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Biopreservation technologies and novel modelling approaches to control the development of *Staphylococcus aureus* in goat's raw milk soft cheeses

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Doctoral Thesis
Food Science and Technology and Nutrition

Work developed under the supervision of
Professor Doctor Ursula Gonzales-Barron
Professor Doctor José António Teixeira
Doctor Mariem Ellouze

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DIREITOS DE AUTOR E CONDIÇÕES DE UTILIZAÇÃO DO TRABALHO POR TERCEIROS

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STATEMENT OF INTEGRITY

I hereby declare having conducted this academic work with integrity. I confirm that I have not used plagiarism or any form of undue use of information or falsification of results along the process leading to its elaboration.

I further declare that I have fully acknowledged the Code of Ethical Conduct of the University of Minho.

RESUMO

Tecnologias de biopreservação e novas abordagens de modelação para controlar o desenvolvimento de *Staphylococcus aureus* em queijos frescos de leite cru de cabra

A presença de *Staphylococcus aureus* em queijos frescos de leite cru de cabra tem sido documentada várias vezes. Nestes queijos, não existe uma fase de maturação prolongada que reduza a carga do patogénico e a pasteurização não é utilizada para garantir as características sensoriais únicas associadas a queijos de leite cru. Assim, esta tese teve como objetivo fornecer soluções para melhorar a qualidade microbiológica deste produto em relação à contaminação por *S. aureus*. Para tal, testou-se a incorporação de extratos de plantas em queijo, a utilização de uma cultura de arranque no leite, e um tratamento térmico moderado do leite (termização), usando modelos preditivos para avaliar o efeito destas estratégias na sobrevivência de *S. aureus*.

Os extratos de plantas avaliados (alfazema, erva-cidreira, estragão, hortelã, manjericão e sálvia) apresentaram várias bioatividades relevantes, entre elas a antimicrobiana, atribuídas aos seus perfis fenólicos. Foi evidente a influência do tipo de solvente e extração no perfil fitoquímico e bioatividades obtidas, o que levou à seleção de três extratos hidroetanólicos (erva-cidreira, hortelã e sálvia) para incorporação em queijo como bioconservantes. Entre as bactérias ácido-láticas isoladas de queijos artesanais, várias demonstraram capacidade antimicrobiana e acidificante. Pelas suas características promissoras, as estirpes *L. mesenteroides, L. paracasei*, *L. cremoris* e *L. lactis* foram selecionadas para a produção de uma cultura de arranque.

As várias temperaturas de termização testadas (55 a 64 °C) foram eficazes na inativação de *S. aureus* em leite, conforme revelaram os modelos, ainda que produzindo efeitos distintos na capacidade de sobrevivência do patogénico. A abordagem de modelação global mostrou estimativa de parâmetros aprimorada, comparando com a modelação em dois passos.

Por último, através de modelos dinâmicos (baseados na equação de Bigelow), o efeito inibitório dos extratos vegetais e da cultura de arranque adicionados ao queijo/leite foi validado, demonstrando que a adição de qualquer um dos bioconservantes reduz o tempo necessário para reduzir em um log a concentração de *S. aureus*.

No geral, os modelos preditivos construídos permitiram confirmar a eficácia da termização e dos métodos de biopreservação testados no controlo de *S. aureus*, oferecendo assim soluções para melhorar a qualidade microbiológica de queijos artesanais de leite cru de cabra.

Palavras-chave: Bactérias ácido-láticas; Compostos bioativos; Extratos de Plantas; Modelação dinâmica; Termização.

ABSTRACT

Biopreservation technologies and novel modelling approaches to control the development of *Staphylococcus aureus* in goat's raw milk soft cheeses

The presence of *Staphylococcus aureus* in goats' raw milk soft cheeses has been documented several times. In these cheeses, there is no prolonged ripening stage that reduces the pathogen load, and pasteurisation is not used to ensure the unique sensory characteristics associated with raw milk cheeses. Thus, this thesis aimed to provide solutions to improve the microbiological quality of this product in terms of *S. aureus* contamination. For that, the incorporation of plant extracts in cheese, the use of a customised starter culture in milk, and a mild thermal treatment of milk (thermisation) were tested, and predictive models were used to evaluate the effect of these strategies on the survival of *S. aureus*.

The evaluated plant extracts (French lavender, lemon balm, tarragon, spearmint, basil and sage) showed several relevant bioactivities, including antimicrobial activity, which were attributed to their phenolic profiles. The influence of the type of solvent and extraction on the phytochemical profile and bioactivities obtained was evident, which led to the selection of three hydroethanolic extracts (lemon balm, spearmint and sage) to be incorporated into cheese as biopreservatives. Among the lactic acid bacteria isolated from artisanal cheeses, several showed antimicrobial and acidifying capacity. Due to their promising characteristics, *L. mesenteroides*, *L. paracasei*, *L. cremoris* and *L. lactis* strains were selected to produce a starter culture.

The various thermisation temperatures tested (55 to 64 °C) were effective in inactivating *S. aureus* in milk, as shown by the adjusted models (Weibull), although producing different effects on the pathogen's ability to survive. The global modelling approach showed improved parameter estimation compared to the two-step modelling.

Finally, through dynamic models (based on the Bigelow equation), the inhibitory effect of plant extracts and starter culture added to cheese/milk was validated, demonstrating that the addition of any of the biopreservatives reduces the time required to reduce the concentration of *S. aureus* by one log.

Overall, the predictive models built allowed confirming the effectiveness of thermisation and the biopreservation methods tested in controlling *S. aureus*, thus offering solutions to improve the microbiological quality of artisanal goats' raw milk cheeses.

Keywords: Bioactive compounds; Dynamic modelling; Lactic acid bacteria; Plant Extracts; Thermisation.

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LIST OF ABBREVIATIONS, ACRONYMS AND SYMBOLS

3-HPA	3-Hydroxy-propionaldehyde
AAPH	2,2′-Azobis(2-methylpropionamidine) dihydrochloride
ABTS	2,2′-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt
AGS	Gastric adenocarcinoma
ATP	Adenosine triphosphate
ATPase	Adenosine triphosphatase
BHI	Brain heart infusion
BSA	Bovine albumin serum
BSAE	Bovine albumin serum equivalents
CaCo-2	Colorectal adenocarcinoma
Carbohyd.	Total carbohydrate content
CE	Catechin equivalents
CFU	Colony-forming unit
Chl-a	Chlorophyll-a
Chl-b	Chlorophyll-b
DAD	Diode array detector
DMEM	Dulbecco's modified eagle medium
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DP	Dry plant
DPPH	2,2-Di(4-tert-octylphenyl)-1-picrylhydrazyl
EO	Essential oil
Et70	Ethanol 70% (v/v)
FRAP	Ferric reducing antioxidant power
GAE	Gallic acid equivalents
GE	Glucose equivalents
GI_{50}	Half-maximal cell growth inhibitory concentration
GRAS	Generally recognized as safe
IC_{50}	Half-maximal inhibitory concentration
HeLa	Cervical carcinoma

hFOB	Human foetal osteoblasts
HPLC	High-performance Liquid Chromatography
ISO	International organization for standardization
LAB	Lactic acid bacteria
LM	*Listeria monocytogenes*
LOD	Limit of detection
LOQ	Limit of quantification
MBC	Minimum bactericidal concentration
MCF-7	Breast adenocarcinoma
MFC	Minimum fungicidal concentration
MIC	Minimum inhibitory concentration
MRS	De Man, Rogosa and Sharpe
MS	Mass spectrometry
NADH	Nicotinamide adenine dinucleotide
NCI-H460	Large cell lung carcinoma
OxHLIA	Oxidative haemolysis inhibition essay
PBS	Phosphate-buffered saline
PCA	Principal component analysis
pKA	Negative log of the acid dissociation constant
RNA	Ribonucleic acid
rpm	Revolutions per minute
SA	*Staphylococcus aureus*
SD	Standard deviation
SE	Standard error
SS	*Salmonella spp.*
STEC	Shiga toxin-producing *Escherichia coli*
TBARS	Thiobarbituric acid reactive substances
TE	TROLOX equivalent
TFC	Total flavonoid content
TPC	Total phenolic content
TProtein	Total protein content

TROLOX	6-Hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid
TSB	Tryptic soy broth
UPLC	Ultra performance liquid chromatography

SCIENTIFIC OUTPUTS

According to the 2nd paragraph of the article 8 of the Portuguese Decree-Law no. 388/70, the scientific outputs of this thesis are listed below. The results presented in this thesis have been partially published elsewhere.

Papers accepted in peer-reviewed journals:

Meta-Regression models describing the effects of essential oils and added lactic acid bacteria on pathogen inactivation in cheese. *Microb. Risk Anal.* 2021, 18, Article 100131. https://doi.org/10.1016/j.mran.2020.100131

Chemical Profile and Bioactivities of Extracts from Edible Plants Readily Available in Portugal. *Foods* 2021, *10*, 673. https://doi.org/10.3390/foods10030673

Phytochemical Composition and Bioactive Potential of *Melissa officinalis* L., *Salvia officinalis* L. and *Mentha spicata* L. Extracts. *Foods* 2023, *12*, 947. https://doi.org/10.3390/foods12050947

Papers submitted to peer-reviewed journals:

Mild heat treatment and biopreservatives for artisanal raw milk cheeses: reducing microbial spoilage and extending shelf-life through thermisation, plant extracts and lactic acid bacteria. Submitted to *Trends in Food Science & Technology.*

Lavandula stoechas L., *Artemisia dracunculus* L. and *Ocimum basilicum* L. polyphenolic extracts as functional food ingredients. Submitted to *Innovative Food Science and Emerging Technologies*.

Modelling the kinetics of *Staphylococcus aureus* in goat's raw milk under different sub-pasteurisation temperatures. Submitted to *Microbial Risk Analysis*.

Dynamic modelling to describe the effect of plant extracts and customised starter culture on the survival of *S. aureus* in goat's raw milk cheeses. Submitted to *Foods.*

Proceedings/Conference papers:

Extraction, chemical characterization, and antioxidant activity of bioactive plant extracts. 1st International Electronic Conference on Food Science and Functional, 2020

Technological Potential of Lactic Acid Bacteria Isolated from Portuguese Goat's Raw Milk Cheeses. 2nd International Electronic Conference on Foods - "Future Foods and Food Technologies for a Sustainable World", 2021

Plant Extracts as Potential Bioactive Food Additives. 2nd International Electronic Conference on Foods - "Future Foods and Food Technologies for a Sustainable World", 2021

Effect of lemon balm and spearmint extracts on the survival of *S. aureus* in goat's raw milk cheese. The 3rd International Electronic Conference on Foods: Food, Microbiome, and Health—A Celebration of the 10th Anniversary of Foods' Impact on Our Wellbeing, 2022

Conference posters:

Effect of lemon balm and spearmint extracts on the survival of *S. aureus* in goat's raw milk cheese. 27th International ICFMH Conference – FoodMicro, Athens, Greece, 2022

Effect of herbal extracts on the survival of *S. aureus* in goat's raw milk cheese. IAFP European Symposium on Food Safety, 2023

Oral communications:

Meta-regression models describing the effects of essential oils and added lactic acid bacteria on *Listeria monocytogenes* inactivation in cheese. 11th International Conference on Predictive Modelling in Food, Portugal, 2019

Meta-regression models describing the effects of added lactic acid bacteria on pathogen inactivation in milk and cheese. FoodSim'2020 Virtual, 2020

Meta-regression models describing the effects of essential oils and added lactic acid bacteria on *Staphylococcus aureus* inactivation in cheese. IAFP Virtual Annual Meeting, 2020

Extraction, chemical characterization, and antioxidant activity of bioactive plant extracts. 1st International Electronic Conference on Food Science and Functional, 2020

Differentiation of six herbal extracts using principal component analysis. 8th Nordic-Baltic Biometrics Virtual Conference, 2021

Technological and antimicrobial properties of indigenous LAB from Portuguese artisanal goat cheeses. Artisanal Foods & Bioactive Biomolecules Seminar (online), 2021

S. aureus inactivation in goat's milk at sub-pasteurisation temperatures. International Seminar Advances in Food Biopreservation, Tunisia, 2022

Omnibus modelling to describe the effects of thermisation on raw milk. International Seminar ArtiSaneFood: Bio-preservation and risk modelling approaches, Portugal, 2023

Effect of herbal extracts on the survival of *S. aureus* in goats' raw milk cheese. International Seminar ArtiSaneFood: Bio-preservation and risk modelling approaches, Portugal, 2023

SECTION I

INTRODUCTION

CHAPTER 1

MOTIVATION AND OUTLINE

This chapter provides the background information that served as motivation for this work, lists the thesis objectives and specifies its outline.

1.1 THESIS MOTIVATION

Staphylococcus aureus, an enterotoxin-producing pathogen, has been described as one of the top causes of foodborne illnesses. In a recent meta-analysis [1], the pooled occurrence of *S. aureus* in goat milk cheeses was found to be high (16%; 95% CI: 7.92-29.8%); while cheeses made of raw milk, regardless of the origin, presented an even higher prevalence of this pathogen (38.7%; 95% CI: 9.28-79.6%). This underscores the importance of improving cheese manufacture and using antimicrobial agents to hinder *S. aureus* development.

Based on the occurring physicochemical changes, fermentation can be regulated to provide microbial stability and safety of cheeses. During fermentation, the organic acid production by lactic acid bacteria (LAB) that decreases the pH, the production of antimicrobial substances by certain LAB strains and the decrease in water activity can act as effective hurdles against pathogens proliferation. However, in faulty fermentations of slow pH drop or short processing periods, which are likely to occur in artisanal cheese production, pathogens can survive. Additionally, as a salt-tolerant pathogen, *S. aureus* can proliferate in salty conditions, making the brining/ripening process insufficient to guarantee the product's quality. The presence of *S. aureus* in various cheese types, including Portuguese soft cheeses, has been reported in several publications [2-5].

Currently, biopreservatives such as functional starter cultures and plant-based antimicrobials have been proposed as hurdles to increase safety and stability of fermented products. LAB strains that produce anti-staphylococcal bacteriocins have been used as functional starter cultures [6] and plant-based antimicrobials have been added to various foods, including low-fat cut cheese and cottage cheese, for instance [7,8], for their effectiveness in delaying degradation and nutritional quality loss and potential improvement of sensory properties [9]. From a technological perspective, to eliminate *S. aureus* during fermentation/ripening, a bacteriocin-producing functional starter culture that causes rapid pH decrease should be used, or, alternatively, a bacteriocinogenic strain in co-culture with an acidogenic LAB, in combination with plant-based antimicrobials that will have a synergetic hurdle effect.

Mild heat treatments have also been suggested and tested by various researchers to reduce the microbial contamination in milk without the need for pasteurisation [10-12]. Thermisation, for example, reduces the number of spoilage and pathogenic bacteria, including *S. aureus*, with minimum collateral heat damage to milk caseins and other constituents, mild effect on the raw milk microflora, and reduced impact on the sensory profile of the cheese [12-15].

Predictive microbiology can be used to understand and model bacterial growth and inactivation, allowing the optimisation of hurdles that provide long term stability and safety. However, most researchers that have studied the effect of plant antimicrobials and LAB against *S. aureus*, have done so in vitro, with few having tested the antimicrobial activity of such biopreservatives in milk or cheese [16,17] or developed models that describe their impact on *S. aureus* growth/survival in those matrices [18,19]. This suggests a gap in using food matrices for antimicrobial testing and in using predictive modelling for this research topic, thus hinting an opportunity for developments. In this sense, this thesis aims to contribute to reduce such gaps, and to provide insight on how plant extracts, LAB and thermisation can be used to ensure the microbial safety of cheeses.

1.2 RESEARCH OBJECTIVES

The main objective of this thesis was to provide specific biopreservation options and novel modelling approaches that could be used to improve the microbiological quality and safety of artisanal goats' raw milk cheeses regarding *S. aureus* contamination. The work focused on the evaluation of different strategies (plant extracts, customised starter culture and thermisation) and their effect on the survival of the pathogen.

To achieve the main objective, the specific aims of this project were:

1. To narrow down the most suitable biopreservation agents by performing a systematic review of biopreservatives suitable for use in cheeses through literature examination and meta-analysis.
2. To evaluate the bacteriostatic and bactericidal effect against *S. aureus* of selected plant extracts and LAB strains isolated and purified from a series of artisanal goat's raw milk soft cheeses.
3. To evaluate the bacteriostatic and bactericidal effect against *S. aureus* of a mild thermal treatment (thermisation) of milk.
4. To develop a functional starter culture with acidogenic and anti-staphylococcal capabilities.
5. To assess the growth or survival of *S. aureus* inoculated in soft cheeses elaborated under the traditional and biopreservation-based manufacturing processes by the conduction of challenge studies.
6. To develop models describing the growth or survival of *S. aureus* in soft cheese produced with biopreservative agents.

1.3 THESIS OUTLINE

This thesis has been structured in four sections, which are divided in a total of ten chapters, six of them reporting experimental results and their discussion (Chapter 4-9).

Section I: Introduction

CHAPTER 1 – The current chapter presents the motivation, research goals and outline of this thesis.

CHAPTER 2 – This chapter comprises a review of the two biopreservation strategies (plant extracts and LAB) and the mild heat treatment (thermisation) selected to be studied in this thesis to improve the microbial safety of artisanal raw milk cheeses.

CHAPTER 3 –This chapter presents the results of meta-regression models describing the effects of essential oils and added LAB on pathogen inactivation in cheese, serving as a review of potential plants with antimicrobial capacity and of good practices for the inoculation of LAB in cheeses for challenge testing.

Section II: Biopreservation Strategies and Mild Heat Treatment

CHAPTER 4 – In this chapter, a study was designed to evaluate the chemical profile and bioactivities of sage, lemon balm, spearmint, tarragon, French lavender and basil extracts produced using two different methodologies (solid-liquid and Soxhlet) and solvents (ethanol 70% (v/v) and water).

CHAPTER 5 – This chapter evaluates the phytochemical composition and bioactive potential of sage, lemon balm and spearmint extracts producing through three distinct methodologies (infusion, decoction, hydroethanolic extraction).

CHAPTER 6 – In this chapter, the phytochemical composition and bioactivities of French lavender, basil and tarragon extracts obtained using three different methodologies (infusion, decoction, hydroethanolic extraction) were evaluated.

CHAPTER 7 – In this chapter, a study was designed to evaluate the antimicrobial capacity and technological properties of LAB isolated from artisanal Portuguese goat's raw milk cheeses.

Section III: Predictive Modelling

CHAPTER 8 – This chapter characterises and validates the heat resistance of *S. aureus* in goats' raw milk at sub-pasteurisation temperatures contrasting two distinct modelling approaches.

CHAPTER 9 – In this chapter, dynamic predictive models are used to describe the effect of plant extracts and of a customised starter culture on the survival of *S. aureus* and on the growth of LAB in goat's raw milk cheeses.

Section IV: Conclusions and Suggestions for Future Work

CHAPTER 10 – This chapter contains the global appraisal of the thesis findings, with the concluding remarks and future perspectives.

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CHAPTER 2

GENERAL INTRODUCTION

This chapter was based on the following submitted paper:

Silva, B.N.; Teixeira, J.A.; Cadavez, V.; Gonzales-Barron, U. Mild heat treatment and biopreservatives for artisanal raw milk cheeses: reducing microbial spoilage and extending shelf-life through thermisation, plant extracts and lactic acid bacteria. *Trends Food Sci. Technol.* (submitted)

2.1 INTRODUCTION

Cheese is a highly nutritious food, with hundreds of varieties that have different colours, odours, flavours and textures, depending on the type of milk used, production and maturation processes, and age, for example.

Artisanal raw milk cheeses are particularly appreciated for their unique sensorial characteristics, which result, among other factors, from the unpasteurised milk. In fact, despite having numerous advantages, such as reducing the bacterial load and extending the shelf-life of milk, pasteurisation causes, among other heat-induced changes, denaturation of whey proteins and complex interactions among denatured whey proteins, casein micelles, minerals and fat globules [1]. These modify the biochemistry and microbiology of milk acidification and cheese ripening and, consequently, the characteristic flavour, aroma and texture of raw milk cheeses cannot be achieved using pasteurised milk [2].

Nonetheless, consumption of raw milk cheeses may pose health safety issues, due to the possible presence of pathogenic bacteria in raw milk that can remain viable during manufacture and through ripening [3–5]. The consumption of this type of dairy product has caused a few outbreaks [6–10], thus highlighting the need for preservation strategies to improve the microbial safety of raw milk cheeses.

Chemical preservatives would not be suitable for artisanal cheeses, as they would disregard the appeal of a traditional product derived from cultural heritage and produced using only natural, healthy ingredients. Furthermore, they would be an outdated preservation strategy, as the mishandling and extensive consumption of some chemical additives have shown to induce gut microbiota dysbiosis, which is a contributing factor to various diseases, including neurodegenerative ones [11–13]. Finally, current consumer expectations are increasingly towards “clean-label”, chemical preservative-free food products, and consequently, the food industry and scientific community are compelled to investigate novel food preservation methods [14].

Between other techniques, advanced non-thermal technologies (high pressure, cold plasma, pulsed light, and ultrasound) and packaging systems (bioactive films, coating, and modified atmospheric packaging) are among the innovative cheese preservation approaches developed to inactivate microorganisms in milk and extend the shelf life of raw milk cheeses [15]. However, these are not easily implementable for artisanal producers, mainly because of the need for specific and costly equipment, as well as the need for training to operate such technologies.

On the other hand, the incorporation of natural antimicrobial agents in artisanal cheese production is more feasible, since starter cultures (lactic acid bacteria, LAB), plant extracts, essential oils and propolis [15,16] can be easily purchased and added directly to the milk, cheese curd, or final product. Another alternative would be to implement a mild thermal process such as thermisation, which uses sub-pasteurisation temperatures, to reduce bacterial load while avoiding large heat-induced changes in milk that would affect the final typical organoleptic characteristics of raw milk cheeses [17,18]. This technology would also be easy for artisanal producers to implement since it does not require specialised equipment.

Considering the above-mentioned possibilities, this review presents an overview of the existing information on LAB and plant extracts as biopreservative strategies, as well as thermisation as a mild heat treatment, to be used in raw milk cheeses.

The main microorganisms involved in cheese spoilage are described, and, for each biopreservation strategy, the various targets, mechanisms of antimicrobial action, limitations and, when applicable, relevant commercial applications are discussed.

2.2 SPOILAGE MICROORGANISM IN RAW MILK AND RAW MILK CHEESES

The most prevalent spoilage fungi genera identified in raw milk and cheeses are *Candida*, *Cryptococcus*, *Debaryomyces*, *Geotrichum*, *Kluyveromyces*, *Trichosporon*, *Pichia*, and *Rhodotorula* spp. (yeasts), and *Penicillium*, *Aspergillus, Cladosporium, Mucor, Fusarium* and *Alternaria* spp. (moulds) [19–22]. *Candida rugosa*, *Geotrichum candidum*, *Torulaspora delbrueckii*, *Kluyveromyces marxianus* and *Yarrowia lipolytica* are among the common yeast species found in raw milk, while *Penicillium commune* is one of the most frequently occurring mould species [20,22,23].

Psychrotrophic bacteria dominate the microflora of raw milk, particularly species of the genera *Pseudomonas*, *Acinetobacter*, *Aeromonas*, *Serratia*, *Bacillus*, *Lactococcus*, *Microbacterium*, and *Staphylococcus* [19,24]. Other bacteria associated with cheese spoilage are *Enterobacteriaceae* and clostridial species (*E. cloacae*, *E. agglomerans*, *E. zakazakii*, C. *tyrobutyricum*, *C. butyricum*, *C. sporogenes*, and *C. beijerinckii* have been isolated from milk) [25,26].

The main pathogens of concern that have been detected in raw milk cheeses include enterotoxin-producing *Staphylococcus aureus*, Shiga toxin-producing *Escherichia coli* (STEC; *E. coli* O157:H7, for example), *L. monocytogenes*, *Salmonella* spp., *Brucella* spp. and *Campylobacter*

spp. [4,8,19,27,28]. These pathogens may be shed directly into milk via the udder by a diseased or infected animal or may enter milk from the external surfaces of animals, the environment, the milking environment, equipment or from personnel (operators' hands, for example) [4,28]. *L. monocytogenes* and STEC have been identified as especially high-risk pathogens owing to the severity of illness and potential lethality associated with each [17].

2.3 BIOPRESERVATION STRATEGIES

2.3.1 Plant extracts

The use of plants and herbs as colouring and flavouring agents in cheese manufacture is not new, with some traditional herb-flavoured cheeses having centuries of history [29]. However, plants may be used for more than their organoleptic and decorative properties, owing to their phytochemical constituents that have been shown to have antimicrobial activity [16,30]. The addition of plants and herbs to cheese can be carried out by incorporating them into milk (before cheese making), into cheese curd, or by rolling the cheese into crushed herbs, for example [16].

