, thiamin), rusk (fortified wheat flour (wheat flour, calcium carbonate, iron, niacin, thiamin), water, salt, raising agent (ammonium carbonates)), potato flakes, gram flour, pea protein, yeast extract, rapeseed oil, garlic, stabiliser (methyl cellulose), salt, yeast, sage, coriander, nutmeg, thyme, black pepper, acidity regulator (citric acid), sausages coated in sodium alginate casing	https://www.goodlife.co.uk/
	Heck Foods	Heck Meat Free Chipolatas	Water, pea flour, meat-free base (thickener (methyl cellulose), pea fibre, modified starch, flavour, beetroot powder (maltodextrin, beetroot juice concentrate, acidity regulator (citric acid)), tea extract, spice extracts, smoke flavour), textured vegetable protein (pea protein isolate, pea flour), pea protein (2%), seasoning (salt, spice, herb, yeast extract, preservative (sodium sulphite), antioxidant (ascorbic acid), spice extract), citrus fibre, sunflower oil. Filled into calcium alginate casing	https://www.heckfood.co.uk/

Origin	Brand	Product	Ingredients	Reference
United Kingdom (cont.)	Heck Foods (cont.)	Vegan Italia Chipolatas	Water, pea flour, meat-free base (thickener (methyl cellulose), pea fibre, modified starch, flavour, beetroot powder (maltodextrin, beetroot juice concentrate, acidity regulator (citric acid)), tea extract, spice extracts), pea protein (4%), oven dried tomato (3%), basil (3%), seasoning (rice flour, salt, sugar, leek powder, preservative (sodium sulphite), antioxidant (ascorbic acid), yeast extract, spice, acidity regulator (citric acid), spice extract, natural flavouring, herb extract), citrus fibre, vegan mozzarella-style cheese (1%) (water, coconut oil (25%), modified potato starch, gluten free oat fibre, maize starch, salt, natural flavourings, modified maize starch, tricalcium citrate, thickeners (carrageenan, guar gum), acidity regulators (lactic acid, sodium lactate), colour (mixed carotenes)), garlic (1%), sunflower oil. Filled into calcium alginate casing	https://www.heckfood.co.uk/
	Just WholeFoods	Organic & Vegan Sausage Mix	Extured soya protein*, wholemeal breadcrumbs* (wheat*, yeast, salt), brown rice flour*, couscous*, onion*, vegetable bouillon* (sea salt, rice flour*, vegetables* [onion*, carrots*, parsnip*], yeast extract, sunflower oil*, parsley*, turmeric*), tomato powder*, celery powder*, leek*, sea salt, parsley*, black pepper*, sage*. *certified organic ingredient	https://www.justwholefoods.co.uk/
	Linda McCartney Foods	Vegetarian Red Onion & Rosemary Sausages	Rehydrated textured soya protein (62%), red onion (19%), water, seasoning (red onion powder, kibbled red onion, salt, sugar, yeast extract, rosemary, sunflower oil, white pepper, ginger), soya protein concentrate, rapeseed oil, stabilizer: methyl cellulose	https://lindamccartneyfoods.co.uk/

Origin	Brand	Product	Ingredients	Reference
United Kingdom (cont.)	No Meat	No Porkies Cumberland Sausages	Water, rehydrated textured soya protein (31%) (textured soya protein, water), sunflower oil, thickener: methyl cellulose; pea fibre, flavouring, modified potato starch, modified maize starch,salt, black pepper, stabiliser: triphosphates; dried sage, ground coriander, yeast extract, beetroot powder (maltodextrin, beetroot juice from concentrate, acidity regulator: citric acid), tea extract, dried parsley, white pepper, garlic extract, preservative: sodium sulphite; ground nutmeg, antioxidant: ascorbic acid; black pepper extract, nutmeg extract, coriander extract, smoke flavouring	https://www.no-meat.co.uk/
	Suma	Suma Sausage Mix - Vegan	Whole wheat flour, palm oil, textured soya protein (21%), thickener: methylcellulose; maltodextrin; seasoning (sugar, potato starch, yeast extract, rapeseed oil, herb & spice extracts), hydrolysed maize protein), salt, onion powder, raising agent: ammonium carbonate; sage, thyme, pepper & beetroot juice powder	www.suma.coop
	Symington's Ltd	Granose Lincolnshire Sausage Mix	Dried textured **soya** protein (45%), breadcrumbs (32%) [fortified wheatflour (wheatflour, calcium carbonate, iron, niacin, thiamin), yeast, salt], flavourings (contain barley), wheat gluten, palm oil, dried parsley, onion powder, stabiliser (methylcellulose), dried sage, ground black pepper	https://www.sainsburys.co.uk/
	The Tofoo	Sage & onion sizzlers	Tofu* (60%) (water, soya beans*, nigari), maize flour*, water, rapeseed oil*, nutritional yeast*, onion powder*(1%), sea salt, sage*(0.5%), ground linseed*, ground white pepper*, ground nutmeg* (* organic ingredients)	https://tofoo.co.uk/
	VBites	Garlic Sausg Slices	Water**,** **wheat** gluten, **soya** protein, coconut oil, rapeseed oil, garlic powder, potato starch, salt, yeast extract, natural flavouring, dried yeast, sugar, thickener: carrageenan, onion powder, preservative: potassium sorbate, black pepper, colour: iron oxide	https://www.vbites.com/

Origin	Brand	Product	Ingredients	Reference
United Kingdom (cont.)	VBites (cont.)	Lincolmmmshire Sausgs	Water, **wheat** gluten, rapeseed oil, onion, **soya** protein, whole meal **wheat** rusk, sugar, salt, dried yeast, dried herbs, yeast extract, thickener: methyl cellulose; natural flavouring, preservative: potassium sorbate; garlic powder, **barley** malt extract	https://www.vbites.com/
		Meat-Free Oregano & Basil Sausages	Water, **wheat gluten,** vegetable oil (rapeseed), **soya** protein, whole meal **wheat** rusk, herbs, salt, dried yeast, yeast extract, sugar, natural flavouring, thickener: methyl cellulose, garlic powder, onion powder, herb & spice extracts, malt extract, black pepper	
		Meat-Free Mini Pork Sausages	Water, **wheat gluten**, vegetable oil (rapeseed), onion, whole meal **wheat** rusk, **soya** protein, dried yeast, yeast extract, salt, herbs & spices, natural flavouring, thickener: methyl cellulose; sugar, preservative: potassium sorbate, garlic powder, malt extract (contains **barley**)	
		Meat-Free Pork and Apple Sausage Rolls	Pork style sausage (water, wheat gluten, onion, vegetable oil (rapeseed), whole meal wheat rusk, soya protein, dried yeast, yeast extract, salt, dried spices, natural flavouring, sugar, preservative: potassium sorbate, thickener: methyl cellulose, garlic powder, malt extract, colour: iron oxide). Puff pastry (wheat flour, non-hydrogenated vegetable oils & fats, water, salt, emulsifier: mono- and diglycerides of fatty acids)	
		Meat-Free Pork Sausage Rolls	Pork style sausage (water, wheat gluten, onion, vegetable oil (rapeseed), whole meal wheat rusk, soya protein, dried yeast, yeast extract, salt, dried spices, natural flavouring, sugar, preservative: potassium sorbate, thickener: methyl cellulose, garlic powder, malt extract, colour: iron oxide). Puff pastry (wheat flour, non-hydrogenated vegetable oils & fats, water, salt, emulsifier: mono- and diglycerides of fatty acids)	