Plant extracts can be obtained from a multitude of plants, using various solvents and extraction methodologies. However, if intended for human consumption, they must be obtained using non-toxic solvents authorised for the industrial production of foodstuffs and food ingredients [31] such as water, ethanol, or their combination.

Conventional extraction procedures include maceration, percolation, infusion, decoction, reflux extraction, Soxhlet extraction and hydro-distillation (which can be subcategorised into steam-, water-distillation, or a combination of both) [32–34]. While these may still be widely used, nowadays, it is crucial to consider the ecological impact of extraction methods, and those that are more sustainable and "green", reducing the amount of solvents used and waste generated, and optimising the recovery of bioactive compounds with high added value, should be preferred [35]. To this, techniques such as subcritical water extraction, supercritical fluid extraction, enzyme-, microwave- and ultrasound- assisted extractions, pulsed electric field extraction and accelerated solvent extraction can be used, among many other modern procedures [32–34,36]. Moreover, as the extraction method, temperature, solvent and pressure, for example, influence the chemical profile of the extracts produced, the most appropriate extraction parameters should be selected, considering the desired compounds and bioactivity [35,37]. In addition, the plant genotype,

geographical location, environmental and agronomic conditions, among other factors, also contribute to variations in the chemical composition of plant extracts [38].

Based on their structure, plant-derived chemicals may be classified as alkaloids, organosulfur compounds, phenolic compounds, coumarins and terpenes [39]. Generally, phenolic compounds are found in higher concentrations in plants [40] and assumed as the main antimicrobial agents [40–42], although the remaining compounds have also shown this capacity [39]. With respect to the chemical structure of the bioactive compounds, it has been demonstrated that functional groups such as hydroxyl groups and the number of double bonds can influence antimicrobial strength [34].

The exact targets of plant antimicrobials are often difficult to define, considering the many interacting reactions taking place simultaneously [30] and the various compounds found in plant extracts, each exerting its own effect [39]. Nonetheless, several mechanisms have been suggested to explain the antimicrobial mode of action of plant extracts. These include inhibition of efflux pumps (implicated in the export of harmful substances from within the cell into the external environment) [39] and permeabilisation or disruption of the cell membrane, which allows, respectively, the passage of compounds or the release of intracellular contents (especially potassium, calcium, and sodium ions [31]), adding to the loss of cellular integrity [30,34,40,42,43]. Disruption of the cell membrane may be prompted, for example, by the interaction of phenolics with membrane proteins, inducing alterations in their structure and function, namely in terms of electron transport, nutrient uptake, synthesis of proteins and nucleic acids, and enzyme activity [34]. Additionally, plant extracts may also inhibit DNA and protein synthesis [39], inactivate cellular enzymes (including ATPase) [42,43], and dissipate cellular energy in ATP form [30].

Different mechanisms of action have been reported for distinct groups of compounds. In fact, while membrane disruption is associated with the action of terpenoids and phenolics, the antimicrobial properties of phenols and flavonoids seem related to their chelating properties complexing metal ions that are essential for the bacterial growth, whereas coumarin and alkaloids seem to produce effects on genetic material [30,38]. In its turn, the antimicrobial activity of some organosulfur compounds, such as onion and garlic isothiocyanates, is due to the inactivation of extracellular enzymes through oxidative dissociation of -S-S- bonds [34]. The mechanism of action may also be dependent on the concentration of the compounds, as it has been shown that at a

low concentration, phenols inhibit microbial enzyme activity, whereas at high concentrations they induce protein denaturation [41].

Irrespective of the mode of action, it is recurrent that Gram-positive bacteria are more susceptible to plant extracts and phenolic compounds than Gram-negative, whose greater resistance is due to the existence of lipopolysaccharides in their outer membranes [34,40,44].

Considering that cheese is a fermented product that contains natural and, sometimes, artificially added microbial populations of LAB, which are a group of Gram-positive organisms, it is reasonable to question if using plants and plant extracts as preservatives may influence bacterial metabolism and/or inhibit this beneficial set of bacteria, potentially compromising the fermentation process. Some studies have reported on this drawback [45–48], including that of Shori et al. that observed a reduction in peptides content and free amino acids of cheeses in the presence of three different types of plant extracts (*I. verum*, *C. longa*, and *P. guajava*), caused by the impairment of LAB growth and, consequently, LAB proteolytic activity [49].

Nonetheless, the ability of herbal extracts to impact LAB is determined by a number of variables, including the genus, species and strain of the LAB, as well as the plant species and the extraction method used, for example [50]. Various studies have shown that when selected plant extracts are employed in appropriate amounts, they may be able to promote the growth of desired microorganisms, or at least not affect them negatively, while avoiding the development of harmful bacteria [50]. For example, Mohamed et al. [51] reported the inhibitory effect of ethanolic and aqueous extracts of *Moringa oleifera* leaves against numerous pathogens in vitro, stressing that these did not inhibit LAB growth. In addition, Ziarno et al. [50] investigated the effect of seven plant extracts (valerian, sage, chamomile, cistus, linden blossom, ribwort plantain and marshmallow) with known antimicrobial activity against pathogens on the activity and growth of LAB and observed that the addition of such extracts up to 3% in milk did not hinder the growth of LAB in fermented milk drinks such as yogurts. Likewise, Chouchouli et al. [52] supplemented yoghurts but with grape seed extracts and did not observe any effect on pH or the viability of *Lactobacilli*.

Considering the distinct results described in the literature, it is important to establish if a particular plant extract can be successfully used in cheeses, by evaluating its impact on the growth and the technological properties of LAB populations, whether they are endogenous raw milk flora or intentionally added starter cultures.

Other issues that should be considered when adding plant extracts to cheeses are, for instance, the influence of the food matrix, the loss of bioactive compounds during cheesemaking and storage, and the organoleptic characteristics of the novel cheese.

The food matrix is an important factor as interactions with food ingredients occur, resulting in reduced biological activities of the natural compounds, when comparing the results of in vitro and in situ (cheese) studies. More specifically, it is generally accepted that high concentrations of lipids or proteins limit the antimicrobial efficacy of plant extracts [53–56]. Studies regarding the effects of carbohydrates on the antimicrobial activity of plant extracts are scarce [57], as most of the literature focuses on the interaction between carbohydrates and plant essential oils. In this case, different authors report contrasting results: Gutierrez et al. [58] observed a reduction in oregano and thyme essential oils efficacy when testing 5% and 10% starch concentration; whereas Shelef et al. [59] reported that carbohydrates in foods do not protect bacteria from the antimicrobial action of essential oils, at least not as much as fat and protein. The complexity of the food structure also plays an important role in the biological activity of plant extracts in food, as well as the changing variables during cheese production (namely water activity, pH, microflora composition, temperature and nutrient composition) [40].

Natural compounds can be lost during cheese making or storage as a result of their sensibility to environmental factors (including light, temperature, oxygen and pH [40,60], which can cause the epimerisation of bioactive components [60]), solubility in whey [61] or solubility of hydrophobic active molecules in lipidic phases [16]. Aqueous phases are generally the preferred ones for cell growth [62], not lipidic phases, although some bacteria have been reported to have a preference for the fat-water interface in emulsion systems [63–65].

Although not as intense as essential oils, plant extracts may still negatively affect the sensory characteristics of the food product, especially if the concentrations needed to inactivate pathogens and ensure food safety are higher than those that lead to acceptable sensory properties of the treated products [58]. Nonetheless, numerous studies have reported improved sensory quality of cheeses containing plant extracts [66–70], thus showing that the sensory issue does not always arise, and that it is dependent on the antimicrobial dose applied.

To avoid interactions with food components, degradation and loss of bioactive compounds, as well as the unpleasant taste of polyphenols, bio-based functional packaging materials incorporating natural active compounds and ingredients may be used (for example, coatings and edible films using nano- and microencapsulation techniques) [38,40,60].

Other concerns that must be taken into account include: (i) the effects of plant extracts and their natural compounds on human health, as typical toxicological information such as "acceptable daily intake" or "no observed adverse effect level" are usually not available [30]; and (ii) the economic costs, legislation, and practical effectiveness [40] of using plant extracts as preservatives in the food industry.

The potential toxicity of plant extracts is generally difficult to define considering the problems in their standardisation, due to the great variability in their composition between batches [30]. In terms of economic costs and legislation, it is crucial that the price of natural preservatives is competitive in comparison to that of synthetic compounds providing comparable antimicrobial effect, and that plant additions in and on foods comply with the existing legislation [71,72], which nonetheless is still limited and must be improved (for example, natural additives are legislated in the same manner as synthetic ones, making it sometimes difficult to understand how production is carried and what is their source [73]).

Overall, it is clear that plant extracts can be useful as antimicrobial agents in foods, including raw-milk cheeses, although further scientific and legal grounds are needed to motivate and simplify the use of such additives.

2.3.2 Lactic acid bacteria

Traditional raw milk cheeses exhibit a complex microbiota, including LAB naturally occurring in milk and purposefully introduced LAB [74]. They comprise a large and heterogeneous group and bacterial communities differ vastly among raw milk cheeses, but, usually, the main genera identified in raw-milk artisanal cheeses include *Lactococcus*, *Lactobacillus*, *Enterococcus*, *Streptococcus* and *Leuconostoc* [74,75].

LAB can be relevant for their role as starter cultures, which promote the rapid acidification of milk (crucial for an adequate fermentation and production of high-quality cheeses) through the production of organic acids (primarily lactic and acetic acids) [74]. Starter cultures and adjunct cultures (also called non-starter LAB) can also contribute to the maturation of cheese and development of desirable texture, flavour, aroma and nutritional value, as a result of their metabolic features [74]. and are commercially available [74,76]. Various selected LAB strains or mixture of strains are commercially available as starter cultures for cheese production, and the most frequently used species are *Lc. lactis* (particularly subspecies *lactis* and *cremoris*), *S. salivarius* subsp. *thermophilus*, *L. helveticus*, and *L. delbrueckii* [74,77].

Furthermore, LAB may also have probiotic potential, meaning that they can offer health-promoting benefits to consumers. These include immune system modulation [78], improvement of mental health via the gut-brain axis [78], degradation of nutrient-damaging compounds, such as biogenic amines [79] and cholesterol [80], and increase of the quantity of beneficial compounds, such as antihypertensive peptides [81], short-chain fatty acids [82], γ-aminobutyric acid and conjugated linoleic acid [83].

Besides their role in successful fermentations, contribution to textural and sensorial characteristics, and health-promoting properties, some LAB species and strains can also act as antimicrobial agents during and after fermentation, throughout the maturation/storage step. This can be due to competition for the adherence site [84], competition for nutrients (i.e., Jameson effect [85]), ability to acidify the environment, and ability to produce antimicrobial metabolites, during fermentation, which remain in the final product (except for volatile compounds) [74,86]. In fact, some studies have screened the antimicrobial properties of these microorganisms as a strategy to improve the safety of cheeses and successfully used cocktails of LAB strains to hinder the growth of pathogenic bacteria [86–88].

The antimicrobial metabolites produced by LAB that reduce the risk of pathogen growth and survival include organic acids, hydrogen peroxide, diacetyl, fatty acids, reuterin and bacteriocins [74].

Acidification of the environment by organic acids creates adverse conditions for the growth of spoilage and pathogenic microorganisms [89]. *S. aureus*, for example, is strongly inhibited by lactic and acetic acids, as most Gram-negative and neutrophilic bacteria [90]. Undissociated organic acids can diffuse across the cell membrane of pathogens when $pH_{environment} < pKa$ and dissociate within the cell (due to the higher cellular pH), which lowers the cytoplasmic pH [89]. This affects various metabolic processes, promotes accumulation of toxic anions, dysregulates cell homeostasis, and neutralises the electrochemical proton gradient, disrupting the substrate transport systems and the cell membrane, which potentially leads to the death of the organism [34,74,89]. The concentrations and types of organic acids produced during fermentation are specie- and strain-dependent, and also vary with matrix composition and growing conditions [91].

Hydrogen peroxide can be produced by LAB in the presence of oxygen through the action of flavoprotein oxidases or NADH peroxidases [91]. Since LAB cannot degrade this compound, it accumulates in the medium, exerting its bactericidal effect through the destruction of basic molecular structures of cell proteins, denaturation of metabolic enzymes (by oxidation of sulfhydryl

groups), and peroxidation of membrane lipids, which increases cell membrane permeability [89,90]. Hydrogen peroxide may also serve as a precursor to the DNA-damaging superoxide ($O_2^{\bullet -}$) and hydroxyl (·OH) free radicals [89]. In milk, hydrogen peroxide activates the lactoperoxidase system, which has proved bacteriostatic and/or bactericidal activity against various Gram-positive and Gram-negative bacteria [91,92].

Diacetyl is an aromatic compound produced by some LAB strains in the presence of organic acids such as citrate, which is converted via pyruvate into diacetyl (citrate fermentation) [89,91]. Lactobacilli and enterococci are the genera associated with high diacetyl production, whereas *Leuconostoc* strains produce none or low amounts of diacetyl from citrate [93]. Jay [94] showed that diacetyl was much more effective against Gram-negative bacteria, yeasts, and moulds than against Gram-positive bacteria, while LAB and clostridia were virtually unaffected. The same study also showed that the inhibitory activity of diacetyl against Gram-negative bacteria was related to its interference with arginine utilisation in the periplasmic space, and that pH has an inverse synergistic effect on diacetyl's bioactivity (lower pH, higher bioactivity) [94], statements corroborated by the research of Tan et al. [90].

LAB can produce various fatty acids that improve the sensory attributes of fermented products while potentially exerting antibacterial and antifungal activity [74]. The antibacterial mechanisms of action of these compounds include DNA/RNA replication inhibition, cell wall biosynthesis inhibition in Gram-positive bacteria, inhibition of protein synthesis, cytoplasmic membrane disruption and inhibition of metabolic pathways [95]. The literature available reports that both unsaturated and saturated fatty acids have antibacterial properties towards Gram-positive and Gram-negative bacteria [95], but that fatty acids with medium and long carbon chains, such as lauric (12C) and capric (10C) acids, provide higher inhibitory effects than short chain fatty acids (< 8C) [95,96].

Lactobacillus reuterin strains can anaerobically convert glycerol into 3-hydroxy-propionaldehyde (3-HPA), which in aqueous solutions exists in equilibrium as a dynamic system of 3-HPA, 3-HPA hydrate, 3-HPA dimer and acrolein [91,97,98]. This multi-compound system is commonly known as reuterin [91,97,98]. Effective against Gram-positive and Gram-negative bacteria, yeasts, moulds and protozoa [41], this broad-spectrum antimicrobial aldehyde can also be produced by other LAB, including *L. brevis*, *L. buchneri*, *L. collinoids*, and *L. coryniformis* [99]. The antimicrobial activity of reuterin has been linked to the ability of 3-HPA to cause depletion of free thiol groups in glutathione, proteins and enzymes, resulting in an imbalance of the cellular

redox status and leading to bacterial cell death [100]. However, the work of Engels et al. [97] suggested, for the first time, that acrolein, and not 3-HPA, is the active compound responsible for the antimicrobial activity attributed to reuterin. The high potential of reuterin as a food biopreservative is supported by its hydrosolubility, stability over a wide range of pH and temperatures, and resistance to degradation by proteolytic and lipolytic enzymes [34,98]. Moreover, reuterin has a wider range of antimicrobial activity than bacteriocins and other non-bacteriocin antimicrobial compounds [98]. However, due to legislative and regulatory requirements, reuterin is not yet commercially available [101].

To that, bacteriocins are extracellularly released bioactive peptides or peptide complexes synthetised in ribosomes [74]. They have narrow-to-broad antimicrobial effect against bacteria in the same species or across genera, respectively [102], and the producer cell exhibits specific immunity to the action of its own bacteriocin [89]. The majority of bacteriocins produced by LAB are active only against LAB and other Gram-positive bacteria [103,104], but some studies reported on their effectiveness also against Gram-negative bacteria [104,105]. Antifungal bacteriocins have also been reported, with *Lactobacillus* species being the most predominant isolates associated with such compounds [106]. Bacteriocin-producing LAB include *Lactococcus*, *Lactobacillus*, *Pediococcus*, *Streptococcus* and *Enterococcus* strains [102]. The mechanism of action of bacteriocins depends on their primary structure [105]. In bacteria, while some bacteriocins can promote the formation of pores in the phospholipidic bilayer of the cytoplasmic membrane, causing the dissipation of the proton motive force and loss of cell contents; others can inhibit cell wall synthesis or enter the cytoplasm, and affect gene expression and protein synthesis [105]. The antifungal mode of action of protein compounds by LAB, however, remains somewhat unclear, requiring further studies [106,107]. Bacteriocins maintain activity at high temperatures and over a large pH range, and as they are rapidly hydrolysed in the human gastrointestinal tract by digestive proteases, they pose no negative impacts to the gut microbiota [74]. Currently, and although other LAB bacteriocins have shown potential to be used as biopreservatives, only nisin A, produced by *Lc. lactis*, and pediocin PA-1, produced by *P. acidilactici*, have been approved as food preservatives for industrial application and are commercially available [74,76].

Considering the vast diversity of LAB species and antimicrobial metabolites available, there are numerous possibilities for improving food safety and preventing microbial food spoilage. Nonetheless, it is important to consider any potential limiting factors that might reduce the antimicrobial activity of LAB or its compounds. In this sense, the food matrix and its inherent

microflora [108], environmental conditions (such as temperature and pH), aerobic conditions, LAB growth phase and load [84], and pathogen content, for example, are among the factors that should not be disregarded when aiming to use such biopreservatives in foods.

2.4 THERMISATION

Thermisation is the standard description for a range of sub-pasteurisation heat treatments of milk, generally from 57 to 68 °C with a holding time between 5 seconds and 30 minutes, that is able to reduce bacterial contamination by 3 to 4 log [17,19,109–112].

Unlike pasteurisation, thermisation causes minimum collateral heat damage to milk constituents, mild effect on the raw milk flora and functionality of milk caseins and salts, and reduced impact on the sensory profile of the final cheeses [18,109–111,113]. For example, since the heat load is lower, compared to that used in pasteurisation, enzymes involved in cheese flavour development, such as lipoprotein lipase, are less inactivated [111]. For this reason, this process may be suitable to produce artisanal cheeses as it reduces microbial counts and simultaneously enables the profile of the heat-treated milk to be closer to that of raw milk, thus allowing the desirable sensorial properties of typical raw milk cheeses to develop [18].

The mechanisms for heat inactivation of mesophilic microorganisms have been extensively studied, and while the ultimate cause leading to cell inactivation by heat remains uncertain, it is clear that heat can affect a wide range of cellular structures and functions, generally known as cellular targets [114,115]. Focusing on non-sporulating bacteria, the cellular targets most affected by heat treatments are the outer and inner membrane, the peptidoglycan cell wall, the nucleoid, the cell's RNA, the ribosomes, and the proteins [114].

Damage to the outer cell layers of bacteria (cell wall for Gram-positive bacteria; outer membrane for Gram-negative bacteria) has been reported by several researchers: in Gram-negative bacteria, damage to the outer membrane after mild thermal treatment can be verified by loss of outer membrane lipopolysaccharides [116] and morphological and structural changes [117] in membrane integrity and permeability, which leads to the release of periplasmic proteins and sensitivity to hydrophobic antibiotics, for example [118,119]. The cell wall of Gram-positive bacteria is also susceptible to heat, but these organisms are generally more heat-resistant due to the high content and extent of cross-linked peptidoglycan of the cell wall [114].

The cytoplasmic or inner membrane (of Gram-positive and Gram-negative bacteria, respectively) damage by heat injury can be detected through the loss of intracytoplasmic material

leaked from the heated cells, including RNA, DNA, proteins, enzymes, amino acids, and potassium ions, for example [120–122]. Furthermore, the formation of membrane vesicles and loss of membrane material and integrity after heat treatments have also been reported [114,123,124].

Although DNA has high thermostability [125], less intense heat treatments can still modify the nucleoid structure and damage the DNA molecule during and after the treatment [114,124]. Heat-induced DNA damage is manifested by single or double strand breaks, as well as increased mutation frequency in surviving populations after heat exposure [126,127]. Moreover, single strand denaturation induces the action of deoxyribonucleases, which further degrades DNA via hydrolysis of its phosphodiester backbone [128].

RNA and ribosomes, on the other hand, are more heat-sensitive than DNA [129]. In that sense, mild temperatures have been reported to cause degradation of ribosomes and ribosomal RNA (rRNA), with associated leakage of substances from the metabolic pool (free amino acids and proteins, for example) [130-132] that precedes loss of cell viability. Denaturation of 70S ribosomes and 30S and 50S ribosomal subunits can be a consequence of membrane heat-damage and subsequent depletion of magnesium ions from within the cell, as they are essential for the maintenance of the coupled ribosome subunits [130].

Proteins, whether structural or functional (enzymes, for example), may undergo denaturation when bacterial cells are thermally stressed [115]. Protein pumps and channels are also heat-sensitive [114], and, as a response to misfolding and denaturation, protein aggregation may also occur [133]. Rosenberg et al. found a correlation between the thermodynamic parameters of protein denaturation and the death rates of several bacteria [134]. Nevertheless, irreversible denaturation of some proteins might not be lethal to the cell if they can be resynthesised after the heat treatment. On the other hand, it is hypothesised that irreversible denaturation of all copies of RNA polymerase, for example, would represent a lethal event, as this enzyme could not be resynthesised by a cell lacking a single copy [135]. Research has shown that proteins irreversibly denatured by heat are governed by chemical modifications, including deamination of Asn/Gln residues, hydrolysis of peptide bonds at Asp-X residues (X being a small hydrophobic residue), and disulphide bond scrambling [135].

To summarise, the most relevant cellular events that can occur after heat exposure include permeabilisation of membranes, DNA and RNA alterations, loss of ribosome or protein conformation and loss of intracellular components [114]. As microbial inactivation by heat is a multi-target phenomenon, these events may be interconnected and are likely to occur

simultaneously [114]. In any case, the lethality of a heat treatment is contingent to the alteration of at least one critical component (one whose destruction triggers cell death) beyond a critical threshold, which can be a result of the direct effect of heat on the critical cellular target itself, or a consequence of a parallel alteration of another cellular target [114]. It is also crucial to consider that the resistance of each cell target depends on the environmental conditions and the type of microorganism (pH and water activity of the medium during the heat treatment, for example; Gram-positive vs. Gram-negative bacteria, as mentioned before in this section) [114]. Additionally, exposure to sublethal thermal stresses can mediate adaptive responses in bacteria, including the induction of heat shock proteins which are determinant for protein folding, repair and degradation, and the prevention of aggregation, thus promoting increased heat resistance and, consequently, bacterial survival [136,137].

Thermisation has been noted for both psychrotroph and pathogen control [28,138,139]. Nevertheless, and as previously described, different microorganisms may respond differently to heat treatments, depending on a variety of factors [17]. In this sense, a few authors have reported the survival of some yeasts [140]; that some pathogens may remain viable at the lower end of the thermisation temperature range, where the lethal effect is more reduced [28,111]; and that thermisation may not be enough to significantly reduce the population of vegetative cells of the more heat resistant bacterial species (*Enterococcus*, for example) [111,139]. Besides the possibility of some bacteria remaining viable in thermised milk, other shortcomings associated with this thermal treatment are the possible germination of spores present in milk during subsequent cold storage (for example, thermisation at 65 °C for 10 seconds may be sufficient to stimulate the germination of *B. cereus* spores [109]) and the possible selection for heat-resistant microorganisms such as *M. tuberculosis* and *C. burnetii*, by enabling their survival while reducing competitive flora [27,110]. Thermisation may also have a negative impact on LAB strains and the biodiversity of raw milk bacteria. To this, Sameli et al. [113] observed that thermisation at 60 °C for 30 seconds reduced the total number of *Leuconostoc*, *Lactococcus* and mesophilic *Lactobacillus*, while producing an enterococcal selecting effect. To avoid such negative effects, it is important that thermisation parameters are carefully selected, aiming to target pathogens while preserving LAB as much as possible. Moreover, the addition of a starter culture post heat treatment to counteract reduction in LAB numbers may also be recommended.

2.5 CONCLUSIONS

Artisanal raw milk cheeses may impose health issues to consumers, considering that the manufacturing processes are not standardised, and good manufacturing practices are not always followed, which can lead to undesirable microbiological quality of the cheeses. To avoid pasteurisation and the use of chemical preservatives, which are unfit for this niche product, this work collected and discussed the main antimicrobial action mechanisms, bacterial targets, advantages, limitations and, whenever possible, relevant commercial applications of two biopreservatives, plant extracts and lactic acid bacteria, as well as a mild heat treatment of milk, thermisation, with the goal of promoting their use in cheese production. The literature currently available is supportive of the use of these strategies for the improvement of the microbiological quality of artisanal raw milk cheeses, although some considerations, such as their impact on the sensory characteristics of the product and on the natural microflora, must be carefully assessed, as referred in this review.

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CHAPTER 3

META-REGRESSION MODELS DESCRIBING THE EFFECTS OF ESSENTIAL OILS AND ADDED LACTIC ACID BACTERIA ON PATHOGEN INACTIVATION IN CHEESE

This chapter was based on the following paper:

Silva, B.N.; Cadavez, V.; Teixeira, J.A.; Gonzales-Barron, U. Meta-Regression models describing the effects of essential oils and added lactic acid bacteria on pathogen inactivation in cheese. *Microb. Risk Anal.* 2021, *18*, 100131. https://doi.org/10.1016/j.mran.2020.100131

3.1 INTRODUCTION

Listeria monocytogenes (LM), *Staphylococcus aureus* (SA) and *Salmonella* spp. (SS) are some of the most common bacterial agents causing foodborne illnesses and are found in numerous food matrices, including different types of cheeses [1-9]. A recent meta-analysis showed pooled prevalence of 12.8% for LM and 16% for SA in goat raw milk cheeses, while the prevalence of SS was lower (5.91%), but still concerning [10]. LM and SS can cause illnesses even when in low numbers in any food product, including cheese [11]. On the other hand, a larger number of SA (above 10^5 log CFU/g) is required for this pathogen to be able to produce enterotoxins and impose a serious health threat [12]. Nevertheless, SA imposes an important contamination issue since, even at low initial contamination levels, many factors can contribute to SA growth to a sufficiently high concentration that enables enterotoxin production in the curd/cheese [13]. Overall, soft and semi-soft cheeses made from different milk kinds and types (pasteurised, raw or low-heat-treated; and from cows, goats, sheep, etc.) sampled at retail level have revealed non-satisfactory results in terms of contamination by pathogens [14] thus underscoring the importance of improving the safety of cheeses to reduce the occurrence of pathogens.

Biopreservatives such as bacteriocinogenic lactic acid bacteria (LAB) used in starter cultures, and plant-based antimicrobials such as essential oils (EOs) are hurdles used to increase microbiological safety cheeses. The microbial inhibition offered by bacteriocinogenic LAB is mostly due to competition for substrates, production of antimicrobial substances (bacteriocins), production of organic acids that drop the pH during fermentation, and production of other non-proteinaceous compounds such as H_2O_2 [15]. The mechanism of action of EOs include a series of events on the cell surface, and, consequently, within the cytoplasm [16]. Modifications of membrane permeability and compromised transport of molecules can lead to degradation of the cell wall (damaging the cytoplasmic membrane), increased permeability (causing the leakage of cell contents), denaturation of enzymes and cellular proteins, loss of metabolites and ions [16], and cytoplasm coagulation [16, 17].

Over the past years, several authors have performed challenge studies of foodborne pathogens inoculated in milk or cheese to assess the antimicrobial capacity of functional starter cultures or selected LAB [18-20] and plant-based antimicrobials [21-25]. Thus, a meta-analysis of the published results on the effect of antimicrobial biopreservatives will help evaluate their usefulness to control foodborne pathogens in cheeses [26]; and more specifically, compare the

effectiveness of the different biopreservatives and modes of application. In this meta-regression study, the *population* is defined as cheeses with added lactic acid bacteria or essential oils, and the *measured outcome* is the mean log reduction of pathogens. This study aims to deliver an insight on the effects of biopreservation methods in cheese for the optimisation of these hurdle technologies to improve the safety of cheeses.

3.2 MATERIALS AND METHODS

3.2.1 Data collection and description of the data set

Electronic literature search was carried out in Scopus, PubMed and Web of Science databases to find original and review articles, published since 2000, summarising biopreservation methods currently tested and/or applied in cheese-making and their efficiencies against pathogens. The search was done systematically and aimed to find quality studies validated by the scientific community.

The bibliographic searches were conducted by properly applying the AND and OR logical connectors to combine terms regarding biopreservation and terms referring to biopreservatives characteristics and capacities in the selected products, as follows: (preservative OR bio-preservati* OR biopreservati* OR "starter culture" OR starter OR "lactic acid bacteria" OR "essential oil" OR extract) AND (antimicrobial OR inhibitory OR natural OR plant OR functional) AND (activity OR capacity OR propert* OR effect) AND (cheese OR "fermented milk"). When studies referenced in the collected articles were not present in the results of the literature search, said references were added. Grey literature (research that has not undergone peer-review for publication) was not acquired to avoid data validity concerns and data duplication, since high-quality theses and reports are likely to be also published in peer-reviewed journals. Other meta-analysis studies and systematic reviews were also excluded. The criteria for inclusion of data were: (i) the inoculum level, temperature of storage and antimicrobial concentration must be reported in the study; (ii) essential oils could not be mixed; (iii) if an antimicrobial film was used, the control must also be coated with the film but without the antimicrobial (as opposed to uncoated); and (iv) each study must have collected mean log reduction values at a certain time point (or, alternatively, it should provide mean microbial concentrations for the treated and control groups, so that reduction could be thereof calculated). This last criterion signifies that microbial reduction was relative to the control and does not necessarily mean that microbial inactivation occurred in the treated group. Even if

bacterial growth occurred in both control and treated groups, if it was lower in the treated group, the observation would be admitted into the meta-analytical data set.

After assessing all the information from all publications, 53 studies published from 2000 until August 2019 were considered appropriate for inclusion [19; 23-25; 27-75]. The study ID, antimicrobial class (EO or LAB) and specific name, pathogen mean log reduction, storage temperature, exposure time (defined as the time at which the log microbial reduction was quantified in the challenge study), application type (defined as the mode of application of the antimicrobial; namely, milk, film, cheese surface and cheese mixture), antimicrobial concentration and pathogen inoculum level (log CFU/g or ml) were collected for the selected studies. The application type "milk" refers to the direct addition of the antimicrobial agent in bulk milk before curding, while the application type "cheese surface" refers to the practice of applying the tested antimicrobial onto the cheese surface. The category "film" was assigned to those challenges studies where the antimicrobial was embedded in the packaging material through micro- or nano-encapsulation. The application type "cheese mixture" was a special category created to accommodate those challenge studies whose experimental methodology consisted of grinding cheese, inoculating it with the pathogen, and adding the antimicrobial. Thus, "cheese mixture" does not reflect a real mode of application of antimicrobials in the cheese manufacturing process context, but an experimental protocol for challenge studies that researchers have probably devised for being handy although not realistic. Moreover, some of these cheeses were not produced in the laboratory under controlled conditions but purchased for subsequent grinding and inoculation. **Table 1** presents summarised information of the types of cheeses, and **Table 2** compiles the study characteristics extracted from each primary study *j* and the distribution of mean log reduction data among the different levels for each pathogen. For simplification, the types of application "cheese mixture" and "cheese surface" will be referred to as "mixture" and "surface," respectively.

Table 1. Distribution of log reduction data in *L. monocytogenes* (LM), *S. aureus* (SA) and *Salmonella* spp. (SS) by cheese descriptive category for the two bio-interventions strategies meta-analysed

Bio-intervention	Categories	Level	LM	SA	SS
Essential oils (N=1305)	Treatment of milk	Pasteurised	487	77	254
		Sterilised	30	3	-
		Not stated	215	138	101
	Milk species	Bovine	446	80	254
		Caprine	131	56	59
		Not stated	155	82	42
	Type of cheese	Cheese based broth	44	43	-
		Semi-hard cheese	6	3	-
		Semi-soft cheese	68	-	-
		Semi-solid cheese model	17	15	-
		Soft cheese	381	83	296
		Not stated	216	74	59
	Label	Coalho cheese	61	58	-
		Cream cheese	168	-	158
		Domiati cheese	24	24	96
		Feta cheese	131	56	59
		Iranian white cheese	70	53	-
		Kashar cheese	6	3	-
		Lor cheese	24	24	-
		Minas cheese	38	-	-
		Mozzarella cheese	68	-	-
		White cheese	72	-	-
		Undefined cheese	70	-	42
	Starters	Present	151	59	96
		Absent	247	34	-
		Not stated	334	125	259

Added LAB (N=505)	Treatment of milk	Pasteurised	316	60	3
		Raw	6	21	-
		Sterilised	60	-	-
		Not stated	39	-	-
	Milk species	Bovine	218	36	-
		Mixed	34	-	-
		Caprine	36	26	3
		Ovine	4	-	-
		Not stated	129	19	-
	Type of cheese	Hard cheese	4	-	-
		Semi-hard cheese	11	17	-
		Soft cheese	365	35	-
		Not stated	41	29	3
	Label	Afuega'l pitu cheese	-	5	-
		Coalho cheese	-	15	3
		Cottage cheese	40	-	-
		Gorgonzola cheese	62	-	-
		Iranian white cheese	32	13	-
		Jben cheese	55	-	-
		Minas cheese	24	-	-
		Munster cheese	7	-	-
		Pecorino siciliano cheese	2	-	-
		Queso fresco	2	-	-
		Tomato cheese spread	20	-	-
		Undefined cheese	177	48	-

Table 2. Distribution of log reduction data in *L. monocytogenes* (LM), *S. aureus* (SA) and *Salmonella* spp. (SS) by moderator for the two bio-interventions strategies meta-analysed

Bio-intervention	Moderators	Level	LM	SA	SS
Essential oils (N=1305)	Application type, *App*	Mixture	61	58	42
		Film	90	83	59
		Milk	221	77	96
		Surface	360	0	158
	Exposure time, *t* (days)	[0, 30[	676	178	323
		[30, 60[	45	29	32
		[60, 75]	11	11	0
	Storage temperature, *T* (°C)	[4, 15[	690	210	355
		[15, 25[	30	0	0
		[25, 35]	12	8	0
	Inoculum level, *Inoc* (log CFU/g or log CFU/ml)	[1.5, 3.5[	137	109	59
		[3.5, 5.5[	447	27	180
		[5.5, 7]	148	82	116
	Antimicrobial concentration, *Conc* (%v/v or w/w)	[5×10^{-3}, 1.5[	663	168	311
		[1.5, 3[	41	22	15
		[3, 4.5]	28	28	29
Added LAB (N=505)	Application type, *App*	Mixture	0	11	3
		Milk	277	70	0
		Surface	144	0	0
	Exposure time, *t* (days)	[0, 20[	345	61	2
		[20, 40[	46	15	1
		[40, 75]	30	5	0
	Storage temperature, *T* (°C)	[4, 10[	259	40	3
		[10, 16[	115	30	0
		[16, 22[	47	11	0
	Inoculum level, *Inoc* (log CFU/g or log CFU/ml)	[2, 4[	173	27	0
		[4, 6[	153	24	0
		[6, 8]	95	30	3
	Antimicrobial concentration, *Conc* (log CFU/g, log CFU/ml, %v/v or %w/v)	[0.5, 4[	78	38	0
		[4, 8[	228	4	0
		[8, 12]	115	39	3

3.2.2 Meta-regression models

Mixed-effects linear models with weights were separately adjusted to the EOs and added-LAB data sets for each pathogen to evaluate their antimicrobial effects on the square-root of log reduction ($\sqrt{R}$). Moderators are study characteristics that can be selected and codified from the primary sources in an attempt to explain the between-study variability in effect size. In this meta-analysis, the moderators defined, encompassed: application type (*App*), exposure time (*t*),

antimicrobial concentration (*C*), storage temperature (*T*) and inoculum level of the pathogen (*Inoc*). Log reduction and antimicrobial concentration were square-root and natural-logarithm transformed, respectively, to normalise data distribution and reduce heteroscedasticity. Due to lack of or uneven data, not all levels could be evaluated in the meta-regression. Due to lack of data, no model was produced to describe the antimicrobial effect of added-LAB on SS.

The five meta-regression models adjusted are described below:

$$\sqrt{R_{ijk}} = (\beta_0 + u_i) + \beta_{1k}App_k + \beta_{2k}\{App_k \times t\} + (\beta_{3k} + v_i)\{App_k \times LnC\} + \beta_4 T + \varepsilon_{ijk} \quad (1)$$

$$\sqrt{R_{ijk}} = (\beta_0 + u_i) + \beta_{1k}App_k + \beta_{2k}\{App_k \times t\} + (\beta_{3k} + v_i)\{App_k \times LnC\} + \beta_4 T + \beta_5 Inoc + \varepsilon_{ijk} \quad (2)$$

$$\sqrt{R_{ijk}} = (\beta_0 + u_i) + \beta_{1k}App_k + \beta_{2k}\{App_k \times t\} + (\beta_{3k} + v_i)\{App_k \times LnC\} + \varepsilon_{ijk} \quad (3)$$

$$\sqrt{R_{ijk}} = (\beta_0 + u_i) + \beta_{1k}App_k + \beta_{2k}\{App_k \times t\} + \beta_4 T + \beta_5 Inoc + \varepsilon_{ijk} \quad (4)$$

$$\sqrt{R_{ijk}} = (\beta_0 + u_i) + \beta_{1k}App_k + \beta_{2k}\{App_k \times t\} + \varepsilon_{ijk} \quad (5)$$

Equations 1, 2 and 3 describe the meta-regression models used to evaluate the antimicrobial effect of EOs on LM, SA and SS, respectively. Equations 1 and 3 contain fewer terms, compared to Equation 2, as some moderators were not introduced to those models because they were either confounded with other variables or were not significant. The models in Equation 4 and 5 were used to evaluate the inhibitory effect of added LAB on LM and SA.

In the above equations, β_0 is an intercept, β_{1k} is the set of fixed effects of the *k* types of application (a class variable consisting of the levels: cheese mixture, cheese surface, milk and film), and β_{2k} is a set of effects representing the interaction between application type (*App*) and exposure time (*t*). In Equations 1 to 3, β_{3k} is a set of fixed effects describing the mean interaction between application type (*App*) and the natural logarithm of the antimicrobial concentration (*LnC*). Said otherwise, the set of parameters β_{2k} and β_{3k} allow the slopes of exposure time and natural logarithm of antimicrobial concentration, respectively, to take different values depending on the type of application *k* used. The term β_4 in Equations 1, 2 and 4 is the mean effect of a 1°C increment in storage temperature on the square-root of log mean reduction, while β_5 in Equations 2 and 4 is the effect of a one-log increase in inoculum level on that same transformed response variable.

The remaining unexplained variability was extracted by placing random-effects u_i due to antimicrobial type *i* in the intercept β_0; and random effects v_i due to antimicrobial type *i* in the concentration slope β_{3k}. These random effects u_i, v_i were assumed to be correlated following a

normal distribution with mean zero and a variance-covariance matrix $[s_u^2, s_{uv}, s_v^2]$ from where the correlation coefficient $\boldsymbol{\rho}$ of the random effects was calculated. The error term ε_{ijk} accounts for the residuals and follows a normal distribution with mean zero and variance s^2. Model parameters, as affected by moderators, were calculated from the fitted meta-regressions, and the significance of moderators was evaluated by analysis of variance (α=0.05).

The antimicrobial effect of EOs was evaluated by analysis of random-effect marginal intercepts and concentration slopes, organising EOs by origin type. In this analysis, the antimicrobial-specific intercept and slope values are interpreted as deviations u_i and v_i from the mean values β_0 and β_{3k}, respectively. Thus, it was assumed that the higher the u_i and v_i, the stronger the antimicrobial effect of the EO$_i$.

In order to obtain precise estimates of the antimicrobial effect on pathogen inactivation and reflect quality of research design, different weights were assigned to each primary study (*j*) according to the sample size (number of replicates, *n*) used along the experiment to evaluate microbial inactivation. When a primary source did not present the number of replicates sampled to calculate the pathogen reduction, *n*=3 was assigned, as this was the modal value in the database.

To evaluate the fraction of variability in $\sqrt{R}$ that could be explained by the moderators (R^2), null model versions (no moderators) of Equations 1 to 5 were fitted, and τ^2 was calculated as $(s_u^2 + s_{uv} + s_v^2)$. From the fitted full models (Equations 1 – 5), τ^2_{res} was calculated as $(s_u^2 + s_{uv} + s_v^2)$, and finally R^2 was estimated as $(\tau^2 - \tau_{res}^2)/\tau^2$. All meta-regression models described were fitted using the lme function from the nlme package implemented in R version 3.6.2 [76].

3.3 RESULTS AND DISCUSSION

The results of the analysis of variance of the five meta-regression models adjusted are presented in **Table 3**. The EOs-SA model allowed for the inclusion of a higher number of moderating variables. The EOs-LM model does not contain inoculum level as fixed effect since this term reveal to be non-significant (p = 0.627). The EOs-SS model did not include storage temperature, nor inoculum level, as the first variable had only two levels (data was collected at either 4 or 10 °C) and the second variable was highly confounded with the antimicrobial application type.

Table 3. Test of fixed effects of the meta-regression models predicting the square-root of log reduction (log CFU/g or ml) of *L. monocytogenes, S. aureus* and *Salmonella* spp. in cheese with incorporated essential oils or LAB as a function of moderating variables

Model	Fixed effects	Num/Den DF	F-value	Pr > F
EOs-LM	App	3/704	20.68	<.0001
	App * Exposure Time	4/704	41.00	<.0001
	App * Antimicrobial Conc.	4/704	15.71	<.0001
	Storage Temperature	1/70	23.70	<.0001
EOs-SA	App	2/199	16.01	<.0001
	App * Exposure Time	3/199	105.8	<.0001
	App * Antimicrobial Conc.	3/199	27.61	<.0001
	Storage Temperature	1/199	30.98	<.0001
	Inoculum Level	1/199	5.577	0.019
EOs-SS	App	3/4	28.76	0.004
	App * Exposure Time	4/339	121.2	<.0001
	App * Antimicrobial Conc.	4/339	236.6	<.0001
LAB-LM	App	1/51	0.130	0.720
	App * Exposure Time	1/364	18.52	<.0001
	Storage Temperature	1/364	31.21	<.0001
	Inoculum Level	1/364	11.23	0.001
LAB-SA	App	1/62	2.945	0.091
	App * Exposure Time	2/62	3.383	0.040

In the EOs models, the significance of all terms reveal that these terms or variables have an impact on the microbial reduction promoted by this type of biopreservative. The significant interaction terms “application*exposure time” and “application*antimicrobial concentration” showed that exposure time and antimicrobial concentration not only have a strong effect on the extent of microbial reduction on their own, yet those effects are dependent upon the mode of application of the antimicrobial in the cheese. These significant interaction terms therefore denote that some modes of EOs application are more effective than others.

Regarding the LAB meta-regressions, the data did not allow for the construction of a model with SS, as only one study referring to the effect of LAB strains on SS growth was retrieved from the literature search. The LAB-SA model did not include terms for storage temperature because of the small range of temperatures (4 to 18 °C), nor inoculum level because it was confounded with the antimicrobial application type. In the LAB-LM and LAB-SA models, “application” by itself was not found to be significant (p = 0.720 and p = 0.091, respectively). Yet, when the

"application*exposure time" interaction was tested, both terms became significant ($p < .0001$ and $p = 0.040$, respectively). Thus, application type has, in fact, an influence on microbial counts when evaluating the interaction but not when the single moderator is evaluated. The practical meaning of this interaction is that to achieve a certain reduction, distinct exposure times are needed according to the mode of application utilised, as was also observed in the EOs models (**Table 3**).

3.3.1 EOs meta-regression models

The antimicrobial effects of EOs against LM, SA and SS are presented in **Table 4, Table 5** and **Table 6**, respectively.

Table 4. Parameter estimates of the meta-regression model predicting the square-root of log reduction (log CFU/g or ml) of *L. monocytogenes* in cheese with incorporated essential oils as a function of moderating variables

Parameters	Mean	St. Error	Pr > |t|	Heterogeneity
Predictors of $\sqrt{R_{ik}}$				
β_0 (intercept)	2.247	0.264	0.000	
β_{1k} (application type)				
App: mixture	0	-	-	
App: film	-1.497	0.197	0.000	
App: milk	-1.530	0.214	0.000	
App: surface	-0.466	0.185	0.012	
β_{2k} (app × exposure time)				
App: mixture	0.236	0.069	0.001	τ^2_{res}=0.508
App: film	0.044	0.004	0.000	R^2 > 95%
App: milk	0.007	0.002	0.000	
App: surface	0.009	0.002	0.000	
β_{3k} (app × antimicrobial conc.)				
App: mixture	0.632	0.117	0.000	
App: film	-0.095	0.115	0.409	
App: milk	0.225	0.072	0.002	
App: surface	0.420	0.071	0.000	
β_4(storage temperature)	-0.013	0.004	0.001	
Variances				
s_u	0.713			
s_v	0.234			
ρ ($s_u s_v$)	0.791			
s (residual)	0.156			

Table 5. Parameter estimates of the meta-regression model predicting the square-root of log reduction (log CFU/g or ml) of *S. aureus* in cheese with incorporated essential oils as a function of moderating variables

Parameters	Mean	St. Error	Pr > |t|	Heterogeneity
Predictors of $\sqrt{R_{ik}}$				
β_0 (intercept)	2.710	0.466	0.000	
β_{1k} (application type)				
App: mixture	0	-	-	
App: film	-1.530	0.187	0.000	
App: milk	-0.316	0.415	0.447	
β_{2k} (app × exposure time)				
App: mixture	0.229	0.046	0.000	τ^2_{res}=0.640
App: film	0.045	0.003	0.000	R^2 > 95%
App: milk	0.014	0.002	0.000	
β_{3k} (app × antimicrobial conc.)				
App: mixture	0.516	0.077	0.000	
App: film	0.223	0.059	0.000	
App: milk	0.405	0.064	0.000	
β_4 (storage temperature)	0.012	0.010	0.246	
β_5 (inoculum level)	-0.134	0.059	0.025	
Variances				
s_u	0.800			
s_v	0.096			
ρ ($s_u s_v$)	0.896			
s (residual)	0.101			

Table 6. Parameter estimates of the meta-regression model predicting the square-root of log reduction (log CFU/g or ml) of *Salmonella* spp. in cheese with incorporated essential oils as a function of moderating variables

Parameters	Mean	St. Error	Pr > |t|	Heterogeneity
Predictors of $\sqrt{R_{ik}}$				
β_0 (intercept)	1.251	0.130	0.000	
β_{1k} (application type)				
App: mixture	0	-	-	
App: film	-0.645	0.180	0.023	
App: milk	-0.028	0.216	0.903	
Application type: surface	1.224	0.152	0.001	
β_{2k} (app × exposure time)				τ^2_{res}=0.010
App: mixture	0.168	0.013	0.000	R^2 > 95%
App: film	0.116	0.007	0.000	
App: milk	0.008	0.002	0.000	
App: surface	0.008	0.007	0.233	
β_{3k} (app × antimicrobial conc.)				
App: mixture	0.167	0.063	0.008	
App: film	0.445	0.057	0.000	
App: milk	0.332	0.067	0.000	
App: surface	0.937	0.032	0.000	
Variance				
s_u	0.100			
s (residual)	0.131			

The effect differed for each pathogen, although, overall, the statistical analysis revealed a clear tendency for microbial reduction when EOs are incorporated in cheese, as revealed by the positive intercepts β_0.

The greater β_0 values for LM and SA (2.247 and 2.710, respectively), compared to SS (1.251), indicate higher antimicrobial effect of essential oils against LM and SA. These results agree with available literature stating that Gram-negative bacteria (*Salmonella* spp.) are more resistant to EOs than Gram-positive bacteria (*L. monocytogenes* and *S. aureus*) [16]. The increased antimicrobial effect against Gram-positive bacteria is likely due to differences in cell membrane composition between Gram-positive and Gram-negative bacteria [16].

Based on the results of the EOs-LM model (**Table 4**), it is possible to observe that distinct application types lead to different microbial reductions, as shown by the β_{1k} values. Notice that the application type "mixture" is considered the "base value", with mean zero, and the remaining application types reflect deviations from that mean. Thus, the incorporation in milk (β_{1k}=-1.530), within films involving the product (β_{1k}=-1.497) or on the cheese surface (β_{1k}=-0.466) attained overall lower microbial reduction than the cheese mixture application. Therefore, caution must be taken by researchers on using the experimental practice of the "cheese mixture" for a challenge study, since it may lead to significantly overestimated values of LM reduction in relation to the real modes of application. From these, applying EO on cheese surface appeared to be more effective against LM than applying in milk or film.