Origin	Brand	Product	Ingredients	Reference
United Kingdom (cont.)	VBites (cont.)	Sage & Marj Sausgs	Water, **wheat** gluten, rapeseed oil, **soya** protein, whole meal **wheat** rusk, herbs, salt, dried yeast, yeast extract, sugar, natural flavouring, thickener: methyl cellulose, preservative: potassium sorbate, garlic powder, onion powder, herb & spice extracts, **barley** malt extract, black pepper	https://www.vbites.com/
USA	365 Everyday Value	Meatless Breakfast Patties	Water, textured soy protein, textured vegetable protein (soy flour, caramel colour), seasoning (spices, organic evaporated cane sugar, paprika, sea salt, autolyzed yeast extract, citric acid), expeller pressed canola oil, wheat gluten, methylcellulose	https://eu.wholefoodsmarket.com/
	Amy's	Meatless Veggie Sausages	Organic onions, organic mushrooms, organic bulgur wheat, wheat gluten, organic celery, organic carrots, organic tofu (filtered water, organic soybeans, magnesium chloride), organic long-grain red rice, organic quinoa, organic oats, expeller pressed high oleic safflower and/or sunflower oil, organic green lentils, organic unbleached wheat flour, organic bell peppers, filtered water, sea salt, organic potatoes, organic garlic, spices, organic flax seed meal, organic extra virgin olive oil, hickory smoke flavour, organic agave syrup, black pepper, organic cane sugar, yeast	https://www.amys.com
	Beyond Meat	Beyond Breakfast Sausage® – Classic	Water, pea protein, expeller-pressed canola oil, refined coconut oil, calcium alginate casing, natural flavours, yeast, rice protein, chicory root fiber, methylcellulose, salt, tapioca syrup solids, tapioca dextrose, apple extract, psyllium husk fiber, yeast extract, potassium chloride, pomegranate extract, vinegar, lemon juice concentrate, pea fiber, sunflower lecithin, beet powder (for colour), spices, carrot	https://www.beyondmeat.com/

Origin	Brand	Product	Ingredients	Reference
USA (cont.)	Beyond Meat (cont.)	Beyond Breakfast Sausage® – Classic Patties	Water, pea protein, expeller-pressed canola oil, refined coconut oil, natural flavours, inactivated yeast, rice protein, methylcellulose, yeast extract (niacin (vitamin B3), pyridoxine hydrochloride (vitamin B6), thiamin hydrochloride (vitamin B1), riboflavin (vitamin B2), folic acid (vitamin B9), cyanocobalamin (vitamin B12)), apple extract, salt, pomegranate extract, vinegar, lemon juice concentrate, sunflower lecithin, beet juice extract (for colour), carrot	https://www.beyondmeat.com/
		Beyond Breakfast Sausage® – Spicy Patties	Water, pea protein, expeller-pressed canola oil, refined coconut oil, natural flavours, inactivated yeast, rice protein, methylcellulose, yeast extract (niacin (vitamin B3), pyridoxine hydrochloride (vitamin B6), thiamin hydrochloride (vitamin B1), riboflavin (vitamin B2), folic acid (vitamin B9), cyanocobalamin (vitamin B12)), apple extract, salt, spices, pomegranate extract, vinegar, lemon juice concentrate, smoked paprika, sunflower lecithin, beet juice extract (for colour), carrot.	
	El Burrito	Non-GMO Soy Longaniza™	Water, textured soy protein (soy flour), distilled vinegar, seasoning blend (paprika, salt, spices, lime juice powder (corn syrup solids, lime juice solids, lime oil), dextrose, garlic powder, spices extractives), soybean oil, xanthan gum, yeast extract, extractives of paprika, caramel colour	https://elburrito.com
	Field Roast	Apple & Maple	Filtered water, vital wheat gluten, expeller pressed safflower oil, unsulfured dried apples, yeast extract (yeast, salt, sugar), wheat protein isolate (wheat gluten, trisodium phosphate, malic acid, L-cysteine), pure maple syrup, onion powder, barley malt extract, garlic, spices, sea salt, black pepper, ground ginger, nutmeg, natural smoke flavour	https://fieldroast.com/

Origin	Brand	Product	Ingredients	Reference
USA (cont.)	Field Roast (cont.)	Caramelized Onions & Beer	Vital wheat gluten, filtered water, caramelized onions (yellow onions, sugar, apple juice concentrate), expeller pressed safflower oil, organic expeller pressed palm fruit oil, beer (malted barley, hops, yeast, water), yeast extract (yeast, salt, sugar), garlic, pea protein, barley malt extract, onion powder, granulated garlic, natural flavour (potato maltodextrin, gum arabic, dried brewer's yeast, natural flavour, organic sunflower oil), spices, cultured cane sugar, vinegar, sea salt, caraway seeds, red pepper, black pepper	https://fieldroast.com/
		Classic Recipe	Water, pea protein, canola oil, spices (black pepper, white pepper, sage, rosemary, marjoram) fava bean protein, brown rice protein, methylcellulose, vinegar, salt, cane sugar, dried garlic, yeast extract, rice concentrate, flavour, beet powder (colour)	
		Classic Smoked	Filtered water, vital wheat gluten, expeller pressed safflower oil, yeast extract (yeast, salt, sugar, natural flavour), organic expeller pressed palm fruit oil, barley malt extract, tomato paste, apple cider vinegar, paprika colour, spices, sea salt, onions, wheat flour, garlic, natural smoke flavour, celery seed, paprika oleoresin (colour)	
		Italian Garlic & Fennel	Filtered water, vital wheat gluten, expeller pressed safflower oil, eggplant, onions, wheat protein isolate (wheat gluten, trisodium phosphate, malic acid, l-cysteine), yeast extract (yeast, salt, sugar), garlic, barley malt extract, onion powder, dried red bell peppers, red cooking wine (red wine, water, salt), fennel seed, dehydrated garlic, sea salt, spices	
		Smoked Apple & Sage	Filtered water, vital wheat gluten, expeller pressed safflower oil, unsulfured dried apples, yukon gold potatoes, yeast extract (yeast, salt), onion powder, barley malt extract, garlic, spices, sea salt, yeast, rubbed sage, natural smoke flavour	

Origin	Brand	Product	Ingredients	Reference
USA (cont.)	Field Roast (cont.)	Spicy Mexican Chipotle	Vital wheat gluten, filtered water, expeller pressed safflower oil, onions, garlic, wheat protein isolate (wheat gluten, trisodium phosphate, malic acid, L-cysteine), apple cider vinegar, yeast extract (yeast, salt, sugar), chipotle peppers, onion powder, granulated garlic, brown sugar, sea salt, barley malt extract, spices, black pepper, paprika oleoresin (colour), chili de arbol peppers, cumin, oregano	https://fieldroast.com/
	Gardein	Original breakfast saus'age patties	Water, soy protein concentrate, canola oil, vital wheat gluten, soy protein isolate, contains 2% or less of: yeast extract, methylcellulose, organic cane sugar, potato starch, sea salt, spices, salt, paprika, garlic powder, natural flavours, malt extract (malted barley, water), onion powder, annatto (colour), oleoresin paprika (colour), spice extract. Contains wheat and soy	https://www.gardein.com/
		Sliced italian saus'age	Water, soy protein concentrate, canola oil, fractionated palm oil shortening, vital wheat gluten, soy protein isolate, contains 2% or less of: yeast extract, methylcellulose, organic cane sugar, potato starch, sea salt, spices, fennel, paprika, garlic powder, salt, natural flavours, dried garlic, malt extract (malted barley, water), lactic acid	
		Spicy breakfast saus'age patties	Water, soy protein concentrate, canola oil, vital wheat gluten, soy protein isolate, contains 2% or less of: yeast extract, methylcellulose, organic cane sugar, potato starch, sea salt, spices, salt, paprika, garlic powder, natural flavours, malt extract (malted barley, water), onion powder, red bell pepper flakes, annatto (colour), oleoresin paprika (colour), spice extract. Contains wheat and soy	
	Lightlife® Foods, Inc	Gimme Lean® Sausage	Water, soy protein concentrate, soy flour, tapioca starch, soy sauce (water, soybeans, salt, wheat), less than 2% of: soy protein isolate, natural flavours (from vegetable sources), wheat gluten, cellulose gum, evaporated cane sugar, sea salt, spices, soy milk powder, barley malt extract, torula yeast, beet powder, salt, yeast extract	https://lightlife.com/

Origin	Brand	Product	Ingredients	Reference
USA (cont.)	Lightlife® Foods, Inc (cont.)	Plant-Based Bratwurst Sausages	Water, pea protein, canola oil, modified cellulose (from plant fiber), less than 2% of natural flavours, cane sugar, salt, tapioca starch, citrus fiber, fava bean protein, brown rice protein, beet powder (colour), yeast extract, dried torula yeast, dehydrated lemon peel, smoked sugar. In a calcium alginate casing	https://lightlife.com/
		Plant-Based Breakfast Links	Water, pea protein, canola oil, brown rice protein, less than 2% of natural flavours, spice, modified cellulose (from plant fiber), cane sugar, vinegar, beet powder (colour), salt, yeast extract. In a calcium alginate casing	
		Plant-Based Italian Sausage	Water, pea protein, canola oil, modified cellulose (from plant fiber), less than 2% of natural flavours, cane sugar, salt, tapioca starch, citrus fiber, fava bean protein, brown rice protein, dried red bell peppers, beet powder (colour), yeast extract, dried torula yeast, paprika extract (colour). In a calcium alginate casing	
		Smart Dogs®	Water, soy protein isolate, soybean oil, evaporated cane sugar, pea protein isolate, tapioca starch, salt, potassium chloride, yeast extract, carrageenan, dehydrated garlic, natural flavour, natural smoke flavour, xanthan gum, fermented rice flour, guar gum, oleoresin paprika (colour), vital wheat gluten	
		Smart Dogs® Jumbo	Water, soy protein isolate, soybean oil, evaporated cane sugar, pea protein isolate, tapioca starch, salt, potassium chloride, yeast extract, carrageenan, dehydrated garlic, natural flavour, natural smoke flavour, xanthan gum, fermented rice flour, guar gum, oleoresin paprika (colour), vital wheat gluten	
		Smart Sausage® Chorizo	Water, textured soy protein concentrate, soybean oil, egg whites, soy protein isolate, less than 2% of: potato starch, yeast extract, sea salt, wheat gluten, paprika, cellulose gum, dried garlic, salt, spices, fermented rice flour, oleoresin paprika (colour), natural flavour (from plant sources), barley malt extract, dried onions	

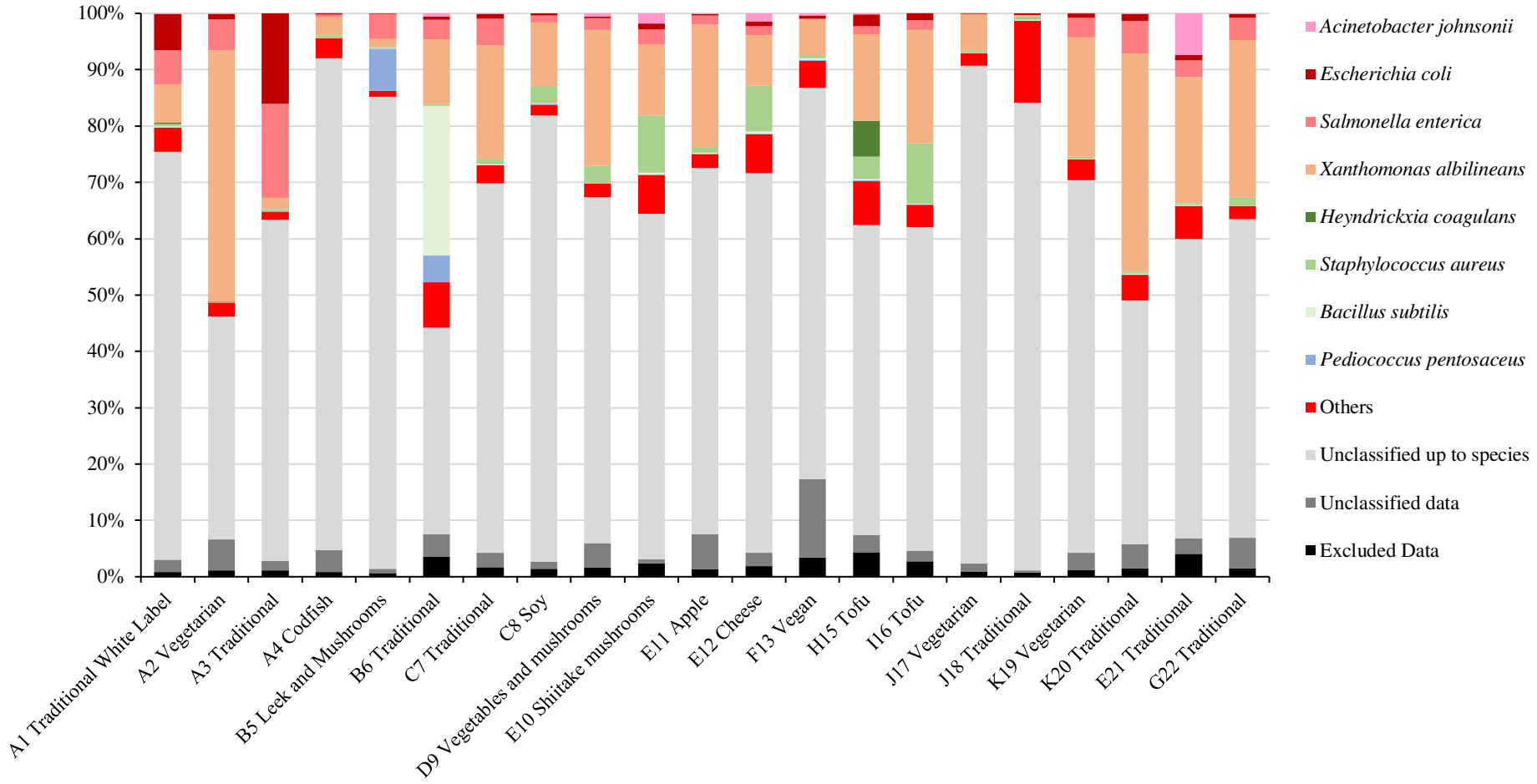
Origin	Brand	Product	Ingredients	Reference
USA (cont.)	Lightlife® Foods, Inc (cont.)	Smart Sausage® Italian	Water, textured soy protein concentrate, soybean oil, egg whites, dried vegetables (onions, garlic, tomato, red and green bell peppers), potato starch, soy protein isolate, wheat gluten, cellulose gum, sea salt, yeast extract, salt, tapioca starch, barley malt extract, spices, natural flavour (from plant sources), evaporated cane syrup, calcium phosphate	https://lightlife.com/
	MorningStar Farm	Incogmeato™ Original Bratwurst	Water, soy protein concentrate, canola oil, wheat gluten, palm oil, methylcellulose. Contains 2% or less of potato starch, salt, onion powder, spices, sugar, yeast extract, fruit juice (colour), natural flavours, sodium alginate, cultured dextrose for freshness, sunflower lecithin, guar gum, citric acid	https://www.morningstarfarms.com/
		Incogmeato™ Italian Sausage	Water, soy protein concentrate, canola oil, wheat gluten, palm oil, methylcellulose. Contains 2% or less of yeast extract, potato starch, spices, salt, fruit juice (colour), sodium alginate, natural flavours, cultured dextrose for freshness, dextrose, paprika (colour), sugar, sunflower lecithin, garlic powder, guar gum, corn oil, citric acid, onion powder, vegetable juice concentrate (colour), soy sauce (water, soybeans, salt)	
		MorningStar Farms® Corn Dogs	Water, wheat flour, sugar, wheat gluten, yellow corn meal, yellow corn flour, corn oil.Contains 2% or less of salt, dextrose, leavening (sodium acid pyrophosphate, sodium bicarbonate), methylcellulose, brown sugar (sugar, molasses), spices, yeast extract, onion powder, pea protein isolate, natural flavours, carrageenan, garlic powder, xanthan gum, red beet juice concentrate (colour), paprika extract (colour), paprika (colour), dried yeast	