Microbial reduction is time and antimicrobial concentration dependent; thus, we analysed the interaction terms "application type*exposure time" (β_{2k}) and "application type*antimicrobial concentration" (β_{3k}). The significant β_{2k} parameter reinforces the impact of application type on microbial reduction by showing that there is an association between this variable and exposure time. The different mean values of β_{2k} denote the need for distinct exposure times, depending on the application type, to obtain the same microbial reduction. In this case, it is possible to observe that, applying the EO to the mixture (β_{2k}=0.236) or within a film (β_{2k}=0.044) results in faster inhibitory effect than applying the EO to the cheese surface (β_{2k}=0.009) or into the milk (β_{2k}=0.007). Regarding β_{3k}, results reveal that, for the same antimicrobial, different concentrations are required to achieve the same inhibitory effect if the EO is added to the cheese, milk, surface, or within a film. The results highlight that for the same concentration of a specific EO, higher microbial reductions are obtained when the application is in cheese mixture (β_{3k}=0.632) or onto the surface (β_{3k}=0.420) than in milk (β_{3k}=0.225). The mean β_{3k} for film was found not significant (p = 0.409) due to the limited concentration range of EOs tested under this application modality. The β_4 value revealed the negative association between microbial inhibition and temperature, meaning that as storage temperature increases, the microbial inhibitory effect of the EOs is counteracted. Once again, for the time and concentration slopes (β_{2k} and β_{3k}), the "cheese mixture" application produced the highest rates of inactivation, which corroborated what was earlier discussed: cheese mixture as a testing procedure that does not mimic the actual manufacturing process, and moreover overestimates microbial reduction, meaning that it is not a suitable methodology for challenge or fate studies. For LM, the increased inhibitory effect of the surface or film application

can be explained by the fact that EOs are applied at a later stage of the production process. With application of EOs into milk, a much earlier phase, it is reasonable to say that the antimicrobial properties of the biopreservative may not endure throughout the processes of curding, fermentation and ripening, as EOs are more susceptible to interaction with cheese components and macronutrients, such as proteins and lipids, than when applied onto cheese surface or incorporated in films.

The EOs-SA model (**Table 5**) showed the recurrent result that the application method of cheese mixture (β_{1k}=0) produces higher reductions in SA than incorporation in films (β_{1k}=-1.530). Since β_{1k} for the application in milk was non-significant (p = 0.447), no comparison could be made with the other modes of application. Nonetheless, comparison between the three methods could be made in terms of exposure time and antimicrobial concentration since all terms were highly significant. Again, applying EO in cheese mixture produced the highest slopes β_{2k}=0.229 and β_{3k}=0.516, therefore leading to the greatest microbial reductions. EO incorporation in films (β_{2k}=0.045) promoted a faster antimicrobial effect than application in milk (β_{2k}=0.014); although, for the same increase in EO concentration applied, higher inactivation effects were found for milk (β_{3k}=0.495) than for film (β_{3k}=0.223). In this meta-regression for SA, storage temperature was not found to affect microbial reduction (p = 0.246); and a negative association between inoculum level and log-reduction was encountered (p = 0.025). This indicates an interesting trend that when higher populations of pathogens are inoculated into milk/cheese, the overall inhibitory effect of EOs will be lower. This finding disagrees with some studies that have shown that inoculum size has no effect on growth kinetics [77, 78]; nonetheless our results may be explained by the fact that an increase in cell numbers increases the probability of growth, even under suboptimal conditions, and thus limits the antimicrobial inactivation [79].

Table 6 presents the results for the EOs-SS model. The application of EOs on cheese surface produced significantly higher reduction in *Salmonella* spp. inoculated populations than EOs containing-films, as can be deduced from the higher values of β_{1k} and β_{3k} (1.224 and 0.937 for cheese surface against -0.645 and 0.445 for films, respectively). The term β_{2k} for cheese surface did not reach significance. Among the realistic EO application types (milk, film and surface), incorporation of EOs in milk produces the lowest inhibitory effect in *Salmonella*, as this application mode rendered overall the slowest reduction in numbers (lower β_{2k} at 0.008), at the same time

that higher concentrations of EO would need to be added in milk in order to attain log reduction levels comparable to the other application modes (lower β_{3k} at 0.332).

Interestingly, some outcomes were consistent across the EOs models built. For the same increase in EO concentration, surface application on cheese is the application method with the greatest inhibitory effect against *L. monocytogenes* and *Salmonella* spp., while EO-incorporated films produce a faster inactivation during fermentation/ripening. According to the models, the direct addition of EO to milk render the lowest antimicrobial effect, while cheese mixture was mostly associated with greater microbial inactivation.

The analysis of random-effect marginal intercepts and natural logarithm of antimicrobial concentration slopes for the three EOs models are presented in **Table 7**.

Overall, results show that the EOs antimicrobial action depends on its origin, and there was greater variability among the antimicrobial effects of the EOs for LM and SA control than for SS, as revealed by the broad interval that intercept values can take for the first two pathogens in comparison to the smaller range of values of the SS model. EOs extracted from lemon balm, sage, and a thyme-like plant (*Zataria multiflora* Boiss) present the greatest bactericidal effects against LM and SA in cheese matrix. Basil also showed high antimicrobial effect against LM, yet its antimicrobial properties against SA have not been tested in cheese. For the control of SS, the best EO was that of clove. All these EOs belong to the *Lamiaceae* family, except for clove (*Myrtaceae)*, and nine out of 16 EOs retrieved from this meta-analysis study belong to this taxonomic family. In the last decades, many promising results have been collected about the antioxidant and health-promoting capacities of *Lamiaceae*'s active compounds, which are predominantly polyphenols and present in large amount [80]. Generally, phenolic compounds are known to show antimicrobial activity against Gram-positive bacteria [16], similarly to the previously discussed results of the EOs meta-regressions, where the greatest bactericidal effect was on LM and SA growth. Overall, it is crucial to consider the bioavailability of essential oils' phenolic compounds and the EO-cheese interaction, as the results from the meta-regressions presented here are specific for cheeses only and may not be accurate if the results are extrapolated to other foods. Moreover, our study gathered information on plant essential oils and not on their specific compounds. Thus, the results are limited by the breadth of the subject itself, and conclusions on the antimicrobial effect of specific plant constituents or compounds may not be appropriate.

Table 7. Random effects of the meta-regression models predicting the square-root of log reduction (log CFU/g or ml) of *L. monocytogenes*, *S. aureus* and *Salmonella* spp. in cheese with incorporated essential oils

Essential oil	LM		SA		SS
	Intercept	Slope	Intercept	Slope	Intercept
Basil	**0.734**	**0.173**	-	-	-
Bay	-0.047	0.144	-	-	**0.014**
Black cumin seed	-0.073	-0.239	-0.753	-0.070	0.000
Cinnamon	-0.061	0.083	-	-	0.001
Cumin	-	-	0.001	0.005	-
Clove	-0.451	-0.064	-	-	**0.106**
Hogweed	-0.020	0.045	-	-	-
Lemon balm	**0.933**	**0.332**	**0.999**	**0.116**	-
Mint	0.354	-0.125	-0.050	-0.012	-
Oregano	-0.397	-0.163	-0.223	-0.048	**0.018**
Pink pepper	-1.186	-0.168	-	-	-
Rosemary	-1.080	-0.281	-	-	-0.018
Sage	**1.103**	**0.414**	**1.328**	**0.150**	-
Tarragon	0.302	0.006	-0.859	-0.076	-
Thyme	-0.817	-0.296	-0.642	-0.046	-0.122
Zataria multiflora Boiss.	**0.706**	**0.143**	**0.199**	-0.020	0.000

(*) Values in bold highlight the EOs leading to greatest pathogen inhibition.

In all three meta-regression models, heterogeneity analysis revealed that more than 95% of the between-EO variability in microbial log reductions could be explained by the moderators introduced in the models. It is possible to state that the microbial reduction observations retrieved from the literature differed not only due to the different EO origin but also due to the distinct application types, concentration applied, exposure time used, storage temperature and inoculum size. This information is valuable as it provides insight on the effectiveness of both application modes and EO origin, which is key when implementing biopreservation hurdle technologies for pathogen control in foods.

In order to evaluate the quality of the meta-regression models built, the goodness-of-fit was assessed, as shown in **Figure 1**. The correlation values R of the goodness-of-fit are particularly high for meta-analysis studies (R=0.824, R=0.943 and R=0.934 for LM, SA and SS, respectively), so it can be stated that the three models are robust.

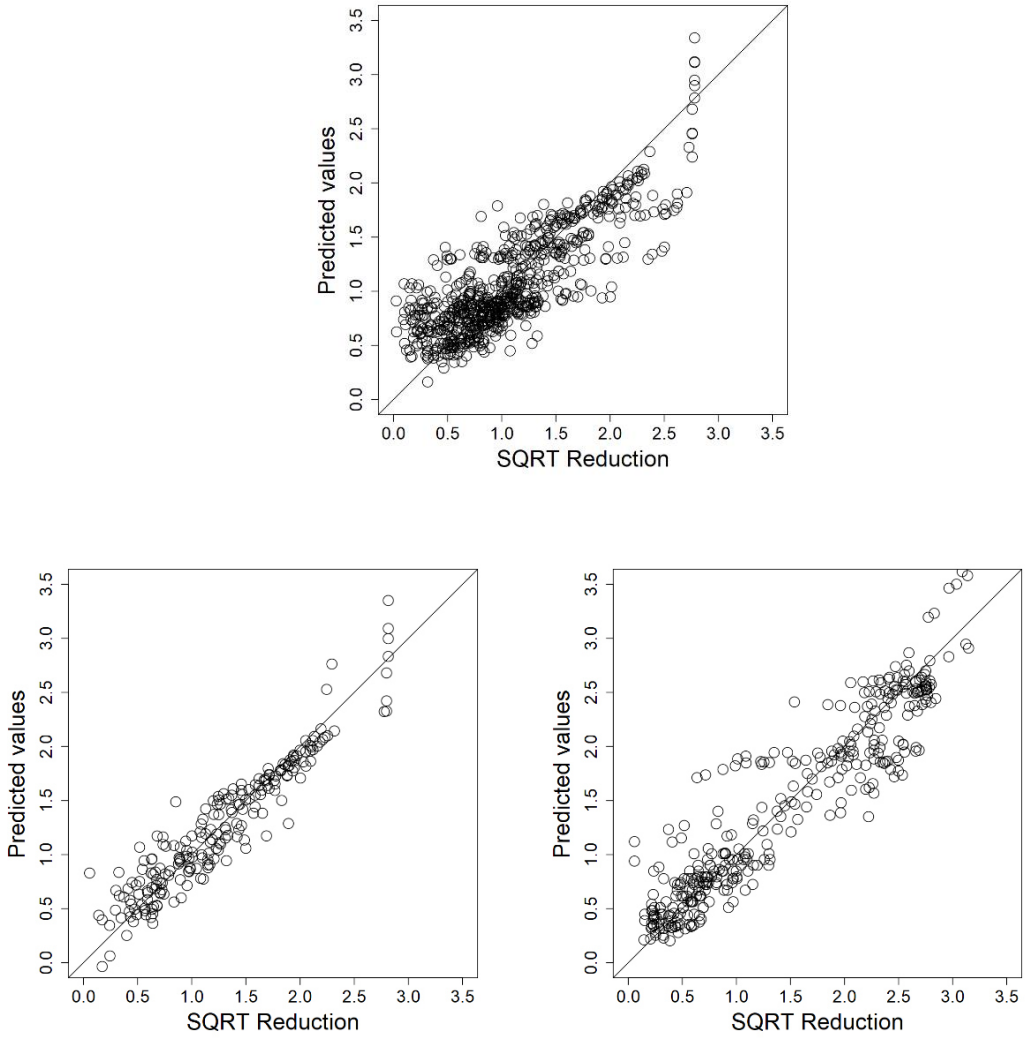

Figure 1. Goodness-of-fit of the meta-regression models predicting the square-root of log reduction (log CFU/g or log CFU/ml) of *L. monocytogenes* (top left; R=0.824), *S. aureus* (top right; R=0.943) and *Salmonella* spp. (bottom; R=0.934) in cheese with incorporated essential oils.

3.3.2 Added-LAB meta-regression models

The results regarding the two models built for the antimicrobial effects of added LAB in LM and SA growth are presented in **Table 8** and **Table 9**, respectively.

Table 8. Parameter estimates of the meta-regression model predicting the square-root of log reduction (log CFU/g or ml) of *L. monocytogenes* in cheese with added LAB as a function of moderating variables

Parameters	Mean	St. Error	Pr > |t|	Heterogeneity
Predictors of $\sqrt{R_{ik}}$				
β_0 (intercept)	0.961	0.125	0.000	
β_{1k} (application type)				
App: milk	0	-	-	τ^2_{res}=0.185
App: surface	-0.017	0.148	0.910	R^2 = 27.61 %
β_{2k} (app × exposure time)				
App: milk	0.052	0.010	0.000	
App: surface	0.046	0.015	0.003	
β_3 (storage temperature)	0.025	0.004	0.000	
β_4 (inoculum level)	-0.067	0.020	0.001	
Variances				
s_u	0.430			
s_v	0.049			
ρ ($s_u s_v$)	0.236			
s (residual)	0.148			

Table 9. Parameter estimates of the meta-regression model predicting the square-root of log reduction (log CFU/g or ml) of *S. aureus* in cheese with added LAB as a function of moderating variables

Parameters	Mean	St. Error	Pr > |t|	Heterogeneity
Predictors of $\sqrt{R_{ik}}$				
β_0 (intercept)	0.601	0.287	0.041	
β_{1k} (application type)				
App: mixture	0	-	-	τ^2_{res}=0.390
App: milk	0.239	0.289	0.411	R^2= 11.80%
β_{2k} (app × exposure time)				
App: mixture	0.001	0.013	0.915	
App: milk	0.008	0.003	0.012	
Variance				
s_u	0.625			
s (residual)	0.173			

As in the EOs models, the positive β_0 intercepts revealed an increase for microbial reduction, thus supporting literature describing the usefulness of bacteriocinogenic LAB strains in cheese. The mean values are, however, lower than those obtained for the EOs models, which could imply that added LAB as biopreservatives have, as a whole, a lower antimicrobial effect than EOs.

The results of the LAB-LM model (**Table 8**) do not reveal significant differences in the application modes milk and surface as intercepts (β_{1k}). However, adding LAB in milk produces a faster inactivation of LM (β_{2k}=0.052) than application onto the cheese surface at the end of processing (β_{2k}=0.046). Higher temperatures of storage (viz. fermentation/ripening) of cheeses in the challenge studies also led to greater microbial reductions ($p < .0001$) which could be a consequence of the more rapid proliferation of LAB and their metabolic products, which quickly inactivate LM populations, at least at the initial stages of fermentation. As observed and discussed before for the EOs-SA model, the parameter β_4, associated with the inoculum level, presents a negative mean value, suggesting that higher microbial reductions are counted when the pathogen's inoculum size is lower ($p = 0.001$). The inverse association between inoculation size and microbial log reduction found in this meta-analysis justifies the need for more research to better understand how the inoculum level affects the microbial kinetics measured.

The conclusions that can be driven from the results of the LAB-SA model (**Table 9**) are quite similar to those derived from the LAB-LM model, in the sense that the two types of application under study (milk and cheese mixture) did not show any significant differences regarding its antimicrobial efficacy in the intercepts β_{1k}; yet adding LAB in milk produces a slightly faster inactivation of SA (β_{2k}=0.008) than the other application mode (β_{2k} for mixture was not significantly different from zero). Thus, in LM challenge studies where the inhibitory effect of LAB is tested, the "cheese mixture" experimental set-up can underestimate the microbial reductions quantified. This is not unexpected since added LAB exert their antimicrobial action right after their incorporation in milk where, at the expense of lactose, they produce lactic acid that drops the pH, bacteriocins and other inhibitory compounds that retard the growth of LM and provoke their decline. Inoculating LAB after curding and draining (or after cheese is made), as the starting point of a challenge study, is therefore not a sensible practice in face of these results.

The results of the heterogeneity analysis performed for the added-LAB meta-regression models showed that the moderators introduced to the LAB-LM and the LAB-SA models explain 27.61% and 11.80% of the variability between the added LAB strains, respectively. These are

considerably lower values when compared to the EO models, revealing, in the first place, that other factors that are not under study here can also have a great impact on the inhibitory effect of LAB strains against pathogens, and could possibly explain a higher percentage of the variability if included in the models. Secondly, it is also plausible that the different added LAB strains studied in the primary studies bring about much more variability in microbial reduction than the various EOs tested.

The goodness-of-fit was also assessed for both models, as shown in **Figure 2**, which shows correlation values considered acceptable for meta-analysis studies (R=0.914 and R=0.943 for LM and SA, respectively) and that support the robustness of the models.

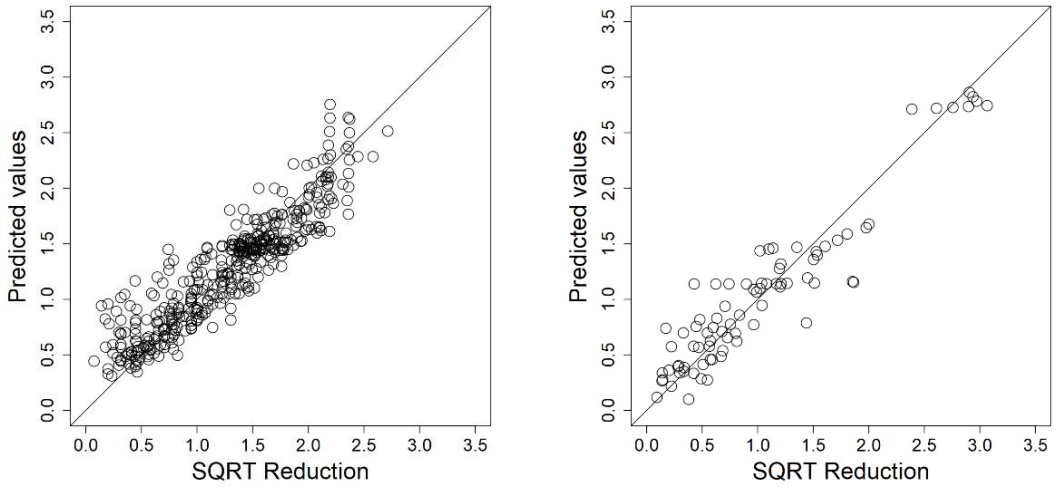

Figure 2. Goodness-of-fit of the meta-regression models predicting the square-root of log reduction (log CFU/g or log CFU/ml) of *L. monocytogenes* (left; R=0.914) and *S. aureus* (right; R=0.943) in cheese with incorporated LAB.

3.4 CONCLUSIONS

Literature data was used to build meta-analytical regression models capable of summarising the reduction in LM, SA and SS populations in cheese attained by added LAB and EOs; and elucidating inhibitory effectiveness by application mode and specific antimicrobial. These meta-regressions showed that the effectiveness of added LAB and EOs were regulated by storage temperature, exposure time, pathogen's inoculum size, antimicrobial concentration and method of application of the biopreservative (cheese mixture, cheese surface, incorporated in film or directly added to milk). EOs-models evidenced that, for a given increase in EO concentration, the

application on cheese surface provides the greatest inhibitory effect against LM and SS, whereas EO-embedded films lead to a more rapid inactivation during maturation/storage. Lemon balm, sage and basil EOs showed the best inhibitory outcomes against LM and SA; whilst clove, oregano and bay EOs presented overall the highest bactericidal effect against SS. In general, the lowest inhibitory effect of EOs against LM, SA and SS is produced when EOs are added to milk. By contrary, and as expected, adding LAB in milk produces a faster inactivation of LM and SA than applying them onto the cheese surface (for LM) or as cheese mixture (for SA).

This meta-analysis has revealed two important issues, one related to experimental design and the other related to directions for further research. The incorporation of the antimicrobial in cheese mixture (this is, using cheese after coagulation, draining and shaping), which has been an experimental practice by many researchers as the starting point of the challenge study, is by no means an adequate practice because it tends to overestimate the capacity of EOs to inactivate pathogens while tends to underestimate the capacity of added LAB to inactivate pathogens. Two meta-analysis models pointed out the trend that higher microbial reductions were quantified when lower concentrations of pathogens are inoculated in the milk, and vice versa. There is a need to further investigate how the pathogen's inoculum size affects the measurement of microbial kinetics in challenge studies.

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SECTION II

BIOPRESERVATION STRATEGIES AND MILD HEAT TREATMENT

CHAPTER 4

CHEMICAL PROFILE AND BIOACTIVITIES OF EXTRACTS FROM EDIBLE PLANTS READILY AVAILABLE IN PORTUGAL

This chapter was based on the following paper:

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4.1 INTRODUCTION

In the food industry, synthetic preservatives have been widely used to improve or maintain the properties of foods and to extend their shelf-life. However, the safety and impact of synthetic food additives on human health has been under discussion for many years. Some studies have reported gastrointestinal, respiratory, dermatological, cardiovascular, musculoskeletal, and neurological adverse reactions, although the cause-and-effect association between food additives and symptoms is not always well documented [1].

In this sense, one of the current trends in food processing is to replace chemical additives with others that are more natural, plant-based, known to be safe from the toxicological standpoint (with GRAS status—Generally Recognised as Safe), in order to satisfy the demand of consumers for "greener" products [2]. For this reason, numerous studies on natural substances, such as plant extracts, have been conducted. These have shown promising results regarding the antimicrobial and antioxidant properties of various natural substances, thus supporting their potential as food preservatives that can be incorporated in the product or its packaging [2-5].

Nonetheless, to assure the safety of herbal extracts for human consumption, it is crucial that these originate from nontoxic solvents, such as water, ethanol, or their binary mixtures (dichloromethane, hexane, ethyl ether, chloroform, and methanol should be avoided), and from herbs with documented traditional use [6]. In this regard, there is a wide variety of plants used in traditional medicine that have been evaluated by researchers on their health-promoting, antimicrobial and antioxidant properties. This is the case of basil, lemon balm, French lavender, sage, spearmint, and tarragon [3,7-11], six plants that, according to recent meta-analyses, can provide protection towards pathogens in cheese [12,13]. Traditionally, the decoctions of basil (*Ocimum basilicum* L.) have been used as an herbal remedy for stomach pains, constipation, and nasal and bronchial catarrh, among other applications [3]. Moreover, basil has shown anti-inflammatory, antidiabetic, cardioprotective, immunostimulatory, anticarcinogenic and hepatoprotective properties [14].

Lemon balm (*Melissa officinalis* L.) has been widely used as a mild sedative and anxiolytic, as well as to prevent and treat gastrointestinal disorders, but other medicinal effects have also been described, including antispasmodic, antiproliferative, anti-cholinesterase and antiviral properties [15,16].

In folk medicine, French lavender (*Lavandula stoechas* L.) is a well-known aromatic plant that has been used for its anti-inflammatory, antispasmodic and carminative properties, as well as for its positive effects against various problems, including eczema, urinary tract infections and heart-burn, for example [10].

Preparations from sage (*Salvia officinalis* L.) leaf have been traditionally used in the treatment of gastrointestinal problems, and mouth and throat inflammations, for example [17]. Additionally, sage has a wide variety of pharmacological activities, such as anticancer, antimutagenic, anti-inflammatory, antinociceptive, hypoglycemic, hypolipidemic, and cognitive and memory-enhancing effects [18].

Mentha species, which include spearmint (*Mentha spicata* L.), have a long history of use in the treatment of respiratory problems (such as bronchitis) and digestive issues (nausea, ulcerative colitis, flatulence, etc.) [11]. The medicinal effects of Mentha species include anticatarrhal, anti-inflammatory, carminative, antiemetic, diaphoretic, antimutagenicity, antispasmodic, antioxidant, and analgesic activities [11].

As for tarragon (*Artemisia dracunculus* L.), this herb is commonly used in traditional medicine to treat insomnia, as a digestive stimulant, and for the treatment of skin wounds, allergic rashes, and dermatitis [19]. The main therapeutic applications reported are for the nervous, digestive and renal systems (due to the anti-epileptic, spasmolytic, laxative, and diuretic properties), for liver function, and as anti-inflammatory, anticancer and antibacterial agents [19].
With proven beneficial effects for human health—and because basil, lemon balm, French lavender, sage, spearmint, and tarragon are readily available in Portugal—further characterisation of these plants was intended.

In this context, our study was designed to evaluate the chemical profiles and bioactivities of a variety of extracts, obtained from different plants, extraction techniques, and solvents. The main goals of this study were the following: (i) to deliver insight on the plant extracts with most potential to be used as food additives, among those cultivated in Portugal; and (ii) to assess the influence of distinct extraction methods and solvents on the chemical profile and antioxidant activities of plant extracts.

4.2 MATERIALS AND METHODS

4.2.1 Plant Material and Extraction Procedures

Basil, lemon balm, French lavender, sage, spearmint, and tarragon dry aerial parts were provided by Pragmático Aroma Lda. ("Mais Ervas", Trás-os-Montes, Portugal), and mechanically ground. The extractions were performed in triplicate (n = 3) using ethanol 70% (v/v) (Et70) and distilled water as solvents, in a shaking water bath (at 150 rpm) at 60 °C for 90 min (solid-liquid extraction); or using a Soxhlet apparatus (at 90 or 120 °C, for Et70 and distilled water, respectively), for 7 recycles (around 3.5 to 4 h). Both methods used a sample/solvent ratio of 1 g/20 mL. After filtration (filter paper of 7–10 μm), the extracts were stored in a refrigerator at 4 °C until use. For the antimicrobial essays, the extracts were frozen and lyophilised.

4.2.2 Extraction Yield

The dry weight method was used to determine the solvent efficiency in extracting compounds from the plant material. The extraction yield (presented in %) was calculated as shown in Equation (1):

$$Extraction\ Yield\ (\%) = \frac{extracted\ solids\ (g)}{initial\ dry\ material\ (g)} \times 100 \quad (1)$$

4.2.3 Chemical Characterisation

4.2.3.1 Total Protein Content

The soluble protein content (TProtein) was analysed using the Bradford essay with some modifications [20]. For this, a subsample of 20 μL plant extract was mixed with 230 μL of Bradford dye reagent. The microplate was placed in the dark for 5 min at room temperature and the absorbance was measured at a wavelength of 595 nm by an UV/Vis spectrophotometer (Synergy HT, BioTek Instruments Inc., Winooski, VT, USA). Bovine albumin serum (BSA) was used to perform the standard curve (1000–33 mg/L, R^2 = 0.98) and the results were expressed as micrograms of BSA equivalents (BSAE) per gram of dry plant (μg BSAE/g dry plant).

4.2.3.2 Total Carbohydrate Content

The carbohydrate content (Carbohyd.) was analysed by the phenol-sulfuric acid method, as described by Masuko et al. [21]. For this, 50 μL of plant extract were mixed with 150 μL of

sulfuric acid (96–98% (v/v)). Then, 30 µL of 5% phenol reagent were added and the final solution was heated for 5 min at 90 °C. After cooling at room temperature for 5 min, the absorbance was measured at 490 nm by an UV/Vis spectrophotometer (Synergy HT, BioTek Instruments Inc.,Winooski, VT, USA). Glucose was used to perform the standard curve (600–10 mg/L, R^2 = 0.99) and the results were expressed as micrograms of glucose equivalents (GE) per gram of dry plant (µg GE/g dry plant).

4.2.3.3 Chlorophyll Contents

The plant extracts were analysed for chlorophyll-a and chlorophyll-b content as described by Sumanta et al. [22]. Briefly, 2 mL of plant extract were centrifuged at 10 000 rpm for 15 min. The supernatant was collected, placed in a cuvette, and the absorbance as measured at 649 and 664 nm by an UV/Vis spectrophotometer (Synergy HT, BioTek Instruments Inc., Winooski, VT, USA). Quantification was done using Equations (2) and (3) for chlorophyll-a (Ch-a) and chlorophyll-b (Ch-b), respectively.

$$Ch - a = 13.36Abs_{664\,nm} - 5.19Abs_{649\,nm} \quad (2)$$

$$Ch - b = 27.43Abs_{664\,nm} - 8.12Abs_{649\,nm} \quad (3)$$

Results were expressed as micrograms of each photosynthetic pigment per gram of dry plant (µg Ch-a or Ch-b/g dry plant).

4.2.3.4 Total Phenolic and Flavonoid Contents

The total phenolic content (TPC) was determined using the Folin-Ciocalteu essay [23]. For all analyses, 5 µL of plant extract (water or ethanol 70% for control) were mixed with 15 µL Folin-Ciocalteu reagent, 60 µL of Na_2CO_3 (75 g/L). The prepared solution was kept at 15 °C for 5 min. Absorbance was measured at 700 nm by an UV/Vis spectrophotometer (Synergy HT, BioTek Instruments Inc., Winooski, VT, USA). A calibration curve was prepared using a standard solution of gallic acid (2500–100 mg/L, R^2 = 0.99), and the final values were expressed as milligrams of gallic acid equivalents (GAE) per gram of dry plant material (mg GAE/g dry plant). The total flavonoid content (TFC) was determined by aluminium chloride colorimetric method [24]. An aliquot (500 µL) of the plant extract was mixed with 2 mL of distilled water and 150 µL of $NaNO_2$ solution (5%). After 6 min, 150 µL of $AlCl_3$ solution (10%) was added and allowed to stand further 6 min; thereafter, 2 mL of NaOH solution (4%) and 200 µL of distilled water were added to the

mixture. Then, the mixture was properly mixed and allowed to stand for 15 min, and the absorbance was measured at 510 nm by an UV/Vis spectrophotometer (Synergy HT, BioTek Instruments Inc., Winooski, VT, USA). A calibration curve (400–0 mg/L, R^2 = 0.99) was prepared using (+)-Catechin, and the results were expressed as milligrams of catechin equivalents (CE) per gram of dry plant (mg CE/g dry plant).

4.2.3.5 Identification and Quantification of Individual Phenolic Compounds

Individual phenolic compounds were analysed by Shimadzu Nexera X2 UHPLC chromatograph equipped with Diode Array Detector (DAD) (Shimadzu, SPD-M20A, Kyoto, Japan) using a previously validated method, as described by Ferreira-Santos et al. [25]. Separation was performed on a reversed phase Acquity UPLC BEH C18 column (2.1mm × 100 mm, 1.7 μm particle size; from Waters) and a precolumn of the same material at 40 °C. The flow rate was 0.4 mL/min. HPLC grade solvents water/formic acid 0.1% (A) and acetonitrile (B) were used. The elution gradient for solvent B was as follows: from 0 to 5.5 min eluent B at 5%, from 5.5 to 17 min linearly increasing from 5 to 60%, from 17 to 18.5 min linearly increasing from 60 to 100%; last, the column is equilibrated at 5% from 18.5 to 30 min. Phenolic compounds were identified by comparing their UV spectra and retention times with those of corresponding standards. Quantification was carried out using calibration curves for each pure phenolic compound standard, using concentrations between 250–2.5 mg/L, and the limit of detection (LOD) and limit of quantification (LOQ) were calculated for as previously reported by Busaranon et al. [26]. In all cases, the coefficient of linear correlation was R^2 > 0.99 (**Table 10**). Compounds were quantified and identified at different wavelengths (209–370 nm). All analyses were made in triplicate.

Table 10. Limit of detection (LOD), limit of quantification (LOQ) and coefficient of linear correlation (R^2) of the different phenolic compounds tested

Phenolic compound	LOD (mg/L)	LOQ (mg/L)	R^2
Chlorogenic acid	17.71	53.67	0.9988
Vanillic acid	9.57	29.00	0.9996
Syringic acid	2.24	16.80	0.9999
Cinnamic acid	13.99	42.40	0.9998
p-coumaric acid + epicatechin	12.78	38.71	0.9986
o-coumaric acid	11.75	35.60	0.9988
Rosmarinic acid	14.42	43.71	0.9970
Ellagic acid	30.65	92.88	0.9912
Naringin	9.89	29.96	0.9992
Hesperidin	36.87	111.7	0.9996
Kaempferol	55.48	107.5	0.9960
Resveratrol	32.95	99.85	0.9909
Ferulic acid	31.19	94.53	0.9916
Quercetin	21.34	64.67	0.9961
3,4-Dihydroxybenzoic acid	9.60	29.07	0.9993

4.2.4 Bioactivities

4.2.4.1 Antioxidant Activity

The antioxidant activity was measured using DPPH, ABTS and FRAP methods, to evaluate distinct mechanisms of action of the extracts. The free radical scavenging (DPPH) and the radical cation decolorisation (ABTS) essays were conducted as described by Ballesteros et al. [27] with some modifications. Calibration curves were prepared with a standard solution of TROLOX (250–15 μM, R^2 = 0.998, for DPPH; and 800–31.25 μM, R^2 = 0.996, for ABTS) and a corresponding control was used for each solvent. The radical scavenging activity for DPPH and ABTS methods (% inhibition) was calculated as Equation (4)

$$\%\ Inhibition = \frac{A_C - A_S}{A_C} \times 100 \quad (4)$$

where A_s is the sample absorbance and A_c the control sample absorbance. The results were expressed as micromoles of TROLOX equivalent (TE) per gram of dry plant (μmol TE/g dry plant).

The ferric reducing antioxidant power (FRAP) essay was performed as described by Meneses et al. [28]. A calibration curve was prepared using an aqueous solution of ferrous sulphate (800–100 μM, R^2 = 0.98). FRAP values are expressed as micromoles of ferrous equivalent per g of dry plant (μmol Fe^{2+}/g dry plant).

4.2.4.2 Antimicrobial Activity

From all the extracts produced, three were selected for presenting distinctive results in terms of chemical profile (specifically, phenolic compounds content) and antioxidant activity. The bacteria tested were *Salmonella enterica* subsp. *enterica* serovar Typhimurium ATCC 43971, *Listeria monocytogenes* WDCM 00019, *Staphylococcus aureus* ATCC 6538, and *Escherichia coli* (clinical isolate), obtained from the Polytechnic Institute of Bragança stock collection. Bacteria strains were subcultured twice by streaking on blood agar and incubated at 37 °C for 48 h and then 24 h to ensure that bacterial cells were in the exponential growth phase. Following incubation in agar, single colonies from the second plate were inoculated into individual tubes containing sterile water and the bacterial suspensions were adjusted to a concentration of approximately 1.5 × 10^8 CFU/mL (0.5 McFarland).

The minimum inhibitory concentrations (MIC) were determined by broth microdilution method according to the Clinical and Laboratory Standards Institute (CLSI) recommendations [29], with some modifications. The minimum bactericidal concentrations (MBC) were also determined by subcultivation of 10 μL of the microplate wells containing extracts at 20 mg/mL and 10 mg/mL into blood agar plates. The lowest concentration that showed no growth after this subculturing was regarded as the MBC. The results were expressed in milligrams per millilitre of the resuspended lyophilised extracts (mg/mL). The MIC experiments were performed four times (n = 4) and the MBC tests were carried in duplicate (n = 2).

4.2.5 Statistical Analysis

Principal component analysis (PCA) was performed using the prcomp function from the factoextra package [30] to evaluate the contribution of variables (essays) and factors (plant, method, solvent) to the discrimination of extracts. Groupwise summary statistics (mean ± standard error) were calculated by plant, extraction method, and solvent, for each attribute (extraction yield,

each chemical characterisation essay and each antioxidant essay) using the summary_by function from the doBy package [31].

To assess the main effects of plant, extraction method, and solvent, and the interactions between those terms on each variable (essay), three-way interaction linear nonparametric models were built using the art function from the ARTool package [32], which applies an aligned rank transformation to every model. This transformation was done to enable a nonparametric analysis of variance ($\alpha = 0.05$), as the normality assumptions were not met. The three-way interaction "plant × method × solvent" was included in the model to provide an adequate fit.

For each variable, pairwise comparisons of levels within single factors were conducted using the emmeans function from emmeans package [33], coupled with the artlm function from the ARTool package [32]. Superscript letters indicating significantly different values ($p < 0.05$) were defined according to the results of the emmeans function.

Statistical analysis was conducted in R software (version 3.6.2) [34].

4.3 RESULTS AND DISCUSSION

With the potential to be used as food additives, basil, lemon balm, French lavender, sage, spearmint, and tarragon were used to produce twenty-four extracts, testing two extraction methods and two solvents per plant. The methods tested were solid-liquid and Soxhlet extractions because they generally offer good extraction results and are easy to implement, thus justifying their widespread use in the food industry to extract bioactive compounds [35]. Water and ethanol 70% (v/v) were selected as extraction solvents because herbal extracts should be produced using water, ethanol, or their binary mixtures, while toxic organic solvents should be avoided [6].

4.3.1 Influence of Extraction Yield, Chemical Characteristics, and Antioxidant Activity on Extracts Differentiation

To visualise the influence of extraction yield, chemical characteristics, and antioxidant activity on the differentiation of extracts, PCA was conducted (**Figure 3**).

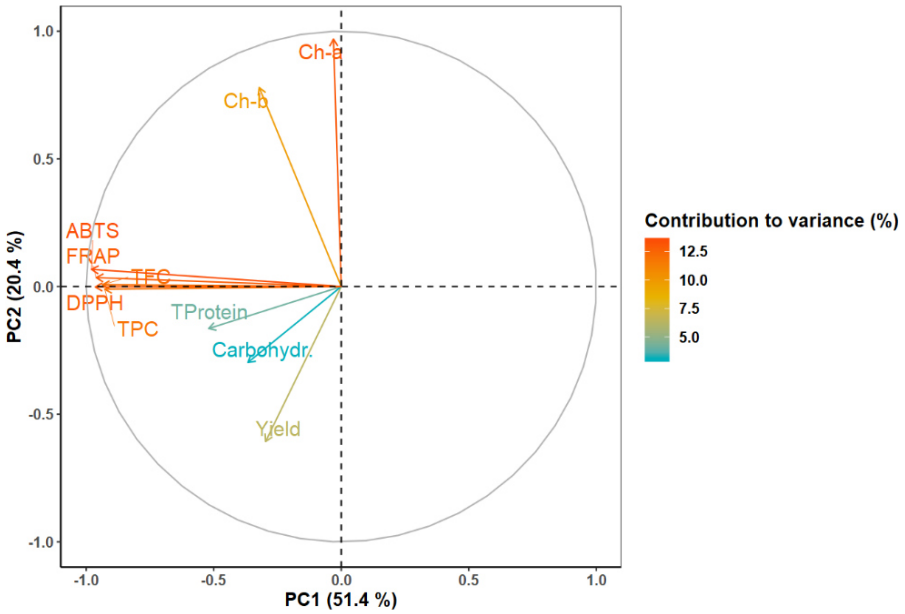

Figure 3. Loading plot of the first two components of the principal component analysis (PCA).

The first two principal components, PC1 and PC2, accounted for most of the variance observed, 51.4% and 20.4%, respectively. While the first component, PC1, indicates that dissimilarities across the horizontal axis are mostly due to distinct phenolic contents and antioxidant activities; the second component, PC2, reflects the contribution of the photosynthetic pigments and extraction yield to the differentiation of samples.

In **Figure 3**, variables with little contribution to extracts distinction will appear closer to the plot origin, whereas variables with greater contribution will be further from the centre of the plot. From this, the variables with the highest contribution to extracts differentiation are those associated with antioxidant activity (ABTS: 13.42%; DPPH: 12.95%; FRAP: 12.86%), TFC (12.20%), TPC (11.98%), and photosynthetic pigments (chlorophyll-a and -b, 13.09% and 9.90%, respectively). The variables with lower contribution were extraction yield, total protein content, and carbohydrate content (6.36%, 4.14% and 3.10%, respectively). These results reveal that extraction yield, total protein content, and carbohydrate content were fairly similar across the samples produced, but divergencies were mainly found in terms of phenolic contents, photosynthetic pigments, and antioxidant activities.

Figure 3 also provides insight on correlations between variables: positively associated variables will have approximately the same loading (i.e., distance from the plot origin) and will appear close to each other on the plot, whereas negatively correlated variables will appear diagonally opposite each other [36]. In this sense, the PCA shows that TPC and TFC are positively

correlated with antioxidant activity (ABTS, DPPH and FRAP), an expected result due to the redox properties of phenolic compounds, which allow for adsorption and neutralisation of free radicals, quenching of singlet and triplet oxygen, or decomposition of peroxides [37]. Several other studies on various plant materials have also reported on the strong correlation between phenolic compounds and antioxidant activity [38,39,40,41].

4.3.2 Influence of Extraction Method, Solvent, and Plant Type on Extracts Differentiation: Principal Component Analysis

Score plots of the first two components of the PCA were also produced to display the grouping of plant extracts by extraction method, solvent, and plant type, as shown in **Figure 4A-C**, respectively.

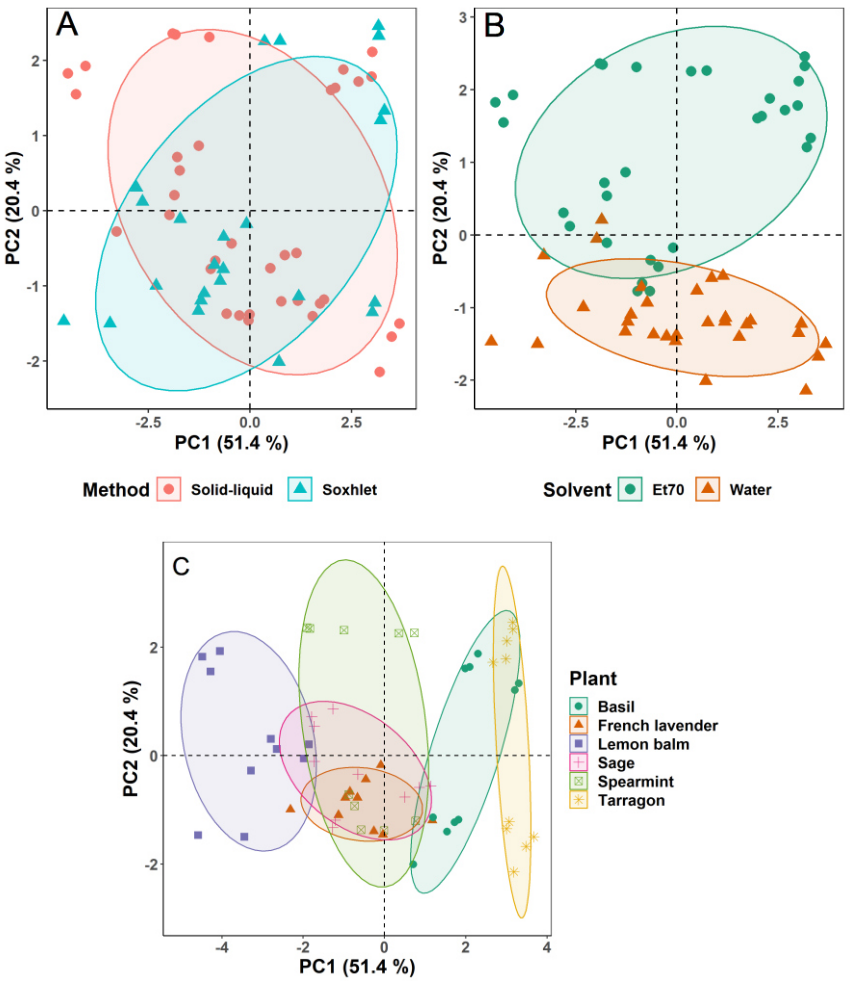

Figure 4. Score plots of the first two components of the principal component analysis (PCA) grouped by extraction method (A), solvent (B) and plant type (C).

While the ellipses aim to group samples according to the method or solvent used, it is noticeable that three aqueous extracts are within the Et70 ellipse on **Figure 4B**. This is a result of three solid-liquid water extracts of lemon balm that revealed high chlorophyll contents, comparable to those of hydroethanolic extracts, unlike other aqueous extracts.

The score plots produced showed that the discrimination between extracts obtained from different solvents (**Figure 4B**) and different plants (**Figure 4C**) is greater than that attained between extraction methods (**Figure 4A**). This better discrimination arises from the greater difference in chemical characteristics and antioxidant properties among extracts obtained using different solvents or feedstocks than the different extraction methods. The influence of the solvent used on the extraction of phenolic compounds and antioxidant potential has also been reported by other research groups: Meneses et al. [28] pointed out the difference in antioxidant activity and total phenols content in brewer's spent grains extracts when using water or organic solvents; Teofilović et al. [42] demonstrated the impact of different polarity solvents on the total phenolic and flavonoid contents of basil extracts; and Martins et al. [43] produced *L. tridentata* extracts with varying antioxidant activity, total phenols and flavonoids contents by using distinct extraction solvents.

Nevertheless, the chemical composition and antioxidant activity of extracts are also influenced by the extraction method used, even if such impact is less noticeable from **Figure 4B**. This effect of the extraction method on phytochemical constituents and antioxidant capacity was also reported by Scollard et al. [44] and Dhanani et al. [45].

Analysing **Figure 4C**, the ellipses of spearmint, French lavender, and sage overlap, indicating similar phenolic contents and antioxidant activities (yet, different amounts of photosynthetic pigments, as revealed by the various heights of the ellipses, in PC2). However, they differentiate from the other three plants: lemon balm, basil, and tarragon. The figure suggests that lemon balm extracts contain the highest phenolic and flavonoid contents and antioxidant activity, as the extracts are in the same direction of the arrows of TPC, TFC, DPPH, ABTS and FRAP (see **Figure 3**). On the other hand, tarragon extracts contain the lowest quantity of phenolic compounds and most reduced antioxidant potential, as samples appear in the opposite direction.

4.3.3 Influence of Extraction Method, Solvent, and Plant Type on Extracts Differentiation: Main Effects and Interactions

In addition to the principal component analysis conducted, to further study the extracts differentiation and characteristics, groupwise summary statistics were calculated by plant, extraction method, and solvent, for each essay. Furthermore, three-way interaction models were built to assess the main effects of plant, extraction method, and solvent, and the interactions between those terms. These results are displayed in **Table 11**.

Despite the improved discrimination achieved due to solvent and plant types (**Figure 4B, Figure 4C**) rather than between extraction methods (**Figure 4A**), the results of the main effects in **Table 11** reveal the significant impact ($p < 0.05$) of all three terms on the extraction yield, chemical characteristics, and antioxidant capacities of the plant extracts.

In most essays, all three terms had a significance level of $p < 0.001$. The plant term, in particular, showed a significance level of $p < 0.001$ for all essays. The exceptions were found for the other two terms, method and solvent. Their impact on the TPC and carbohydrate essays appears to be less significant than that of plant type, therefore suggesting a greater difference in phenolic and carbohydrate contents between extracts obtained from distinct plants than from distinct methods or solvents—a result otherwise expected due to the specificities of each plant. The effect of plant type ($p < 0.001$) on total protein content and DPPH essays was also found to be stronger than the effect of solvent type ($0.001 < p \leq 0.01$). The results of the DPPH and ABTS essays were the only ones found to be independent from one of the factors, namely the extraction method ($0.05 < p \leq 1$), which is also indicated by the same superscript letter, in both columns.

The groupwise summary statistics in **Table 11** provide information on the overall means and standard errors of each level of the main effects. From these statistics, it appears that the solid-liquid technique improves extraction yields and results in extracts with high levels of chlorophyll, carbohydrates and phenolic compounds ($p < 0.05$ for these essays), whereas Soxhlet extraction produces extracts with greater content in proteins and flavonoids, associated with a high reducing antioxidant power (determined by the FRAP test) ($p < 0.05$ for these essays). Evaluating the results by solvent type, water seems to be more effective ($p < 0.05$) in extracting proteins, phenolic compounds, and carbohydrates, whereas ethanol 70% (v/v) appears to be more efficient ($p < 0.05$) in chlorophylls and flavonoids recovery.