Origin	Brand	Product	Ingredients	Reference
USA (cont.)	MorningStar Farm (cont.)	MorningStar Farms® Hot & Spicy Sausage Patties	Water, wheat gluten, soy protein concentrate, corn oil, soy flour, egg whites, sodium caseinate, modified tapioca starch.Contains 2% or less of soy protein isolate, soybean oil, lactose, spices, methylcellulose, autolyzed yeast extract, natural and artificial flavours, sodium tripolyphosphate, salt, modified corn starch, hydrolyzed wheat gluten, caramel colour, disodium inosinate, crushed red pepper, whey, hydrolyzed corn gluten, red bell peppers, hydrolyzed soy protein, potassium chloride, dextrose, onion powder, disodium guanylate, tetrasodium pyrophosphate, tricalcium phosphate, hydrolyzed wheat protein, sodium hexametaphosphate, succinic acid, niacinamide, monosodium phosphate, lactic acid, brewer's yeast, torula yeast, calcium phosphate, soy lecithin, iron (ferrous sulfate), magnesium carbonate, vitamin B1 (thiamin mononitrate), vitamin B6 (pyridoxine hydrochloride), vitamin B2 (riboflavin), vitamin B12	https://www.morningstarfarms.com/
		MorningStar Farms® Maple Flavoured Sausage Patties	Water, wheat gluten, soy protein concentrate, sugar, corn oil, soy flour, egg whites, sodium caseinate, modified tapioca starch. Contains 2% or less of soy protein isolate, lactose, soybean oil, natural and artificial flavours, methylcellulose, autolyzed yeast extract, spices, sodium tripolyphosphate, salt, modified corn starch, hydrolyzed wheat gluten, disodium inosinate, caramel colour, whey, hydrolyzed corn gluten, hydrolyzed soy protein, potassium chloride, dextrose, onion powder, disodium guanylate, tetrasodium pyrophosphate, tricalcium phosphate, hydrolyzed wheat protein, sodium hexametaphosphate, succinic acid, niacinamide, monosodium phosphate, lactic acid, brewer's yeast, torula yeast, calcium phosphate, soy lecithin, iron (ferrous sulfate), magnesium carbonate, vitamin B1 (thiamin mononitrate), vitamin B6 (pyridoxine hydrochloride), vitamin B2 (riboflavin), vitamin B12	

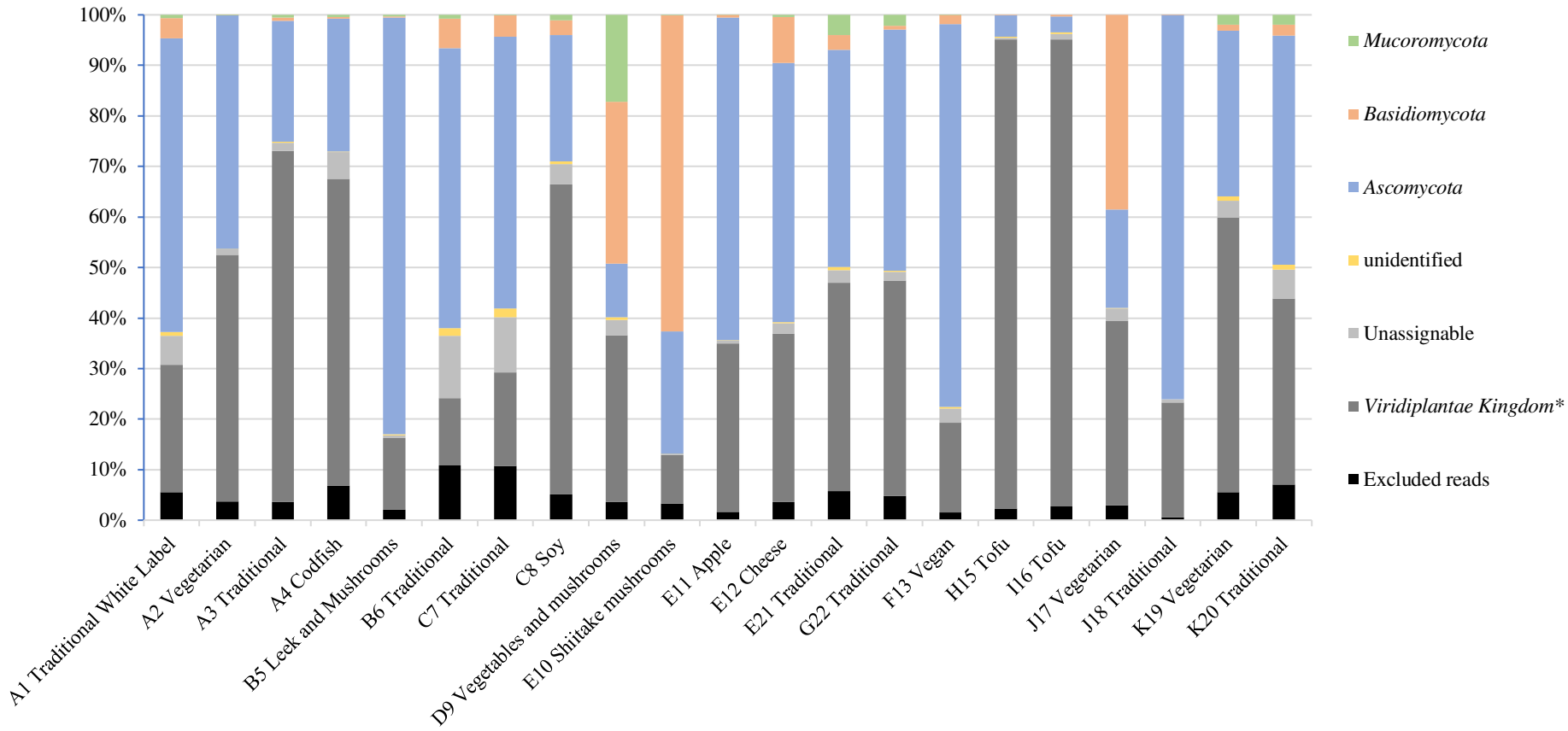
Origin	Brand	Product	Ingredients	Reference
USA (cont.)	MorningStar Farm (cont.)	MorningStar Farms® Original Sausage Patties	Water, wheat gluten, soy protein concentrate, vegetable oil (corn, canola and/or sunflower), soy flour, soy protein isolate.Contains 2% or less of maltodextrin, methylcellulose, yeast extract, salt, fruit juice for colour, natural flavours, spices, sugar, potato starch, konjac flour, onion powder, soy sauce (water, soybeans, salt, wheat)	https://www.morningstarfarms.com/
		Morningstar Farms® Veggie Breakfast Sausage Links	Water, wheat gluten, corn oil, egg whites, soy protein concentrate, contains 2% or less of potato starch, salt, sodium caseinate, soy protein isolate, methylcellulose, sugar, canola oil, spices, hydrolyzed vegetable protein (soy, wheat, and corn), yeast extract, caramel colour, guar gum, natural and artificial flavours, autolyzed yeast extract, onion powder, soy sauce powder (soy sauce [soybeans, salt, wheat], maltodextrin), disodium inosinate, disodium guanylate, soybean oil, sunflower oil, xanthan gum, sesame oil.Vitamins and minerals: niacinamide, iron (ferrous sulfate), vitamin B1 (thiamin mononitrate), vitmain B6 (pyridoxine hydrochloride), vitamin B2 (riboflavin), vitamin B12	
		MorningStar Farms® Veggie Dogs	Water, wheat gluten, corn syrup solids.Contains 2% or less of methylcellulose, dextrose, salt, egg whites, natural flavours, brown sugar (sugar, molasses), hydrolyzed vegetable protein (corn protein, soy protein), hydrolyzed corn protein, soy protein isolate, carrageenan, mustard flour, onion powder, maltodextrin, spices, xanthan gum, hydrolyzed soy protein, autolyzed yeast, paprika, garlic powder, soybeans, disodium guanylate, disodium inosinate, hydrolyzed torula and brewers yeast, wheat, gum arabic, hydrolyzed vegetable protein (corn gluten, soy protein, wheat gluten), soybean oil, thiamin hydrochloride, paprika extract for colour, autolyzed yeast extract, lactic acid, nonfat milk, red 40, sunflower oil, citric acid, blue 1	