Table 11. Groupwise summary statistics (mean ± standard error) by plant, method, and solvent, for extraction yield, chemical characterisation and antioxidant essays, and significance of the principal effects and interactions of the models

	Yield (%)	Ch-a (µg/g DP)	Ch-b (µg/g DP)	TProtein (µg BSAE/g DP)	TFC (mg CE/g DP)	TPC (mg GAE/g DP)	Carbohydr. (µg GE/g DP)	DPPH (µg TE/g DP)	ABTS (µg TE/g DP)	FRAP (µmol Fe^{2+}/g DP)
Plant										
Tarragon	23.1 ± 1.34 ^{bc}	96.9 ± 28.7 ^{a}	132 ± 33.0 ^{bc}	4.19 ± 0.83 ^{c}	8.05 ± 0.78 ^{c}	20.0 ± 0.95 ^{e}	17.6 ± 2.44 ^{b}	61.8 ± 6.59 ^{c}	107 ± 6.07 ^{c}	191 ± 11.5 ^{e}
Spearmint	21.5 ± 1.33 ^{d}	92.6 ± 23.8 ^{a}	149 ± 35.0 ^{ab}	8.91 ± 0.81 ^{b}	30.5 ± 1.23 ^{b}	44.0 ± 3.16 ^{c}	13.7 ± 1.39 ^{cd}	259 ± 14.4 ^{b}	361 ± 20.4 ^{b}	722 ± 31.0 ^{b}
Lemon balm	26.2 ± 1.24 ^{a}	96.1 ± 14.8 ^{a}	209 ± 33.1 ^{a}	10.4 ± 0.77 ^{a}	45.6 ± 4.86 ^{a}	74.4 ± 3.90 ^{a}	22.0 ± 1.76 ^{a}	345 ± 11.0 ^{a}	507 ± 28.6 ^{a}	1013 ± 75.5 ^{a}
Basil	22.2 ± 1.87 ^{cd}	68.9 ± 16.8 ^{b}	108 ± 22.1 ^{d}	6.02 ± 0.94 ^{c}	16.2 ± 1.07 ^{c}	26.9 ± 2.07 ^{d}	11.8 ± 1.21 ^{d}	149 ± 12.0 ^{c}	194 ± 12.6 ^{c}	376 ± 28.0 ^{d}
French lavender	25.2 ± 0.78 ^{ab}	43.5 ± 6.60 ^{c}	115 ± 9.18 ^{cd}	10.7 ± 1.54 ^{a}	32.2 ± 0.94 ^{b}	43.1 ± 2.45 ^{c}	21.6 ± 0.88 ^{a}	241 ± 20.5 ^{b}	326 ± 25.4 ^{b}	614 ± 51.4 ^{c}
Sage	22.4 ± 0.41 ^{cd}	59.8 ± 12.4 ^{bc}	99.9 ± 13.4 ^{d}	8.98 ± 1.16 ^{b}	30.9 ± 2.18 ^{b}	49.5 ± 3.87 ^{b}	16.5 ± 1.65 ^{bc}	265 ± 13.8 ^{b}	358 ± 23.1 ^{b}	752 ± 44.5 ^{b}
Method										
Solid-liquid	24.8 ± 0.55 ^{a}	80.8 ± 9.85 ^{a}	163 ± 16.6 ^{a}	6.15 ± 0.46 ^{b}	26.3 ± 2.39 ^{b}	44.5 ± 3.46 ^{a}	18.0 ± 0.92 ^{a}	216 ± 17.0 ^{a}	310 ± 24.6 ^{a}	579 ± 47.8 ^{b}
Soxhlet	21.4 ± 0.91 ^{b}	69.7 ± 12.7 ^{b}	93.6 ± 8.46 ^{b}	11.3 ± 0.71 ^{a}	28.6 ± 2.83 ^{a}	40.7 ± 3.68 ^{b}	16.1 ± 1.44 ^{b}	226 ± 20.5 ^{a}	307 ± 28.7 ^{a}	660 ± 65.7 ^{a}
Solvent										
Water	21.1 ± 0.73 ^{a}	28.1 ± 4.41 ^{b}	86.6 ± 12.2 ^{b}	8.43 ± 0.70 ^{a}	25.5 ± 2.23 ^{b}	44.3 ± 3.54 ^{a}	18.1 ± 1.29 ^{a}	212 ± 16.1 ^{a}	291 ± 23.2 ^{b}	577 ± 49.4 ^{b}
EtOH 70%	25.8 ± 0.50 ^{b}	124 ± 8.06 ^{a}	184 ± 14.5 ^{a}	7.97 ± 0.75 ^{b}	29.0 ± 2.88 ^{a}	41.7 ± 3.67 ^{b}	16.3 ± 0.95 ^{b}	229 ± 20.6 ^{b}	327 ± 29.0 ^{a}	646 ± 60.2 ^{a}
Principal effects										
Plant	***	***	***	***	***	***	***	***	***	***
Method	***	***	***	***	***	**	*	.	NS	***
Solvent	***	***	***	**	***	*	*	**	***	***
Interactions										
Plant × Method	**	***	***	***	***	***	***	.	*	*
Plant × Solvent	***	***	***	***	***	***	**	***	***	***
Method × Solvent	***	NS	***	***	***	***	NS	***	***	***
Plant×Method×Solvent	*	**	***	***	***	**	.	*	*	**

DP: dry plant; Values with different superscript letters in a column are significantly different (CI 95%); "NS": $p < 1$; ".": $p < 0.1$; "*": $p < 0.05$; "**": $p < 0.01$; "***": $p < 0.001$

In terms of feedstock, lemon balm did not only exhibit higher and distinctive ($p < 0.05$) results, compared to the remaining plants, on TPC, TFC and antioxidant essays, but it also presented the highest values in extraction yield, chlorophyll-b, and carbohydrate contents ($p < 0.05$). Moreover, lemon balm also revealed the second highest values in total protein and chlorophyll-a content, although it not significantly different from the plant displaying the highest outcome in such essays (namely, French lavender presented the greatest total protein content, whereas tarragon showed the highest chlorophyll-a content). Such distinctive results ($p < 0.05$) in terms of flavonoids and total phenolic contents suggest the great potential of lemon balm to be used as a food preservative against oxidation and microbial spoilage because phenolic compounds have been associated with antioxidant and antimicrobial activities [5,46].

Interaction terms were also included in the models to provide information on whether the effect of one term depends on the level of one or more terms. In this sense, when they are statistically significant, it would not be correct to generalise the trends pointed out by the main effects without considering the interactions.

Apart from the interaction "method × solvent" in the case of chlorophyll-a and carbohydrate content (both $0.1 < p \leq 1$), and the interaction "plant × method" in the DPPH essay ($p < 0.1$), all two-way interactions were found significant ($p < 0.05$) for all essays. This means, for example, for the significant interactions "plant × solvent" and "plant × method" on TPC (both $p < 0.001$), that the effects of solvent and extraction method on the total phenolic content, respectively, are different for each plant. These interactions can be visualised in **Figure 5A** and **Figure 5B**, respectively.

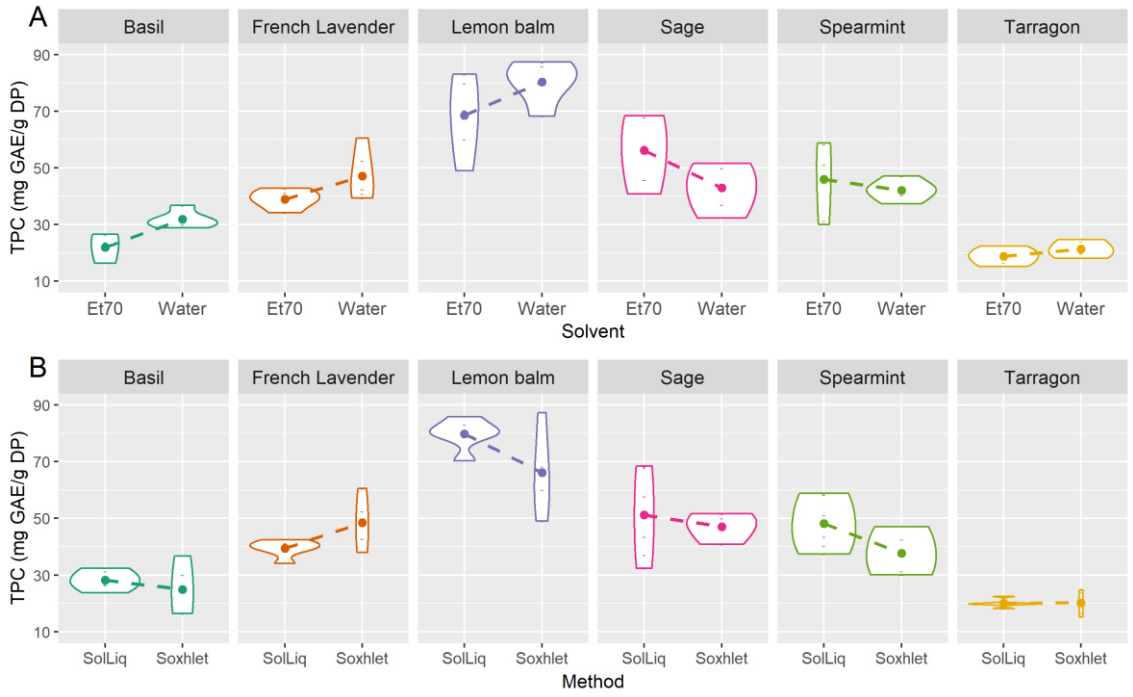

Figure 5. Interaction plots "plant × solvent" (A) and "plant × method" (B) on the total phenolic content of plant extracts.

In **Figure 5A**, each violin plot displays the distribution of TPC values obtained from both extraction methods, for each solvent and plant combination tested. Similarly, in **Figure 5B**, each violin plot displays the distribution of TPC values obtained from both solvents, for each extraction method and plant combination tested. The height of the violin plot indicates the distribution of the values obtained, while the varying width indicates the frequency of data points in each region.

The interactions are indicated by the different slopes of the dashed lines, across the six plants. For example, from **Figure 5A**, it is clear that the effect of solvent type is very different for lemon balm than it is for sage. In case there was no significant interaction, the dashed lines of the various plants would be practically parallel to each other.

The significance of these interactions also indicates the distinct abilities of different solvents and extraction techniques in retrieving various compounds from the raw material and their influence on the antioxidant capacity of extracts (as discussed earlier, and according to results previously reported by other research groups [28,42,43,44,45]), for each plant type.

The "method × solvent" term reveals that, for the same extraction method, the results of each essay will depend on the solvent used. Moreover, it implies that, for the same solvent, the results of each essay will vary according to the extraction technique selected. This suggests that

the four method/solvent combinations tested must be assessed to identify the one leading to the best or worst outcomes, in each essay.

In this sense, considering the results in **Table 12** and adding to the discussion on the main effects, the solid-liquid technique does improve extraction yields and carbohydrates contents, particularly when the solvent is water, while high levels of chlorophylls and phenolic compounds can be achieved using ethanol 70% (v/v). On the other hand, Soxhlet extracts have the greatest content in proteins when using ethanol 70% (v/v), while the greatest flavonoid content and high reducing antioxidant power (FRAP) is obtained from aqueous extracts. Furthermore, water does seem to be more effective than ethanol 70% (v/v) in extracting proteins and phenolic compounds, when Soxhlet extraction is used, compared to the solid-liquid one. Oppositely, ethanol 70% (v/v) is more efficient than water in flavonoids recovery, but only when solid-liquid extraction is conducted. Nonetheless, these are overall results that vary depending on the plant selected, due to its specific characteristics.

Table 12. Extraction yield, chemical profile and antioxidant activity of the extracts produced (mean ± standard deviation)

		Yield (%)	**Ch-a** (μg/g DP)	**Ch-b** (μg/g DP)	**TProtein** (μg BSAE/g DP)	**TFC** (mg CE/g DP)	**TPC** (mg GAE/g DP)	**Carbohydr.** (μg GE/g DP)	**DPPH** (μmol TE/g DP)	**ABTS** (μmol TE/g DP)	**FRAP** (μmol Fe^{2+}/g DP)
Soxhlet H_2O	Tarragon	26.8 ± 0.55	11.9 ± 0.1	40.9 ± 0.33	5.54 ± 0.07	8.78 ± 0.18	24.3 ± 0.41	5.94 ± 0.04	94.9 ± 1.50	130 ± 4.48	232 ± 3.71
	Spearmint	23.6 ± 0.90	24.0 ± 0.17	72.9 ± 0.24	13.1 ± 0.20	32.8 ± 1.62	44.8 ± 2.29	8.91 ± 0.64	268 ± 0.50	379 ± 6.22	777 ± 8.57
	Lemon balm	25.4 ± 1.40	25.3 ± 2.50	81.8 ± 7.15	11.3 ± 0.76	53.5 ± 1.51	77.8 ± 9.56	29.0 ± 2.79	363 ± 19.51	533 ± 31.1	1182 ± 126
	Basil	26.1 ± 1.70	13.4 ± 4.50	44.2 ± 12.7	10.7 ± 0.34	18.7 ± 1.03	33.4 ± 3.38	14.8 ± 4.18	193 ± 1.20	227 ± 3.48	509 ± 19.7
	F. lavender	25.5 ± 2.10	34.0 ± 10.5	106 ± 23.8	13.5 ± 0.34	34.8 ± 4.44	56.5 ± 4.06	20.5 ± 2.41	293 ± 37.5	389 ± 51.9	818 ± 65.5
	Sage	22.5 ± 0.10	19.5 ± 0.30	62.2 ± 1.10	11.7 ± 0.07	34.8 ± 0.57	50.8 ± 0.83	23.0 ± 2.55	266 ± 0.50	383 ± 4.72	791 ± 35.1
Soxhlet EtOH 70%	Tarragon	16.3 ± 0.40	194 ± 0.44	175 ± 0.12	7.22 ± 0.48	12.3 ± 0.74	15.9 ± 0.71	13.1 ± 0.37	62.9 ± 1.08	102 ± 19.0	204 ± 19.1
	Spearmint	15.0 ± 0.25	177 ± 5.25	137 ± 2.32	9.85 ± 0.08	26.7 ± 0.62	30.7 ± 0.60	8.70 ± 0.96	232 ± 12.8	289 ± 10.1	689 ± 36.3
	Lemon balm	20.6 ± 0.05	86.1 ± 5.56	99.7 ± 12.7	12.5 ± 1.41	47.9 ± 1.07	54.5 ± 5.56	15.6 ± 0.27	360 ± 2.36	492 ± 8.13	1094 ± 22.9
	Basil	13.4 ± 0.15	112 ± 4.58	84.9 ± 1.69	7.57 ± 0.46	10.8 ± 0.02	16.6 ± 0.19	10.7 ± 0.74	85.4 ± 0.97	123 ± 0.99	256 ± 1.52
	F. lavender	21.4 ± 0.40	55.0 ± 9.31	136 ± 31.8	18.2 ± 1.86	30.1 ± 0.43	40.4 ± 2.37	21.2 ± 1.05	213 ± 41.6	277 ± 52.9	511 ± 80.6
	Sage	20.6 ± 0.50	83.6 ± 10.3	83.1 ± 18.6	14.2 ± 1.45	32.5 ± 1.71	43.3 ± 2.50	21.2 ± 1.03	281 ± 23.8	357 ± 27.6	857 ± 66.7
Solid-liquid H_2O	Tarragon	26.2 ± 1.87	11.0 ± 0.99	34.7 ± 3.20	0.63 ± 0.15	6.59 ± 0.62	19.3 ± 0.85	24.4 ± 3.27	57.8 ± 1.54	87.0 ± 2.24	143 ± 2.72
	Spearmint	25.4 ± 2.12	20.1 ± 5.84	55.2 ± 13.4	7.84 ± 0.44	28.5 ± 3.81	40.4 ± 2.47	17.4 ± 0.37	220 ± 28.0	324 ± 30.9	621 ± 65.6
	Lemon balm	31.0 ± 0.22	95.4 ± 11.2	271 ± 26.3	11.5 ± 1.56	25.5 ± 7.15	81.9 ± 2.90	24.8 ± 2.29	303 ± 29.0	410 ± 75.3	693 ± 97.7
	Basil	27.9 ± 0.32	22.6 ± 0.99	67.5 ± 2.26	4.97 ± 0.18	18.9 ± 0.56	30.9 ± 1.48	12.1 ± 2.76	157 ± 10.6	197 ± 129	400 ± 9.76
	F. lavender	25.9 ± 0.35	20.5 ± 0.29	87.7 ± 1.89	8.32 ± 0.21	31.7 ± 0.65	40.9 ± 1.30	21.4 ± 3.68	183 ± 54.8	262 ± 63.3	478 ± 112
	Sage	22.1 ± 0.22	26.6 ± 1.33	77.6 ± 3.13	7.15 ± 0.51	21.6 ± 0.88	37.6 ± 4.54	13.3 ± 2.07	210 ± 9.79	265 ± 3.16	562 ± 14.7
Solid-liquid EtOH 70%	Tarragon	22.2 ± 0.42	175 ± 9.58	260 ± 1.21	4.82 ± 0.21	6.22 ± 0.26	20.6 ± 1.28	21.4 ± 1.26	43.0 ± 14.1	114 ± 3.71	203 ± 6.45
	Spearmint	20.6 ± 0.90	155 ± 0.06	303 ± 10.6	6.55 ± 0.55	33.4 ± 1.95	56.1 ± 3.52	16.7 ± 1.61	312 ± 21.3	434 ± 31.0	808 ± 57.2
	Lemon balm	25.8 ± 0.52	151 ± 0.31	306 ± 2.84	7.40 ± 0.38	59.1 ± 4.62	77.7 ± 5.38	18.9 ± 2.35	365 ± 0.98	596 ± 15.4	1166 ± 34.3
	Basil	19.6 ± 0.91	124 ± 0.58	207 ± 4.68	2.95 ± 0.16	15.3 ± 1.26	25.6 ± 1.29	10.3 ± 3.95	153 ± 10.0	217 ± 6.84	342 ± 11.7
	F. lavender	27.3 ± 0.71	65.4 ± 4.28	133 ± 8.87	6.16 ± 0.16	32.2 ± 2.28	38.1 ± 2.93	22.7 ± 2.66	283 ± 11.0	381 ± 15.4	682 ± 39.8
	Sage	23.9 ± 0.40	104 ± 3.17	158 ± 8.87	5.49 ± 0.17	36.7 ± 2.89	64.7 ± 4.91	12.3 ± 0.82	310 ± 7.84	437 ± 11.4	846 ± 44.7

Despite the nonsignificant main effects "method" and "solvent" on the results of the DPPH and ABTS essays, the significance of the "method × solvent" term in both cases ($p < 0.001$) reveals the existence of an interaction between these variables that affects the outcomes of such essays. These interactions on the outcomes of the DPPH and ABTS essays are shown by the distinct slopes of the dashed lines in **Figure 6A** and **Figure 6B**, respectively.

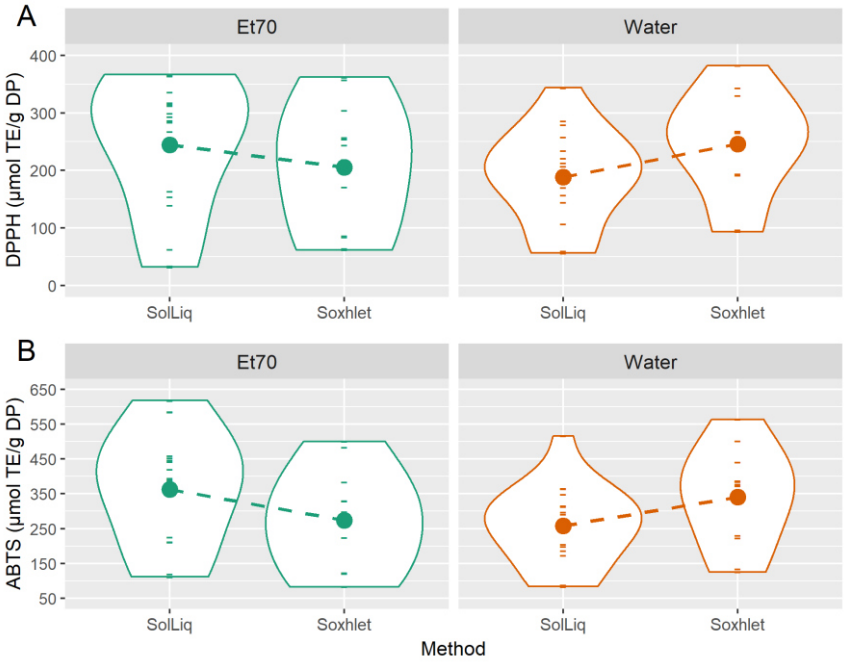

Figure 6. Interaction plots "method × solvent" on the outcomes of the DPPH (A) and ABTS (B) essays of plant extracts.

From **Table 12**, the highest values on the DPPH essay were largely derived from the aqueous Soxhlet extracts, followed by the hydroethanolic solid-liquid ones. The exceptions were sage hydroethanolic Soxhlet extract and basil and tarragon aqueous solid-liquid extracts. As for the ABTS essay, the combination leading to the highest overall values was solid-liquid extraction using ethanol 70% (v/v), and the second-highest was Soxhlet extraction with water as solvent.

Focusing on the results of the total phenolic contents in **Table 12**, hydroethanolic Soxhlet extracts generally presented the lowest (or among the lowest) TPC, when contrasted with the other method/solvent combinations (aqueous and hydroethanolic solid-liquid extracts, and aqueous Soxhlet extracts). This result is likely a consequence of alcoholic solvents being generally very effective in extracting phenolic compounds, as they improve the solubility of such compounds from the raw material to the solvent medium [47,48,49]; however, because this study used a higher

temperature (90 °C) during the Soxhlet extraction – compared to the one used for solid-liquid extraction (60 °C) – it may have promoted thermal degradation and oxidation of the compounds of the hydroethanolic Soxhlet extracts [48]. Chin et al. observed a similar outcome in tea, reporting a decrease in total polyphenols concentration from Soxhlet extraction carried out at 70 °C, compared to those obtained from maceration at 40 °C [50]. In this sense, it is possible to assume that, for Soxhlet extractions carried for seven recycles (3.5 to 4 h) at such high temperature, water may be the most appropriate solvent, whereas for solid-liquid extractions, either water or a mixture of water/ethanol is adequate. Otherwise, if Soxhlet extractions are carried out using ethanol as solvent, the appropriate extraction time and temperature must be assessed for optimum recovery of phenolic compounds from the plant matrix, as also suggested by Alara et al. [51].

The reported effect of the interaction "method × solvent" on the TPC can be visualised in **Figure 7**. In this sense, and given the results from **Table 12**, the method/solvent combinations that could be selected for their potential in producing extracts of increased phenolic and flavonoid contents, and high antioxidant activities are Soxhlet extraction using water as solvent, and solid-liquid extraction using ethanol 70% (v/v). Between the two, the latter combination is highly promising for industrial applications as it is less time-consuming than Soxhlet extractions and it does not require any specific equipment.

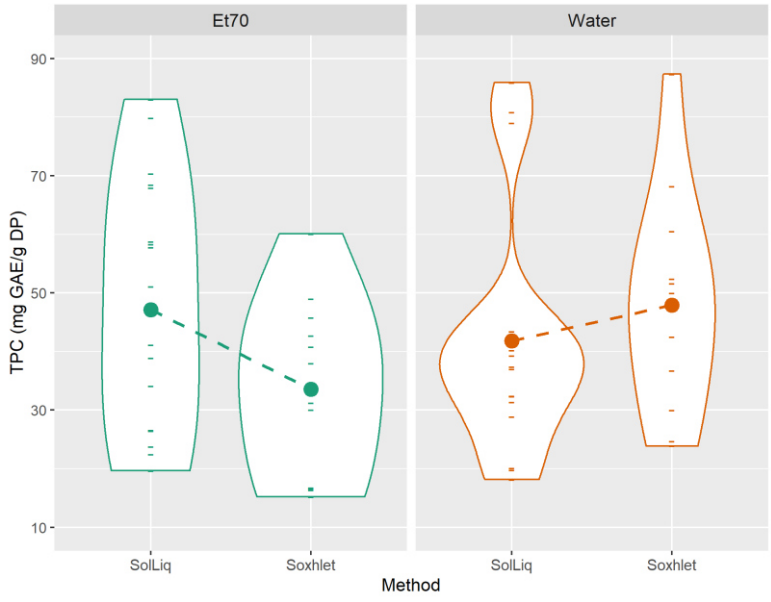

Figure 7. Interaction plot "method × solvent" on the total phenolic content of plant extracts.

4.3.4 Influence of Extraction Method and Solvent on Phenolic Profile of Plant Extracts

To further study the phenolic profile of the extracts, tentative identification and quantification of compounds was performed by UPLC. In total, fifteen compounds were identified in this study (**Table 13**).

The results showed that rosmarinic, ferulic and ellagic acids, naringin, hesperidin, resveratrol and quercetin were present in all plant extracts. They also showed that chlorogenic, vanillic, syringic, 3,4-dihydroxybenzoic, *o*-coumaric and ferulic acids, *p*-coumaric acid/epicatechin, kaempferol, resveratrol, and quercetin were undetected or found in concentrations below 250 mg/L extract, depending on the extract (**Table 13**). On the other hand, the compounds found in higher concentrations (between 267 and 1369 mg/L extract) were cinnamic, rosmarinic and ellagic acids, naringin and hesperidin.

Cinnamic acid was present at concentrations between 280–487 mg/L extract in the hydroethanolic solid-liquid extracts of spearmint, lemon balm and sage.

Rosmarinic acid was found at high concentrations in the aqueous (324–448 mg/L extract) and hydroethanolic (523–679 mg/L extract) Soxhlet extracts of spearmint, lemon balm and sage; in the aqueous solid-liquid extract of basil and tarragon (274 and 341 mg/L extract, respectively); and in the hydroethanolic solid-liquid extract of basil, spearmint, and tarragon (292–355 mg/L extract).

Ellagic acid showed high concentrations only in Soxhlet extracts, namely in the hydroethanolic extracts of tarragon, spearmint, and French lavender (416–554 mg/L extract) and the aqueous ones of tarragon, spearmint, lemon balm, basil, and sage (279–645 mg/L extract).

Naringin was present at high concentrations (267–894 mg/L extract) in both aqueous extracts of tarragon, in both Soxhlet extracts of sage, in the hydroethanolic solid-liquid extract of sage, and in the aqueous and hydroethanolic solid-liquid extract of basil and lemon balm, respectively.

Hesperidin was found in high amounts (279–996 mg/L extract) in all sage extracts, in spearmint and lemon balm Soxhlet extracts (both aqueous and hydroethanolic), and in the hydroethanolic Soxhlet and solid-liquid extracts of tarragon and French lavender, respectively.

Table 13. Identification and quantification of phenolic compounds present in the extracts produced

	Phenolic compound (mg/L extract)	Chlorogenic acid	Vanillic acid	Syringic acid	Cinnamic acid	*p*-coumaric acid + epicatechin	*o*-coumaric acid	Rosmarinic acid	Ellagic acid	Naringin	Hesperidin	Kaempferol	Resveratrol	Ferulic acid	Quercetin	3,4HBA
Soxhlet H_2O	Tarragon	27.4 ± 0.79	nd	nd	9.61 ± 1.11	165 ± 10.0	62.6 ± 6.38	45.9 ± 2.60	645 ± 31.0	270 ± 18.9	99.4 ± 7.57	nd	15.5 ± 2.74	111 ± 2.83	3.31 ± 0.64	9.16 ± 0.56
	Spearmint	8.77 ± 0.22	nd	nd	nd	nd	nd	324 ± 32.4	279 ± 21.9	55.7 ± 9.32	561 ± 45.9	nd	25.7 ± 2.12	55.1 ± 1.24	5.21 ± 0.26	nd
	Lemon balm	12.2 ± 1.76	nd	nd	nd	nd	nd	448 ± 109	373 ± 179	105 ± 31.1	901 ± 232	nd	59.3 ± 21.8	18.2 ± 6.79	12.6 ± 5.26	nd
	Basil	nd	nd	nd	nd	12.3 ± 1.72	nd	128 ± 2.73	420 ± 126	69.6 ± 3.30	206 ± 1.53	nd	5.94 ± 5.52	51.6 ± 2.89	4.13 ± 0.07	nd
	F. lavender	nd	nd	nd	nd	33.4 ± 12.1	nd	198 ± 0.75	75.9 ± 5.61	71.8 ± 10.8	85.0 ± 13.5	93.7 ± 6.69	103 ± 6.35	74.3 ± 12.1	10.3 ± 2.01	nd
	Sage	nd	nd	nd	nd	184 ± 16.6	nd	435 ± 41.8	587 ± 423	523 ± 33.8	900 ± 71.4	nd	4.92 ± 0.62	161 ± 11.7	5.53 ± 0.04	nd
Soxhlet EtOH 70%	Tarragon	nd	nd	nd	nd	93.3 ± 0.75	2.45 ± 0.25	38.9 ± 1.44	472 ± 41.6	133 ± 8.66	61.4 ± 1.37	nd	16.3 ± 0.42	68.6 ± 2.55	5.79 ± 2.04	1.27 ± 0.14
	Spearmint	nd	nd	nd	nd	nd	nd	555 ± 30.7	416 ± 32.4	92.7 ± 9.53	1131 ± 63.2	63.2 ± 2.70	68.1 ± 5.23	49.1 ± 4.78	19.4 ± 1.87	nd
	Lemon balm	nd	nd	nd	nd	nd	nd	679 ± 61.8	238 ± 0.48	93.5 ± 2.66	1369 ± 105	63 ± 3.37	97.9 ± 7.57	0.81 ± 0.06	8.60 ± 0.65	nd
	Basil	nd	nd	nd	nd	nd	nd	143 ± 5.48	150 ± 5.07	34.3 ± 0.72	242 ± 10.4	nd	10.8 ± 1.21	9.00 ± 0.05	6.39 ± 2.55	nd
	F. lavender	nd	nd	nd	nd	nd	nd	244 ± 27.9	554 ± 69.2	116 ± 0.61	495 ± 45.5	77.1 ± 8.35	127 ± 7.71	88.8 ± 10.1	27.2 ± 6.52	nd
	Sage	nd	nd	nd	nd	4.14 ± 0.84	nd	523 ± 3.85	249 ± 11.0	537 ± 8.05	996 ± 113	98.8 ± 4.43	44.5 ± 5.01	163 ± 3.85	33.0 ± 4.47	nd
Solid-liquid H_2O	Tarragon	nd	nd	nd	25.0 ± 3.32	57.6 ± 4.27	44.2 ± 5.08	341 ± 28.8	42.9 ± 5.33	267 ± 32.5	43.3 ± 6.59	nd	33.6 ± 1.21	34.9 ± 2.51	15.9 ± 1.01	nd
	Spearmint	nd	nd	nd	150 ± 3.79	53.7 ± 6.40	20.5 ± 0.14	204 ± 53.2	28.3 ± 2.58	22.8 ± 0.53	110 ± 26.6	nd	75.2 ± 8.25	42.8 ± 1.28	71.4 ± 10.9	nd
	Lemon balm	nd	nd	nd	56.3 ± 8.94	nd	99.9 ± 59.9	129 ± 11.9	82.1 ± 3.43	116 ± 5.43	31.6 ± 0.14	nd	90.9 ± 8.22	80.7 ± 1.71	43.1 ± 0.81	nd
	Basil	nd	17.2 ± 0.30	10.5 ± 0.24	80.5 ± 1.32	103 ± 11.8	33.9 ± 1.35	274 ± 10.9	40.2 ± 3.54	287 ± 1.17	144 ± 9.77	nd	46.4 ± 0.95	39.0 ± 0.17	34.2 ± 0.62	nd
	F. lavender	nd	nd	nd	80.5 ± 6.93	nd	30.9 ± 6.91	120 ± 42.9	39.2 ± 0.32	63.5 ± 27.0	116 ± 5.51	nd	95.2 ± 2.01	64.5 ± 11.1	29.5 ± 0.39	nd
	Sage	nd	12.4 ± 0.05	9.33 ± 0.63	53.3 ± 1.26	nd	36.7 ± 0.79	173 ± 101	43.3 ± 14.5	78.0 ± 12.0	279 ± 30.8	nd	93.9 ± 3.53	35.9 ± 3.55	54.6 ± 7.35	nd
Solid-liquid EtOH 70%	Tarragon	nd	9.92 ± 0.03	nd	37.2 ± 4.66	nd	33.2 ± 4.99	355 ± 5.15	38.5 ± 7.58	123 ± 0.52	292 ± 5.89	2.73 ± 0.38	95.6 ± 2.38	47.0 ± 8.11	33.7 ± 0.29	nd
	Spearmint	nd	nd	nd	280 ± 10.6	69.0 ± 3.17	27.8 ± 0.61	333 ± 57.3	28.6 ± 0.40	62.9 ± 2.16	223 ± 12.7	1.13 ± 0.32	111 ± 7.50	60.7 ± 13.9	55.7 ± 2.67	nd
	Lemon balm	71.2 ± 0.48	nd	nd	487 ± 15.8	123 ± 1.36	111 ± 92.4	185 ± 27.2	50.5 ± 4.25	894 ± 51.8	3.71 ± 3.34	nd	126 ± 9.33	108 ± 35.9	41.2 ± 0.11	nd
	Basil	64.9 ± 1.25	12.9 ± 0.18	nd	79.9 ± 4.15	84.4 ± 10.7	25.5 ± 2.01	292 ± 4.31	31.0 ± 2.62	65.1 ± 7.86	188 ± 13.5	nd	85.4 ± 1.27	50.2 ± 7.47	18.1 ± 0.21	nd
	F. lavender	32.2 ± 0.22	nd	nd	121 ± 2.21	68.5 ± 0.85	31.6 ± 0.59	127 ± 18.4	49.9 ± 14.7	77.8 ± 2.31	123 ± 8.73	nd	96.2 ± 0.86	55.1 ± 0.56	27.3 ± 0.03	nd
	Sage	nd	nd	nd	485 ± 66.3	119 ± 3.22	94.9 ± 4.13	170 ± 13.6	52.2 ± 9.61	279 ± 16.1	805 ± 40.0	nd	200 ± 13.0	78.5 ± 4.35	129 ± 4.76	16.0 ± 0.30

3,4HBA: 3,4-Dihydroxybenzoic acid; nd: not detected

Other researchers have also studied the phenolic profile of the plant materials used in our work. Nunes et al. performed the characterisation of phenolic compounds from *L. stoechas* L. methanolic extracts [52]. Their research identified rosmarinic, ferulic, chlorogenic and vanillic acids, among other compounds. From our French lavender extracts, rosmarinic, ferulic and chlorogenic acids were also detected (the latter in only one of the extracts), while vanillic acid was never detected. Zgórka and Głowniak studied the phenolic profile of sage, basil and lemon balm extracts [53]. Their work indicated the presence of vanillic acid in sage and basil (approximately 25 and 6 μg/g dry plant, respectively). In our study, vanillic acid was detected in the aqueous solid-liquid sage extract at 250 μg/g dry plant (12.4 mg/L extract) and in two basil samples obtained by solid–liquid extraction at 260 and 340 μg/g dry plant (12.9 and 17.2 mg/L extract). Their research also revealed the existence of ferulic acid in sage (around 50 μg/g dry plant); and rosmarinic acid (the most predominant compound) in basil, lemon balm and sage (approximately 11650, 9690 and 5120 g/g dry plant, respectively). Our study also identified ferulic acid in all sage extracts, and rosmarinic acid in all basil, lemon balm and sage extracts. Zgórka and Głowniak did not identify chlorogenic acid in any of the tested plant extracts, which was also the case in our study, depending on the extraction method and solvent used [53].

Kivilompolo et al. also performed the characterisation of phenolic acids from sage, basil and spearmint extracts [54]. Their research identified rosmarinic acid in basil, spearmint and sage (3080, 5620 and 9960 μg/g dry plant)—like we did in our study—as well as chlorogenic acid in basil; vanillic acid in sage and spearmint; and syringic, *p*-coumaric and ferulic acids in all herb extracts. With some exceptions, most of these outcomes agree with those presented in **Table 13**. In contrast, Kivilompolo et al. [54] reported the presence of vanillic acid in basil (140 μg/g dry plant) and chlorogenic acid in sage and spearmint (230 and 310 μg/g dry plant). In our study, vanillic acid was only detected in two basil extracts, as previously referred; chlorogenic acid was never identified in sage, and only detected in one spearmint extract (180 μg/g dry plant; 8.77 mg/L extract).

Slimestad et al. investigated the phenolic profile of tarragon extracts, reporting a limited number of phenolic compounds in this herb, of which chlorogenic acid stands out as one of the main constituents (1607 μg/g dry plant) [55]. In another study, by Mumivand et al. [56], HPLC analysis of twelve tarragon extracts (from different origins) indicated that chlorogenic acid (5.73 to 37.07 μg/g dry plant) and syringic acid (3.17 to 29.01 μg/g dry plant) were present in all extracts,

and that such compounds were generally found in higher quantities (except in a few samples). Quercetin, vanillic, ferulic and *p*-coumaric acids were also identified, but not in all extracts, and usually in lower amounts (also, with some exceptions).

Overall, our results agree with the findings of Slimestad et al. and Mumivand et al. [55,56] in the sense that the compounds reported in those studies were also identified in some of our extracts, except for syringic acid. Another difference is that chlorogenic acid was not the predominant compound in our tarragon extracts (in fact, it was only found in one of them).

The work of Mumivand et al. highlights that, for the same plant species, the origin of the plant has an impact on the phenolic profile of the extracts, hence the variability of the results [56]. For this reason, it is not unexpected to observe discrepancies among outcomes of different studies, even for the same plant species, as seen here in some cases.

In this sense, it should be stated that comparison of results is important but must be done carefully because the outcomes are dependent on various factors, including the plant characteristics. Climate, cultivation method, stage of development of the plant and time of harvesting, etc., are likely to influence the phenolic composition of the extract produced [54].

In addition to the impact of plant specificities, to visualise the influence of extraction methods and solvents on the phenolic profile of extracts, principal component analysis was carried out (**Figure 8A** and **Figure 8B**, respectively).

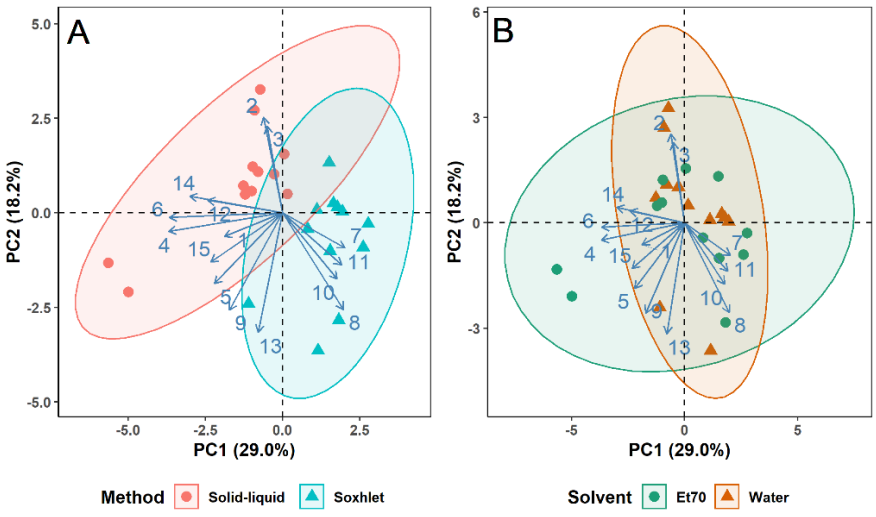

Figure 8. Score plots of the first two components of the principal component analysis (PCA) of phenolic compounds grouped by extraction method (**A**) and solvent (**B**). Phenolic compounds: 1–Chlorogenic acid, 2-Vanillic acid, 3-Syringic acid, 4-Cinnamic acid, 5-*p*-coumaric acid + epicatechin, 6-*o*-coumaric acid, 7-Rosmarinic acid, 8-Ellagic acid, 9–Naringin, 10–Hesperidin, 11–Kaempferol, 12–Resveratrol, 13-Ferulic acid, 14–Quercetin, 15-3,4-Dihydroxybenzoic acid.

4.3.5 Antimicrobial Activity

After considering the outcomes of the principal component analyses and the distinctive results in terms of chemical profile (more specifically, phenolic content) and antioxidant activity of some extracts, three were selected for the determination of MIC and MBC against four pathogens. The extracts chosen were all hydroethanolic, produced by solid-liquid extraction, using spearmint, sage, and lemon balm. The results obtained are displayed in **Table 14**.

Table 14. Minimum inhibitory concentration (mg/mL) of hydroethanolic solid-liquid extracts obtained from spearmint, sage, and lemon balm, against *L. monocytogenes*, *S. aureus*, *S. enterica* ser. Typhimurium, and *E. coli*.

Plant	*L. monocytogenes*	*S. aureus*	*S.* Typhimurium	*E. coli*
Sage	2.5–5	0.625	10	1.25
Spearmint	2.5	1.25	20	1.25
Lemon balm	5	2.5	20	2.5

The MIC is defined as the lowest concentration of an antimicrobial agent that completely inhibits growth of the organism in the microdilution wells as detected by the unaided eye [29]. In this study, all extracts examined showed promising results, with sage extract revealing the greatest potential. In fact, the sage extract resulting from hydroethanolic solid-liquid extraction produced the lowest MIC, 0.625 mg/mL, against *S. aureus*. This extract also revealed greater inhibitory action against *S. enterica* ser. Typhimurium, and equivalent or higher inhibitory action against *L. monocytogenes* and *E. coli*, compared to the remaining extracts.

While Gram-negative bacteria are generally more resistant to natural extracts than Gram-positive bacteria, with some exceptions [57], this was only evident in the case of *S. enterica* ser. Typhimurium, which revealed to be the least susceptible bacterium regardless of the bioactive extract. *E. coli*, on the other hand, did not reveal greater resistance to the plant extracts than the Gram-positive bacteria tested.

All plant extracts failed to kill the bacteria at the tested concentrations of 20 mg/mL and 10 mg/mL. Therefore, the MBC of these extracts were greater than 20 mg/mL.

In a study by Btissam et al. [58], hydroethanolic sage extracts obtained through maceration showed generally lower inhibitory effects than those determined for our extracts, with MIC values of 3.12 mg/mL for *L. monocytogenes*, 1.56 mg/mL for *S. aureus*, 25 mg/mL for *S. enterica* and 50 mg/mL for *E. coli*. Contrarily to our work, in this study, determination of the MBC was possible,

with values ranging from 3.12 to 100 mg/mL. In another work, by Stanojević et al. [59], aqueous sage extracts obtained by maceration revealed MIC values of 20 and 40 mg/mL for *S. aureus* and *E. coli*, respectively.

Scherer et al. [60] tested the antibacterial activities of spearmint extracts obtained by maceration with methanol, acetone, and dichloromethane against strains of *E. coli* and *S. aureus*; however, none of the extracts revealed significant antimicrobial activity. Another study, by Caleja et al. [61], analysed the antimicrobial activity of spearmint aqueous infusions, which presented higher inhibitory activity than the extracts of this study: the MIC values were 0.5 mg/mL for L. monocytogenes, 0.25 mg/mL for *S.* Typhimurium, and 0.5 mg/mL for *E. coli* (MBC values of 1, 0.5 and 1, respectively).

The same work by Caleja et al. also determined the antibacterial activity of lemon balm aqueous infusions against *L. monocytogenes*, *S.* Typhimurium and *E. coli*: all strains were equally affected by the extract, with MIC and MBC values of 1 mg/mL and 2 mg/mL, respectively [61]. These results indicate higher antimicrobial capacity of such infusion than that of our extracts. Oppositely, another study, by Ceyhan et al. [62], also testing the inhibitory effects of lemon balm, revealed MIC values against *S. aureus* of 3.12 and 6.25 mg/mL for the aqueous and hydroethanolic extracts, respectively; and MIC values against *E. coli* O157:H7 of 50 and 6.25 mg/mL for the aqueous and hydroethanolic extracts, respectively.

The large variety of MIC values described in literature for a single plant material may be explained by the impact of multiple factors on the chemical profile of the extracts (extraction method, solvent, and plant specificities, as mentioned before). Moreover, despite the existence of a standard protocol for antimicrobial susceptibility testing, the results may also vary depending on the bacterial strain selected, for example.

The potential of plant extracts as antimicrobials in food products is also dependent on the matrix selected. In this sense, it is crucial to perform experimental trials in food matrices to attest the functionality of the extract because the bioactivities determined in vivo will most likely be different that those found in vitro, a possible consequence of interactions between the plant extract and the food components and properties. Additionally, tests that can determine appropriate doses in foods, dose-response effects, and therapeutic dosages must be conducted, and the cost of usage of plant extracts must be assessed (to validate its commercial potential), as well as the impact on the sensory attributes of the food product (appearance, aroma, taste, texture, etc.).

4.4 CONCLUSIONS

The outcomes of this study provide insight on the phytochemical profile, antioxidant activity and antimicrobial potential of various plant extracts; they also provide insight on the effect of edible plant type, extraction methods and solvents on such characteristics.

The results show that both extraction method and solvent have an impact on most of the chemical characteristics and antioxidant profile of the extracts. Nonetheless, a greater difference is observed between extracts obtained using distinct solvents than distinct methods. The results also indicate the existence of interactions between the factors plant type, extraction method and solvent, affecting some of the chemical and antioxidant characteristics of extracts.

Overall, hydroethanolic solid-liquid extracts showed great potential as biopreservatives, due to their high phenolic contents, antioxidant activities and antimicrobial capabilities. Lemon balm, spearmint, and sage extracts presented the highest phenolic and flavonoid contents and strong antioxidant activities. Additionally, they revealed antimicrobial activity against four important foodborne pathogens (*S. enterica* ser. Typhimurium, *E. coli*, *L. monocytogenes* and *S. aureus*). These outcomes support the potential of lemon balm, spearmint, and sage extracts to be incorporated in foods as preservatives against oxidation and microbial spoilage. Nevertheless, further trials must be carried out to attest the functionality of these extracts, which is likely influenced by the food matrix and their cost of usage (to validate its commercial potential), as well as tests that can determine, for instance, appropriate doses in foods, dose-response effects, and therapeutic dosages.

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CHAPTER 5

PHYTOCHEMICAL COMPOSITION AND BIOACTIVE POTENTIAL OF *MELISSA OFFICINALIS* L., *SALVIA OFFICINALIS* L. AND *MENTHA SPICATA* L. EXTRACTS

This chapter was based on the following paper:

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5.1 INTRODUCTION

Over recent years, with the increasingly negative perception of consumers towards artificial food additives [1] and the higher demand for nutritious foods with additional health benefits, two major trends in the food industry have been to replace synthetic additives, which may be harmful to human health [2], and to develop nutraceuticals/functional foods [3].

In line with these trends, modern science has shown that plant matrices are sources of valuable molecules (for example, phenolic compounds) with promising biological value (e.g., antioxidant, anti-inflammatory, antibacterial and antifungal), thus encouraging their use for the development of functional foods and nutraceuticals, and as possible substitutes for artificial additives in foods or their packaging [4–6]. However, it is necessary to guarantee that the herbal extracts are safe for human consumption, and, among other considerations, it is crucial that they are obtained: (i) using nontoxic solvents authorised for the industrial production of foodstuffs and food ingredients, which do not leave residues or derivatives in the product after removal (or leave them in technically unavoidable quantities that pose negligible risk to human health) [7,8]; and (ii) from herbs with documented traditional use, commonly used in cooking as aroma and/or flavour enhancers [6,8,9].

To this, lemon balm (*Melissa officinalis* L., Lamiaceae), sage (*Salvia officinalis* L., Lamiaceae) and spearmint (*Mentha spicata* L., Lamiaceae) are among the various plants widely used in traditional Mediterranean cuisine and medicine, and for which several researchers have reported health-promoting capacities and potential as natural food additives [10–12]. Lemon balm has many beneficial capacities, such as spasmolytic, sedative, antitumoral, antimicrobial and antioxidant effects [13]. Furthermore, this plant has shown therapeutic effects for the treatment of the cognitive disturbance of Alzheimer's disease, and has been traditionally used to reduce anxiety, sleep disturbance, depression and gastrointestinal disorders [13,14]. In relation to sage, this herb has been used as a gargle for throat inflammations, to reduce perspiration, improve regularity of menstrual cycle, decrease hot flashes in menopause, battle gastrointestinal problems, prevent neurodegenerative diseases and improve mental capacity [11,15]. Furthermore, sage has shown anti-inflammatory, antimicrobial, hypoglycemic, antidiabetic, antioxidant and antitumor activities [15]. As for spearmint, it is frequently used in folk medicine against gastrointestinal and respiratory complications, haemorrhoids, stomach-ache, memory dysfunction, and can be used as a carminative, antispasmodic, diuretic, antibacterial, antifungal and antioxidant agent [12,16,17].

Considering the recognised beneficial effects for human health of lemon balm, sage and spearmint, the goal of this research was to chemically characterise and appraise the bioactivities of extracts from such plants, produced through different environmentally friendly extraction methods (decoction, infusion and maceration), using water and 80% ethanol (v/v) as solvents. More specifically, the extracts' cytotoxicity, antibacterial, antifungal, anti-inflammatory and antioxidant capacities were evaluated to assess their safety and preservative effects.

5.2 MATERIALS AND METHODS

5.2.1 Plant Material and Extraction Procedures

Sage, lemon balm and spearmint dry aerial parts were supplied by Pragmático Aroma, Lda. ("Mais Ervas", Trás-os-Montes, Portugal), mechanically milled and submitted to the following extraction methods: infusion, decoction and dynamic maceration.

For the infusions, 2 g of plant material was mixed with 200 mL of boiling distilled water and left to rest for 5 min without additional heating. For the decoctions, 2 g of plant material was mixed with 200 mL of distilled water, heated to boiling and boiled for 5 min. Infusions and decoctions were then filtrated (7–10 μm), frozen and lyophilised (FreeZone 4.5, Labconco, Kansas City, MO, USA). To obtain hydroethanolic extracts, dynamic macerations were conducted by incorporating 1 g of plant material in 30 mL of ethanol at 80% (v/v) and stirring at room temperature for 1 h. The supernatants were filtrated (7–10 μm), another 30 mL of ethanol 80% (v/v) was added to the extraction residues, and the maceration was repeated for 1 h. Finally, the ethanolic portion was evaporated (Büchi R-210, Flawil, Switzerland) and the resulting extracts were frozen and lyophilised. The extractions were carried out in triplicate (n = 3).

5.2.2 Identification and Quantification of Individual Phenolic Compounds

Individual phenolic compounds were investigated using a previously validated method, as described by Restivo et al. [18]. First, the samples were dissolved in ethanol 20% (v/v) up to a final concentration of 10 mg/mL and filtered through disposable 0.22 μm filters. The phenolic profiles were then determined by a liquid chromatography system (Dionex Ultimate 3000 UPLC, Thermo Scientific, San Jose, CA, USA) equipped with a quaternary pump, an automatic injector at 5 °C, a degasser and a column compartment with an automated thermostat. Compound detection was carried out with a diode-array detector (at wavelengths of 280 nm, 330 nm and 370 nm), coupled

to a mass spectrometry (MS) detector. Separation was performed on a reverse phase Waters Spherisorb S3 ODS-2 C18 column (4.6 mm × 150 mm, 3 µm) at 35°C. The flow rate was 0.5 mL/min. The mobile phase used was water/formic acid 0.1% (A) and acetonitrile (B). The elution gradient for solvent B was as follows: 10–15% eluent B up to 5 min, 15–20% B up to 5 min, 20–25% B 10 min, 25–35% B 10 min, 35–50% B 10 min and column re-equilibration for 10 min. For MS detection, a Linear Ion Trap LTQ XL spectrophotometer equipped with an electrospray ionization source was used. Nitrogen (50 psi) was used as a carrier gas, and the system worked with an initial temperature of 325 °C, a spray voltage of 5 kV and a capillary voltage of −20 V. The tube lens offset voltage remained at −66 V. Spectra were recorded in negative ion mode 100–1500 *m/z*.