Origin	Brand	Product	Ingredients	Reference
USA (cont.)	No Evil Foods	El Capitán - Chorizo	Filtered water, non-GMO vital wheat gluten, organic tomato paste, apple cider vinegar, organic chili powder, sea salt, organic smoked paprika, organic onion powder, nutritional yeast, organic ground cumin, organic garlic powder, mexican oregano, organic cayenne, organic shoyu (water, organic soybeans, organic wheat, salt)	https://www.noevilfoods.com/
		The Stallion – Italian Sausage	Vital wheat gluten, filtered water, organic red kidney beans, organic shoyu, organic chickpea flour, nutritional yeast, sea salt, organic smoked paprika, organic garlic powder, organic chili flakes, organic fennel seed, organic thyme, organic rosemary, organic cayenne, organic black pepper	
	The Meatless Farm Co	Plant-based Breakfast Sausages	Water, pea protein, canola oil, shea oil, methylcellulose. Less than 2% of: seasoning (spices (white pepper, nutmeg, coriander), onion powder, sage, ascorbic acid (antioxidant), sun flower oil), coconut oil, pea fiber, potato fiber, rice protein, natural flavour, salt, vegetable and fruit extracts for colour (beetroot, radish, tomato), yeast extract, ascorbic acid (acidity regulator), caramelized carrot concentrate, sage, coriander, nutmeg, carrot concentrate. sausages filled into sodium alginate casings	https://meatlessfarm.com/
		Plant-based Sausages Patties	Water, pea protein, canola oil, shea oil, methylcellulose. Less than 2% of: seasoning (spices (white pepper, nutmeg, coriander), onion powder, sage, ascorbic acid (antioxidant), sun flower oil), coconut oil, pea fiber, potato fiber, natural flavour, salt, rice protein, vegetable and fruit extracts for colour (beetroot, radish, tomato), yeast extract, ascorbic acid (antioxidant), caramelized carrot concentrate, sage, coriander, nutmeg, carrot concentrate	
	Tofurky	Beer Brats	Water, vital wheat gluten, expeller pressed canola oil, tofu (water, soybeans, magnesium chloride, calcium chloride), onions, soy flour and/or concentrate, amber ale(water, malted barley, hops, yeast), contains less than 2% of sea salt, cane sugar, spices, dehydrated onion, granulated garlic, garlic puree, carrageenan, dextrose, konjac, potassium chloride	https://tofurky.com/

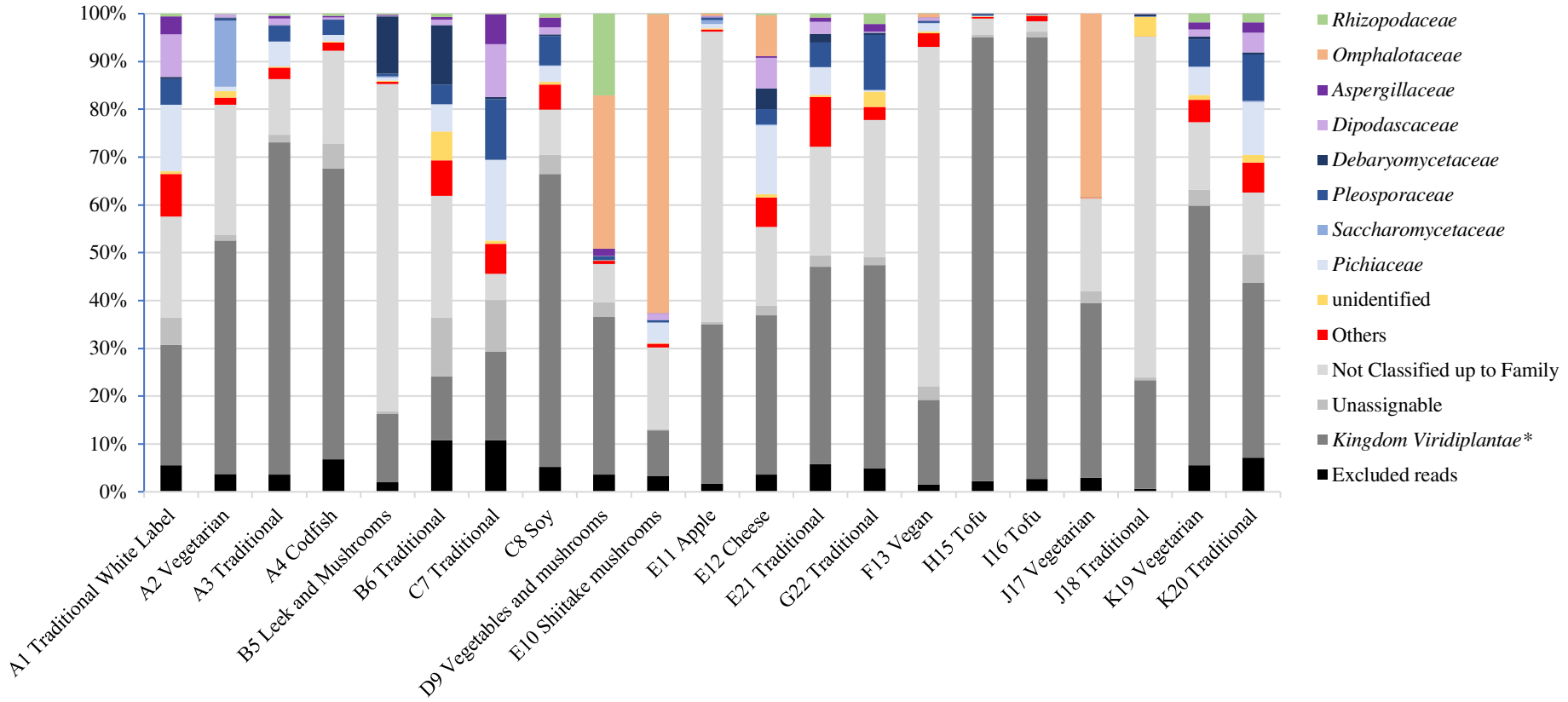
Origin	Brand	Product	Ingredients	Reference
USA (cont.)	Tofurky (cont.)	Spinach Pesto	Water, vital wheat gluten, tofu (water, soybeans, magnesium chloride, calcium chloride), expeller pressed canola oil, soy flour and/or concentrate, soy sauce (water, soybean, wheat, salt), wheat berries, contains less than 2% of spinach, basil, garlic puree, granulated garlic, dried onion, spices, sea salt, oat fiber, natural flavours, potassium chloride, lactic acid	https://tofurky.com/



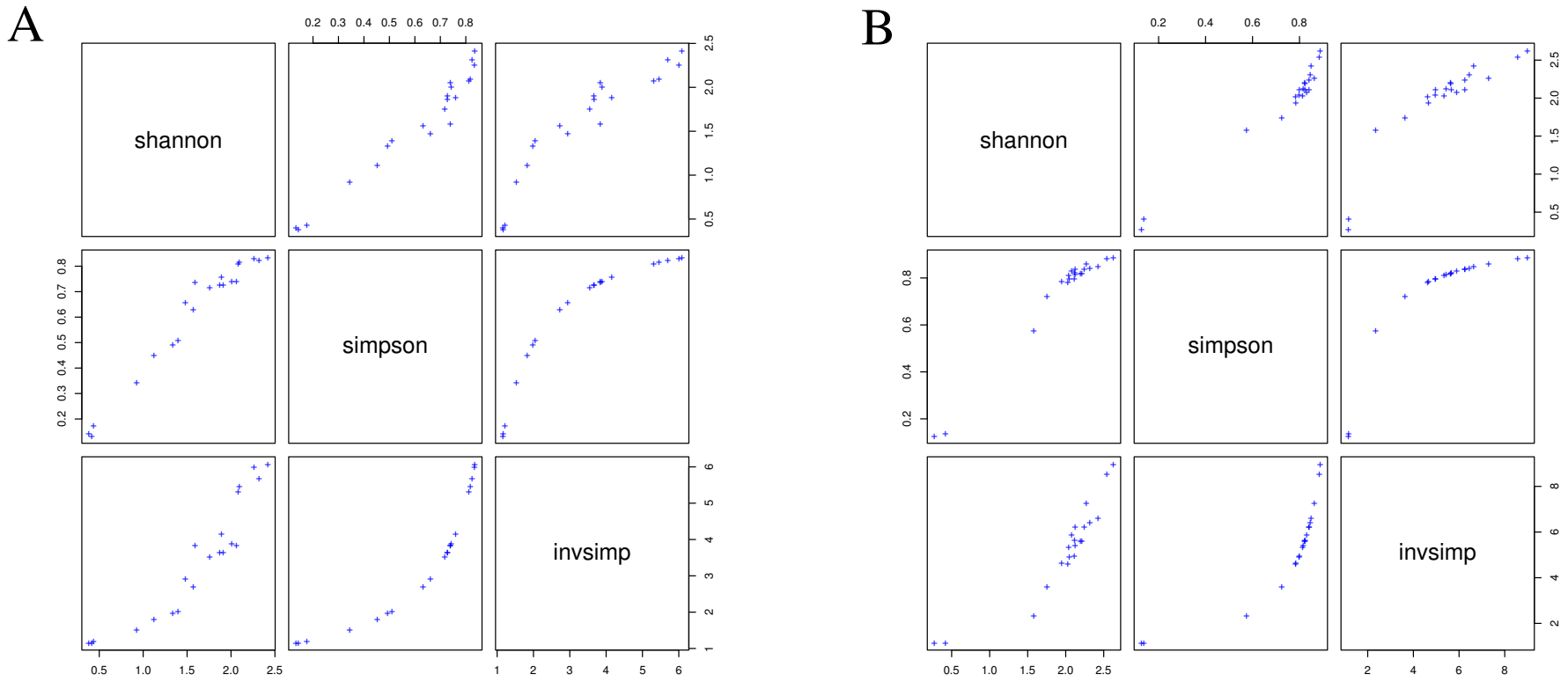
Supplementary Figure 3.1 – Relative abundance of bacterial communities at the species level determined by 16S metagenomic analysis. OTUs with an incidence above 5% are shown.



Supplementary Figure 3.2 – Relative abundance of fungal communities at the phylum level determined by ITS2 metagenomic analysis. OTUs with an incidence above 5% are shown.



Supplementary Figure 3.3 – Relative abundance of fungal communities at the family level determined by ITS2 metagenomic analysis. OTUs with an incidence above 5% are shown.



Supplementary Figure 3.4 – Alpha diversity metrics of all 21 *alheira* samples based on 16S rRNA (**A**) and ITS2 (**B**) sequences, arranged using *R* software.

Supplementary Table 5.1 – Target microorganisms used in the antimicrobial activity by competition screening

Microorganism		Source
Campylobacter jejuni	ATCC 33560	American Type Culture Collection (ATCC; Manassas, EUA)
Enterococcus faecalis	ATCC 29212	
Escherichia coli	ATCC 25922	
Salmonella Enteritidis	ATCC 13076	
Staphylococcus aureus	ATCC 29213	
Enterococcus casseliflavus	DSMZ 20680	Leibniz Institute DSMZ – German Collection of Microorganisms and Cell Cultures (DSMZ; Brunsvique, Germany)
Enterococcus faecalis	DSMZ 12956	
Enterococcus faecium	DSMZ 13590	
Enterococcus flavescens	DSMZ 7370	
Enterococcus gallinarum	DSMZ 20628	
Listeria monocytogenes	2542	Isolates from Culture Collection of *Listeria* Research Center of Escola Superior de Biotecnologia (LRCESB)
	7946	
	7947	
	CECT 911 (serovar 1/2c)	
	CECT 936 (serovar 1/2b)	
	CEP 104794 (serovar 1/2a)	
	FSL J1-177	
	FSL N1-227	
	FSL N3-013	
	FSL R2-499	
	FSL J1-031	
	MF4077	
	NCTC 11994 (serovar 1/4b)	
	SCOTT A	
Acinetobacter baumannii (resistant) (ESB028)		Isolates from Culture Collection of Escola Superior de Biotecnologia
Acinetobacter baumannii (sensitive) (ESB029)		
Acinetobacter calcoaceticus (resistant) (ESB030)		
Acinetobacter calcoaceticus (sensitive) (ESB031)		
Bacillus cereus (ESB014)		
Bacillus stearothermophilus (ESB016)		
Bacillus subtilis (ESB015)		
Candida albicans (ESB025)		
Clostridium perfrigens	1.16 (ESB053)	
	1.19 (ESB054)	
	1.22 (ESB055)	
Clostridium sporogenes	1.31 (ESB050)	
	1.34 (ESB051)	
	1.61 (ESB052)	
Klebsiella pneumoniae (ESB011)		
Listeria innocua 2030c (ESB023)		
Proteus spp. (mirabilis) (ESB027)		
Proteus vulgaris (ESB012)		
Pseudomonas aeruginosa (ESB013)		
Saccharomyces cerevisiae (ESB026)		
Salmonella Braenderup (ESB007)		
Salmonella Tiphymurium (ESB009)		
Staphylococcus aureus 18N (MRSA) (ESB020)		
Staphylococcus aureus 2037 M1 (MSSA) (ESB021)		
Yersinia enterocolitica (ESB024)		

Supplementary Table 5.2 – Virulence genes primers and PCR conditions

Gene	Primer (5' to 3')	PCR	PCR conditions	Size (bp)	Positive controls	Source
ace	GAA TTG AGC AAA AGT TCA ATC G GTC TGT CTT TTC ACT TGT TTC	0.5 µl dNTPs (10 mM*); 2.5 µl buffer NH_4**; 2.5 µl $MgCl_2$ (25 mM**); 0.25 µl primer F/R (10 pM***); 0.4 µl Taq polimerase (5U**)	95 °C (1 min); 30 x [94 °C (1 min), 55 °C (1 min), 72 °C (1 min)]; 72 °C (10 min); 4 °C	1008	*E. faecalis* DS16; *E. faecalis* F2; *E. faecalis* P1; *E. faecalis* P36; *E. faecalis* 29212	Martín-Platero *et al.*, (2009)
agg	AAG AAA AAG AAG TAG ACC AAC AAA CGG CAA GAC AAG TAA ATA	0.25 µl dNTPs (10 mM*); 2.5 µl buffer NH_4**; 2.5 µl $MgCl_2$ (25 mM**); 1.25 µl primer F/R (10 pM***); 0.25 µl Taq polimerase (5U**)	94 °C (1 min); 35 x [94 °C (1 min),55 °C (1 min), 72 °C (2 min)]; 72 °C (7 min); 4 °C	1553	*E. faecalis* P1	Eaton and Gasson (2001)
esp	TTG CTA ATG CTA GTC CAC GAC C GCG TCA ACA CTT GCA TTG CCG AA			933	*E. faecalis* P36	
gelE	ACC CCG TAT CAT TGG TTT ACG CAT TGC TTT TCC ATC			419	*E. faecalis* P1	
efaAfs	GAC AGA CCC TCA CGA ATA AGT TCA TCA TGC TGT AGT A			705	*E. faecalis* F2	
efaAfm	AAC AGA TCC GCA TGA ATA CAT TTC ATC ATC TGA TAG TA			735	*E. faecalis* F10	
hyl	CAG AAG AGC TGC AGG AAA TG GAC TGA CGT CCA AGT TTC CAA	0.5 µl dNTPs (10 mM*); 2.5 µl buffer NH_4**; 2.5 µl $MgCl_2$ (25 mM**); 0.35 µl primer *hyl* e *asa1* F/R (10 pM***); 0.4 µl Taq polimerase (5U**)	95 °C (1 min); 30 x [94 °C (1 min), 56 °C (1 min), 72 °C (1 min)]; 72 °C (10 min); 4 °C	276	*E. faecalis vanB*	Vankerckhoven *et al.*, (2004)
asa1	GCA CGC TAT TAC GAA CTA TGA TAA GAA AGA ACA TCA CCA CGA			375	*E. faecalis* DS16; *E. faecalis* F2; *E. faecalis* P1; *E. faecalis* P36; *E. faecalis* 29212	

Y = C or T; R = A or G; N = A, C, G or T; PCR volumes detailed to reactions of 25µl

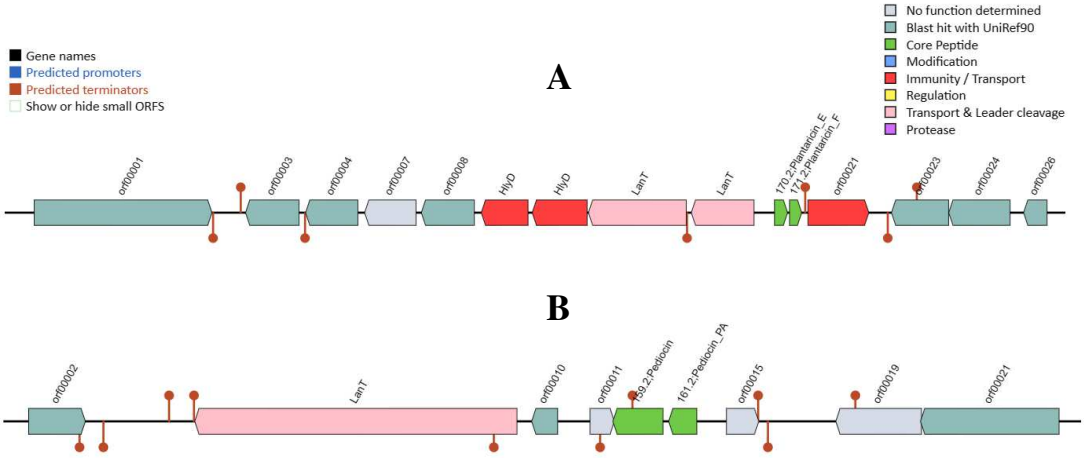
* Bioron, Römerberg, Germany; ** Thermo Fisher Scientific, Massachusetts, USA; *** Stabvida, Caparica, Portugal

Supplementary Table 5.2 – Virulence genes primers and PCR conditions (continuation)

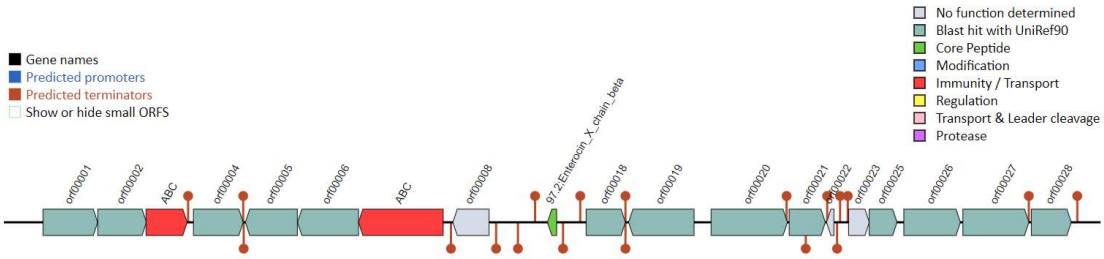
Gene	Primer (5' to 3')	PCR	PCR conditions	Size (bp)	Positive controls	Source
cylA	TGG ATG ATA GTG ATA GGA AGT TCT ACA GTA AAT CTT TCG TCA	0.25 µl dNTPs (10 mM*); 2.5 µl buffer NH_4**; 2.5 µl $MgCl_2$ (25 mM**); 1.25 µl primer F/R (10 pM***); 0.25 µl Taq polimerase (5U**)	95 °C (1 min); 35 x [94 °C (1 min),55 °C (1 min), 72 °C (2 min)]; 72 °C (7 min); 4 °C	517	*E. faecalis* F2	Semedo *et al.*, (2003)
cylB	ATT CCT ACC TAT GTT CTG TTA AAT AAA CTC TTC TTT TCC AAC			843	*E. faecalis* F2	
cylM	CTG ATG GAA AGA AGA TAG TAT TGA GTT GGT CTG ATT ACA TTT			742	*E. faecalis* F2	
cylLL	GAT GGA GGG TAA GAA TTA TGG GCT TCA CCT CAC TAA GTT TTA TAG			253	*E. faecalis* DS16	
cylLS	GAA GCA CAG TGC TAA ATA AGG GTA TAA GAG GGC TAG TTT CAC			240	*E. faecalis* DS16	
hdc1	AGA TGG TAT TGT TTC TTA TG AGA CCA TAC ACC ATA ACC TT	2.5 µl Tris HCl; 0.5 µl dNTPs (10 mM*); 5 µl buffer KCl**; 2.5 µl $MgCl_2$ (25 mM**); 0.75 µl primer *hdc1* F/R (10pM***), 2 µl primer *tdc* F/R (10 pM***) e 1 µl primer *odc* F/R (10 pM***); 0.4 µl Taq polimerase (5U**)	95 °C (1 min); 30 x [95 °C (30s), 52 °C (30s), 72 °C (2 min)]; 72 °C (10 min); 4 °C	367	*E. faecalis* DS16; *E. faecalis* F2; *E. faecalis* P1; *E. faecalis* P36	De las Rivas *et al.*, (2005)
tcd	GAY ATN ATN GGN ATN GGN YTN GAY CAR G CCR TAR TCN GGN ATA GCR AAR TCN GTR TG			924		
odc	GTN TTY AAY GCN GAY AAR CAN TAY TTY GT ATN GAR TTN AGT TCR CAY TTY TCN GG			1446		

Y = C or T; R = A or G; N = A, C, G or T; PCR volumes detailed to reactions of 25µl

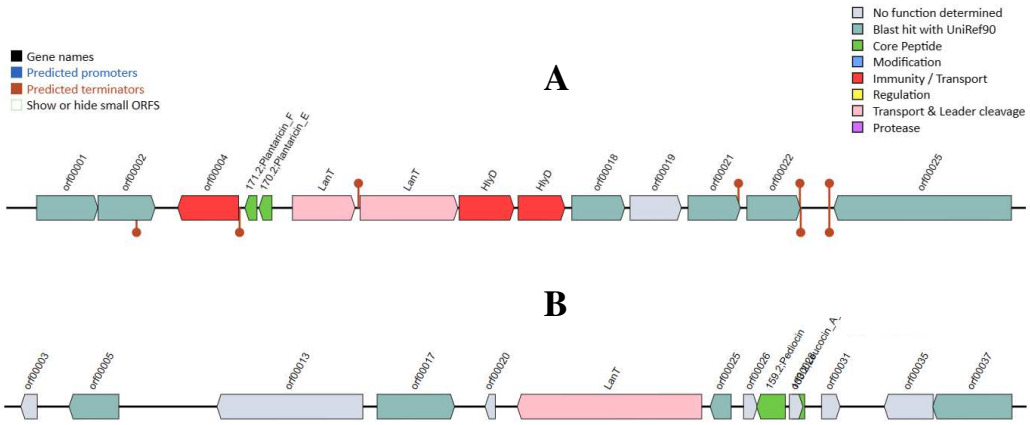
* Bioron, Römerberg, Germany; ** Thermo Fisher Scientific, Massachusetts, USA; *** Stabvida, Caparica, Portugal



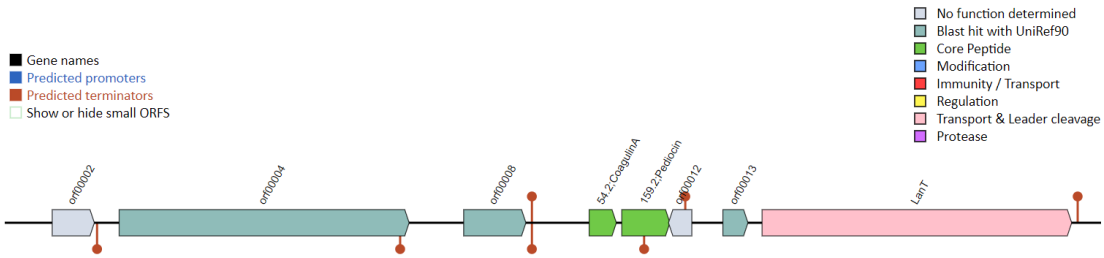
Supplementary Figure 5.1 – Genetic organization map of Plantaricins (**A**) and Pediocins (**B**) gene clusters of *Lactiplantibacillus plantarum* 1A5 strain.



Supplementary Figure 5.2 – Genetic organization map of Enterocin_X gene cluster of *Leuconostoc mesenteroides* 4-8 strain.



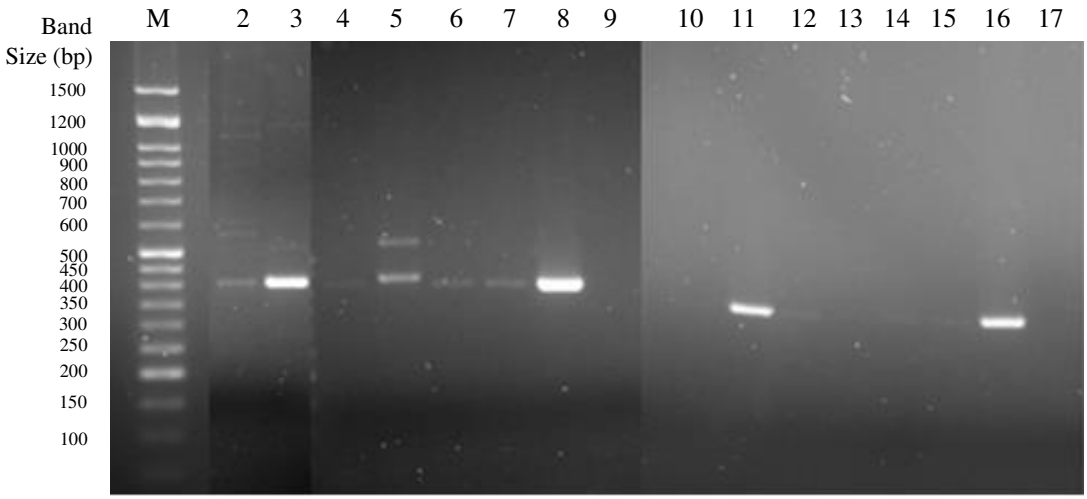
Supplementary Figure 5.3 – Genetic organization map of Plantaricins (**A**), Pediocin and Leucocin A (**B**) gene clusters of *Lactiplantibacillus plantarum* 9A3 strain.



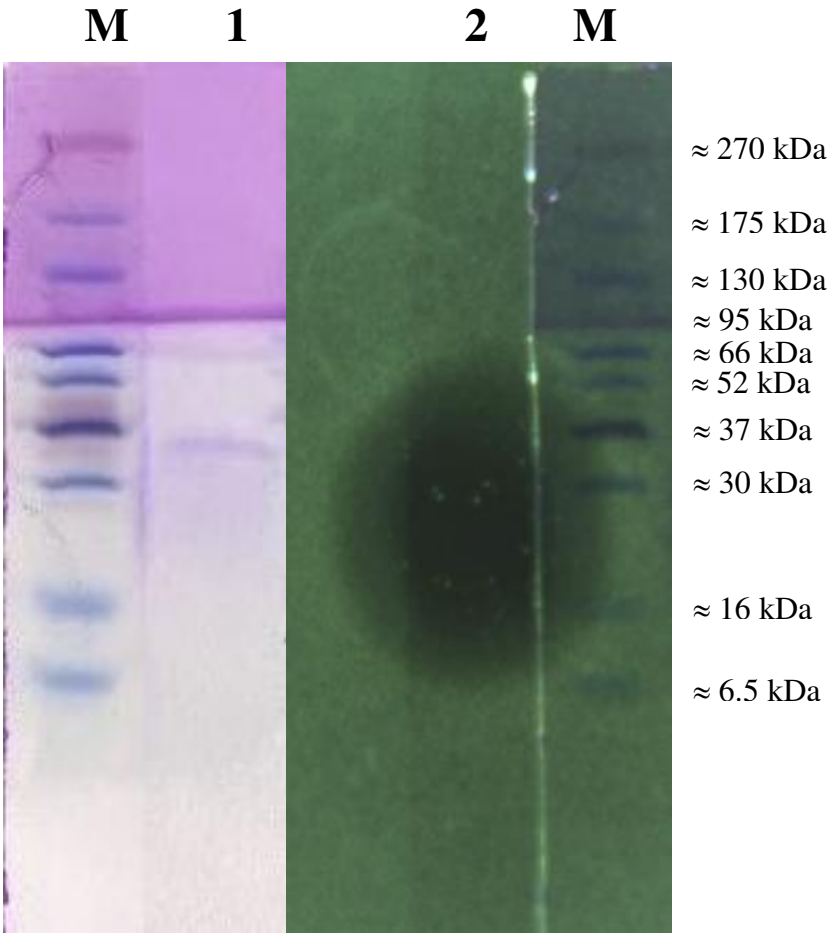
Supplementary Figure 5.4 – Genetic organization map of CoagulinA and Pediocin gene cluster of the *Pediococcus acidilactici* 10A2 strain.

Supplementary Figure 5.5 – Genetic organization map of Enterolysin A gene cluster of *Pediococcus acidilactici* 18-8 strain.

Supplementary Figure 5.6 – Genetic organization map of Enterolysin A gene cluster of *Pediococcus acidilactici* 21/2-2 strain.



Supplementary Figure 5.7 – Agarose gel electrophoresis for amplified products of some virulence genes. Lane 1: Molecular marker (Nzytech Ladder VI); Lane 2 to 9: Gene *gelE* (419bp) – samples1A5, 4-8, 9A3, 10A2, 18-8, 21/2-2, positive control P1 and zero, respectively; Lane 10 to 17: Gene *asa1* (375bp) – samples 1A5, 4-8, 9A3, 10A2, 18-8, 21/2-2, positive control ATCC 29212 and zero, respectively.



Supplementary Figure 5.8 – Tricine/SDS-PAGE of *Lpb. plantarum* 9A3 bacteriocin: Lanes 1 and 4: Molecular mass marker (M); Lane 2: peptide bands in the stained gel with Coomassie Blue R250; Lane 3: growth inhibition zone, corresponding to the position of the peptide band in lane 2 (the gel was covered with viable cells of *L. monocytogenes* CECT 936 (10^6 CFU/mL), embedded in BHI soft agar and incubated at 37 ◦C for 24h.