The phenolic compounds were identified through their chromatographic characteristics by comparison to the obtained standard compounds (4-hydroxybenzoic acid, apigenin-6-*C*-glucoside, apigenin-7-*O*-glucoside, caffeic acid, chlorogenic acid, naringenin and rosmarinic acid) and with the literature [19–21]. For quantitative analysis, calibration curves prepared with appropriate standards (between 100 and 2.5 mg/L) were used. Limits of detection and quantification were also calculated, and, in all cases, the coefficient of linear correlation was $R^2 > 0.99$ (**Table 15**). All analyses were made in triplicate (n = 3). The results were expressed in mg per g of dry extract (mg/g).

Table 15. Limit of detection (LOD), limit of quantification (LOQ) and coefficient of linear correlation (R^2) of the different standards used to obtain the calibration curves required for phenolic compound quantification

Compound Standard	LOD (µg/mL)	LOQ (µg/mL)	R^2
4-Hydroxybenzoic acid	0.17	1.22	0.999
Apigenin-6-*C*-glucoside	0.19	0.63	0.9989
Apigenin-7-*O*-glucoside	0.10	0.53	0.999
Caffeic acid	0.78	1.97	0.994
Chlorogenic acid	0.20	0.68	0.9999
Naringenin	0.20	0.64	0.9998
Rosmarinic acid	0.15	0.68	0.999

5.2.3 Biological Evaluation

5.2.3.1 Antibacterial and Antifungal Activity

For the antibacterial and antifungal activity screening, six bacterial strains were used: *Escherichia coli* (ATCC 25922), *Salmonella enterica* serovar Typhimurium (ATCC 13311), *Enterobacter cloacae* (clinical isolate), *Staphylococcus aureus* (ATCC 11632), *Bacillus cereus* (food isolate) and *Listeria monocytogenes* (NCTC 7973), and six micromycetes: *Aspergillus fumigatus* (human isolate), *Aspergillus niger* (ATCC 6275), *Aspergillus versicolor* (ATCC 11730), *Penicillium funiculosum* (ATCC 36839), *Penicillium verrucosum* var. *cyclopium* (food isolate) and *Trichoderma viride* (IAM 5061).

Minimum inhibitory (MIC), minimum bactericidal (MBC) and minimum fungicidal (MFC) concentrations were determined using a broth microdilution method and 96-well microplates [22]. The streak plate culture method, conducted on tryptic soy agar (Torlak, Belgrade, Serbia) incubated at 37 °C for 24 h, was used to obtain bacterial cells in the exponential growth phase. Then, an adequate number of individual colonies were placed in tubes with sterile water to achieve bacterial suspensions with a concentration of approximately 1.0×10^5 CFU/well in the microplates. For the antifungal activity essay, fungal spores were washed from the surface of malt agar plates (Neogen, Heywood, UK) with sterile 0.85% saline added with 0.1% Tween 80 (v/v) (Zorka pharma, Šabac, Belgrade). Sterile saline was then used to adjust the spore suspension to a concentration of approximately 1.0×10^5 in a final volume of 100 µL per well.

For the antibacterial and antifungal essay, resuspended extracts were obtained by dissolving them in ethanol 30% (v/v) to obtain a final concentration of 10 mg/mL. The liquid media (90 µL) used in the microplate wells was tryptic soy broth (Torlak, Belgrade, Serbia) for the antibacterial essay, or malt extract broth (Neogen, Heywood, UK) in the case of the antifungal essay.

After placing the inoculum, resuspended extract and liquid media in the microplate wells as appropriate, the microdilution plates were incubated at 37 °C for 24 h for the determination of the antibacterial activity, or 28 °C for 72 h for the determination of the antifungal activity. After that, 40 µL of iodonitrotetrazolium (Sigma-Aldrich, St. Louis, MO, USA), at a concentration of 0.2 mg/mL, was added to each well, and the microplate incubated again at 37 °C for 1 h. Afterwards, the microplates were evaluated, and the lowest concentrations without visible growth were determined as the MICs. The MBCs were determined as the lowest concentration with no visible

growth after serial sub-cultivation of 10 µL into microdilution plates containing 100 µL of tryptic soy broth per well and further incubated for 24 h at 37 °C.

For the antifungal essay, MICs were determined under binocular microscope using the same procedure as described above. After that, the MFC was determined by serial sub-cultivation of 2 µL of the content of the wells and further incubation at 28 °C for 72 h. The lowest concentration of this sub-culture with no visible growth was defined as the MFC.

Two commonly used artificial food preservatives, sodium benzoate (E211) and potassium metabisulfite (E224), were also tested to evaluate the sensitivity of the microorganisms to such additives. The MIC, MBC and MFC were expressed in mg/mL of the resuspended lyophilised extracts.

5.2.3.2 Antioxidant Activity

The antioxidant activity was evaluated through two in vitro essays, using previously described methodologies [23,24]: inhibition of lipid peroxidation by decrease in the formation of thiobarbituric acid reactive substances (TBARS), and the oxidative haemolysis inhibition essay (OxHLIA). The extracts were initially diluted in distilled water (for TBARS) or phosphate-buffered saline (PBS, pH 7.4) (for OxHLIA) to different concentrations. TROLOX was used as a positive control in both essays.

For TBARS essay: the extracts were examined for their power to inhibit the ferrous sulphate-induced lipid peroxidation, using porcine brain cell homogenates, through monitorisation of the colour strength (at 532 nm) provided by malondialdehyde-thiobarbituric acid complexes. The results were expressed as the extract concentration (µg/mL) required to inhibit 50% of the TBARS formation (half-maximal inhibitory concentration, IC_{50}).

For OxHLIA essay: 200 µL of an erythrocyte solution at 2.8% prepared in PBS was added to 400 µL of either: extract solution (13–800 µg/mL in PBS), PBS solution (negative control), distilled water (baseline), or TROLOX (7.81–250 µg/mL). After incubation for 10 min at 37 °C with agitation, 200 µL of 2,2′-azobis(2-methylpropionamidine) dihydrochloride (AAPH, 160 mM in PBS) was added, and the optical density (at 690 nm) was measured in a microplate reader (Bio-Tek Instruments, ELx800, Winooski, VT, USA) every 10 min until complete haemolysis. The percentage of the erythrocyte population that remained undamaged (P) was calculated using Equation (1), where S_t and S_0 are the optical density of the sample at t and 0 min, respectively, and CH_0 is the optical density of the complete haemolysis at 0 min.

$$P\% = 100 \times \left(\frac{S_t - CH_0}{S_0 - CH_0}\right) \quad (1)$$

The delayed time of haemolysis (Δt) was calculated using Equation (2), where the 50% haemolytic time (min) graphically obtained from the haemolysis curve of each sample concentration is represented by Ht_{50}:

$$\Delta t\,(\text{min}) = Ht_{50}\,(\text{sample}) - Ht_{50}\,(\text{control}) \quad (2)$$

Lastly, the Δt values were correlated to the various sample concentrations. From that correlation, the concentrations able to promote Δt haemolysis delays of 60 min and 120 min were calculated. The results were expressed as IC_{50} values (μg/mL) at Δt = 60 min and Δt = 120 min, i.e., the sample concentration required to protect 50% of the erythrocyte population from the haemolytic action of AAPH for 60 min and 120 min, respectively.

5.2.3.3 Anti-Inflammatory Activity

The anti-inflammatory activity was evaluated using a previously described essay, with modifications [25]. First, cells from the mouse macrophage-like cell line RAW264.7 were seeded in plates of 96-wells, and their attachment was allowed overnight. Subsequently, cells were subjected to different extract concentrations (6.25–400 μg/mL) for 1 h, and then stimulated with lipopolysaccharides (1 μg/mL) for 18 h. This procedure enabled observation of the occurrence of induced changes in nitric oxide basal levels, using a Griess Reagent System kit (Promega, Madison, WI, USA). The nitrite level produced was determined in a microplate reader (Bio-Tek Instruments, ELx800, Winooski, VT, USA) by assessing the optical density at 540 nm and comparing it with the standard calibration curve. The positive control used was dexamethasone (50 μM). The results are stated as the sample concentration (μg/mL) necessary to inhibit 50% of the nitric oxide production (IC_{50}).

5.2.3.4 Cytotoxic Activity

The lyophilised extracts were dissolved in water and successively diluted to obtain the stock solutions. The cytotoxic activity was then assessed against six human tumour cell lines, namely AGS (gastric adenocarcinoma), CaCo-2 (colorectal adenocarcinoma), HeLa (cervical carcinoma), MCF-7 (breast adenocarcinoma), NCI-H460 (large cell lung carcinoma) and non-tumour hFOB (human foetal osteoblasts), using the previously described sulforhodamine B essay [25]. For this, each of the cell lines (190 μL, 10^4 cells/mL) was incubated with the plant extracts at various

concentrations (6.25–400 μg/mL). Ellipticine was used as a positive control. The results were expressed as the extract concentration required to inhibit 50% of the net cell growth (half-maximal cell growth inhibitory concentration, GI_{50}).

5.2.4 Statistical Analysis

Data were presented as mean ± standard deviation (SD) values. One-way analysis of variance (ANOVA, $\alpha = 0.05$) was used to assess statistical differences between the means. Clustered heatmaps were generated using the pheatmap function from the pheatmap package [26]. Statistical analysis was conducted in R software (version 4.1.0, R Foundation for Statistical Computing, Vienna, Austria).

5.3 RESULTS AND DISCUSSION

5.3.1 Phenolic Profile

The peak characteristics (retention time, wavelength of maximum absorption and mass spectral data), tentative identification and quantification of the phenolic compounds detected in the extracts produced are reported in **Table 16**, **Table 17** and **Table 18** (sage, lemon balm and spearmint, respectively). Heatmaps for a fast visualisation of the phenolic compounds identified and their concentrations were produced and are shown in **Figure 9**, **Figure 10** and **Figure 11** (sage, lemon balm and spearmint, respectively).

The dendrograms of each clustered heatmap arrange the information on phenolic composition in terms of similarities, where the lower the height at which any two objects are joined, the greater the similarity. In this sense, one dendrogram (left) offers insight regarding compounds detected in similar concentrations across extracts obtained through different methodologies (infusion, decoction and hydroethanolic maceration), whereas the other dendrogram (upper) informs about similar total phenolic compound content across the extracts produced, for each plant.

Table 16. Phenolic compound content (mg/g dry extract) of sage (*Salvia officinalis* L.) extracts.

Peak	Rt	λ_{max}	[M-H] *m/z*	MS^2	Tentative Identification	Decoction	Infusion	Hydroethanolic
1	4.87	267sh319	311	179(60),149(100),135(9)	Caftaric acid [1]	0.24±0.01	0.27±0.01	0.26±0.00
2	5.15	325	503	341(17),281(15),221(8),179(34),161(38),135(5)	6-*O*Caffeoyl-fructosyl-glucoside [4]	0.28±0.02	0.27±0.02	0.23±0.01
3	5.74	328	341	179(100)	Caffeic acid hexoside [4]	0.15±0.01	0.14±0.00	0.12±0.00
4	7.30	325	387	369(26), 207(100), 163(47)	Caffeic acid acetylhexoside [4]	0.11±0.01	0.10±0.00	0.12±0.00
5	7.77	282sh323	377	207(100),191(5),163(38),137(7)	3-*p*-Coumarouylquinic acid [5]	0.10±0.01	0.10±0.00	0.09±0.00
6	8.8	337	593	473(18), 383(6), 353(12)	Apigenin-6,8-di-*C*hexoside [2]	1.23±0.09	1.42±0.08	0.89±0.02
7	10.96	323	537	519(84),341(10),179(32),161(48),135(10)	Salvianolic acid I [7]	0.70±0.02	0.67±0.03	0.55±0.01
8	13.14	325	637	285(100)	Luteolin-*O*di-glucuronide [3]	1.10±0.07	1.33±0.08	1.05±0.05
9	14.87	341	477	301(100)	6-Hydroxyluteolin-7-*O*-glucuronide [7]	3.54±0.25	3.63±0.17	3.05±0.03
10	15.93	327	597	359(30),295(26),197(16),179(15),135(8)	Yunnaneic acid F [7]	0.93±0.02	0.87±0.07	0.66±0.01
11	17.18	341	593	473(21), 383(9), 353(16)	Apigenin 6-*C*-glucose-8-*C*-glucose [7]	1.00±0.03	1.47±0.01	0.84±0.00
12	17.81	345	461	285(100)	Luteolin-7-*O*-glucuronide [3]	18.1±0.71	15.7±0.98	12.1±0.41
13	19.41	337	717	537(78),519(100),493(53),339(27),321(45)295(62)	Salvianolic acid B [7]	1.66±0.04	2.88±0.11	2.08±0.02
14	20.66	336	359	359(47),197(73),179(71),161(100)	*cis*-Rosmarinic acid [7]	28.4±1.25	24.7±1.29	22.7±0.71
15	21.22	289sh326	555	493(100),359(16),225(5)	Salvianolic acid K isomer I [7]	4.46±0.13	3.64±0.15	3.34±0.09
16	22.09	329	555	493(100),359(16),225(5)	Salvianolic acid K isomer II [7]	2.98±0.12	3.34±0.24	2.31±0.08
17	22.64	334	447	285(100)	Luteolin-*O*hexoside isomer I [3]	6.20±0.10	5.33±0.34	3.48±0.10
18	23.36	332	359	359(45),197(69),179(75),161(100)	*trans*-Rosmarinic acid [7]	1.50±0.08	2.05±0.12	1.39±0.05
19	24.26	330	555	493(100),359(26),225(7)	Salvianolic acid K isomer [7]	1.71±0.02	1.66±0.02	1.36±0.05
20	25.77	336	503	285(100)	Luteolin-acetyl-*O*-glucuronide [3]	3.52±0.08	3.07±0.07	2.55±0.20
21	27.22	328	537	493(100),359(43),313(8),295(5)	Lithospermic acid A [7]	3.32±0.12	2.69±0.11	1.99±0.02
22	27.9	327	563	545(27),503(40),473(100),443(83),383(90),353(85)	Apigenin 6-*C*-pentosyl-8-*C*hexoside [2]	1.14±0.01	0.88±0.00	0.73±0.04
23	30.86	337	447	285(100)	Luteolin-*O*hexoside isomer II [3]	1.16±0.02	0.90±0.01	0.87±0.05
24	32.87	283sh323	717	537(25),519(44),493(18),339(24),321(25),313(9),295(100)	Salvianolic acid E [7]	0.58±0.04	0.54±0.01	0.44±0.01
					Total Phenolic Acids	47.12±1.38	43.93±0.61	37.64±0.77
					Total Flavonoids	36.95±1.18	33.74±1.71	25.53±0.24
					Total Phenolic Compounds	84.07±2.56	77.67±1.1	63.17±1.01

Rt: Retention time (min), λmax: wavelengths of maximum absorption in the visible region (nm); MS^2: second stage of mass spectrometry. Superscript numbers indicate the compound standard used for the quantification: (1) 4-Hydroxybenzoic acid; (2) Apigenin-6-*C*-glucoside; (3) Apigenin-7-*O*-glucoside; (4) Caffeic acid; (5) Chlorogenic acid; (6) Naringenin; (7) Rosmarinic acid. Content values expressed as mean ± SD (n= 3).

Table 17. Phenolic compound content (mg/g dry extract) of lemon balm (*Melissa officinalis* L.) extracts.

Peak	Rt	λ_{max}	[M-H] *m/z*	MS^2	Tentative Identification	Decoction	Infusion	Hydroethanolic
1	4.67	328	311	179(61),149(100),135(8)	Caftaric acid [1]	0.22±0.01	0.39±0.01	nd
2	8.86	288sh324	179	135(100)	Caffeic acid [4]	0.10±0.00	0.04±0.00	0.37±0.00
3	9.27	281sh325	539	495(63),359(21),295(45),279(4),197(34),179(16)	Yunnaneic acid D [7]	0.32±0.01	0.31±0.01	0.15±0.01
4	11.56	323	537	519(84),341(10),179(32),161(48),135(10)	Salvianolic acid I [7]	1.31±0.07	1.18±0.02	0.33±0.00
5	15.53	284sh326	623	461(100), 285(85)	Luteolin-*O*hexosyl-*O*-glucuronide [3]	0.62±0.04	0.59±0.01	0.61±0.00
6	16.35	330	521	359(51), 197(21),179(37),161(100)	Rosmarinic acid hexoside [7]	2.36±0.01	1.83±0.08	1.36±0.01
7	16.9	278sh319	717	519(100), 339, 321	Salvianolic acid A [7]	1.27±0.05	1.78±0.06	0.53±0.02
8	18.59	283sh325	719	539(12),521(9), 359(100), 197(9), 179(12), 161(51),135(5)	Sagerinic acid [7]	2.35±0.15	2.03±0.06	1.08±0.01
9	19.91	330	439	359(10), 179(8),161(40),135(28)	Sulphated rosmarinic acid [7]	1.37±0.07	0.95±0.07	0.67±0.03
10	20.57	316sh331	359	197(81),179(90),161(100), 135(28)	Rosmarinic acid [7]	41.7±2.00	34.4±0.20	40.4±0.22
11	24.3	326	537	493(100),359(35),313(4),295(2)	Lithospermic acid A [7]	11.5±0.81	5.78±0.36	4.54±0.04
12	27.17	328	537	493(100),359(32),313(4),295(5)	Lithospermic acid A isomer [7]	16.1±0.06	9.44±0.51	6.04±0.41
13	30.05	328	829	667(82), 535(100), 491(21),311(50), 293(5), 197(3), 179(11)	Salvianolic acid C derivative [7]	3.76±0.10	1.51±0.04	1.43±0.03
14	33.16	287sh324	717	537(25),519(44),493(18),339(24),321(25),313(9),295(100)	Salvianolic acid E [7]	1.53±0.04	0.75±0.02	0.86±0.01
					Total Phenolic Acids	83.9±1.74	60.4±1.41	57.7±0.31
					Total Flavonoids	0.62±0.04	0.59±0.01	0.61±0.00
					Total Phenolic Compounds	84.5±1.77	61.00±1.42	58.4±0.31

Rt: Retention time (min), λ_{max}: wavelengths of maximum absorption in the visible region (nm); MS^2: second stage of mass spectrometry. Superscript numbers indicate the compound standard used for the quantification: (1) 4-Hydroxybenzoic acid; (2) Apigenin-6-*C*-glucoside; (3) Apigenin-7-*O*-glucoside; (4) Caffeic acid; (5) Chlorogenic acid; (6) Naringenin; (7) Rosmarinic acid. Content values expressed as mean ± SD (n= 3); nd: not detected.

Table 18. Phenolic compound content (mg/g dry extract) of spearmint (*Mentha spicata* L.) extracts.

Peak	Rt	λ_{max}	[M-H] *m/z*	MS^2	Tentative Identification	Decoction	Infusion	Hydroethanolic
1	3.95	281	191	173(100),111(23)	Quinic acid [1]	0.61±0.01	0.22±0.00	nd
2	4.67	283sh321	387	193(100),179(3),161(8),133(4)	Caffeic acid methyl ester dimer [4]	nd	0.01±0.01	0.10±0.00
3	5.91	291sh314	353	191(20), 179(61), 173(100). 135(12)	5-*O*-Caffeoylquinic acid [5]	0.86±0.03	0.03±0.03	0.05±0.00
4	8.96	299sh321	179	135(100)	Caffeic acid [4]	0.25±0.00	1.01±0.03	1.23±0.07
5	12.67	284sh326	623	461(100), 285(84)	Luteolin-*O*-hexosyl-*O*-glucuronide [3]	0.57±0.01	0.07±0.00	0.07±0.00
6	13.89	285sh331	595	287(100)	Eriodictyol-7-*O*-rutinoside [5]	1.84±0.03	3.99±0.11	4.25±0.09
7	16.87	347	593	285(100)	Luteolin-*O*-rutinoside [3]	9.29±0.06	1.26±0.03	2.34±0.11
8	17.91	347	461	285(100)	Luteolin-*O*-glucuronide [3]	15.3±0.05	3.56±0.09	4.91±0.18
9	19.55	285sh338	717	519(100), 339, 321	Salvianolic acid A [7]	2.20±0.01	1.22±0.04	1.19±0.03
10	20.62	331	359	197(87), 179(91), 161(100), 135(48)	Rosmarinic acid [7]	31.3±0.35	19.6±0.49	32.1±1.18
11	22.58	282sh326	719	539(12),521(9), 359(100), 197(9), 179(12), 161(51),135(5)	Sagerinic acid [7]	3.29±0.14	2.24±0.08	2.68±0.01
12	24.34	299sh327	537	493(100),359(35),313(4),295(2)	Lithospermic acid A [7]	5.89±0.41	2.83±1.30	5.57±0.02
13	27.08	289sh324	533	489 (100), 285 (51)	Luteolin-7-*O*-malonylglucoside [3]	2.19±0.11	0.39±0.08	0.58±0.04
14	28.96	298sh325	537	493(70), 359(15), 313(30), 295(100),269(25),197(16),179(72)	Lithospermic acid A isomer [7]	3.64±0.22	2.35±0.01	2.87±0.05
					Total Phenolic Acids	48.0±0.41	29.5±0.78	45.8±1.24
					Total Flavonoids	29.2±0.1	9.28±0.16	12.2±0.34
					Total Phenolic Compounds	77.2±0.51	38.8±0.62	57.9±1.59

Rt: Retention time (min), λ_{max}: wavelengths of maximum absorption in the visible region (nm); MS^2: second stage of mass spectrometry. Superscript numbers indicate the compound standard used for the quantification: (1) 4-Hydroxybenzoic acid; (2) Apigenin-6-*C*-glucoside; (3) Apigenin-7-*O*-glucoside; (4) Caffeic acid; (5) Chlorogenic acid; (6) Naringenin; (7) Rosmarinic acid. Content values expressed as mean ± SD (n= 3); nd: not detected

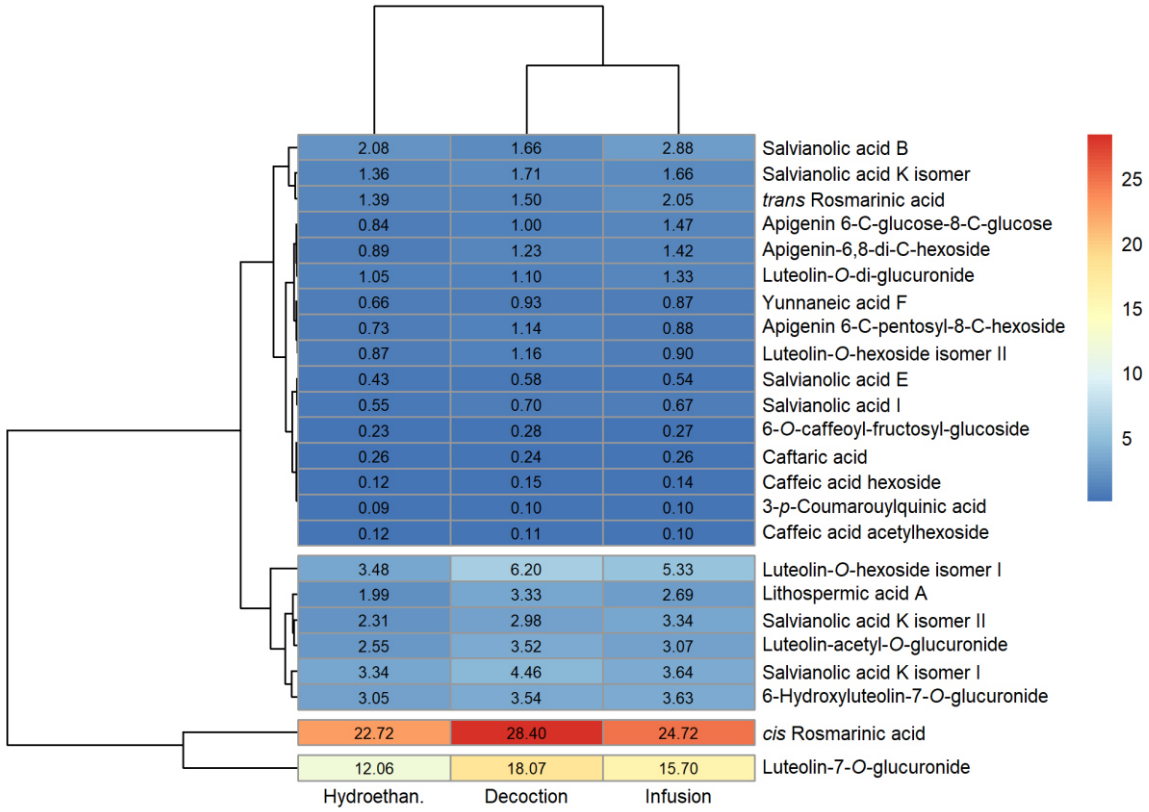

Figure 9. Clustered heatmap visualisation of phenolic compounds detected in sage infusion, decoction and hydroethanolic extract (units: mg/g).

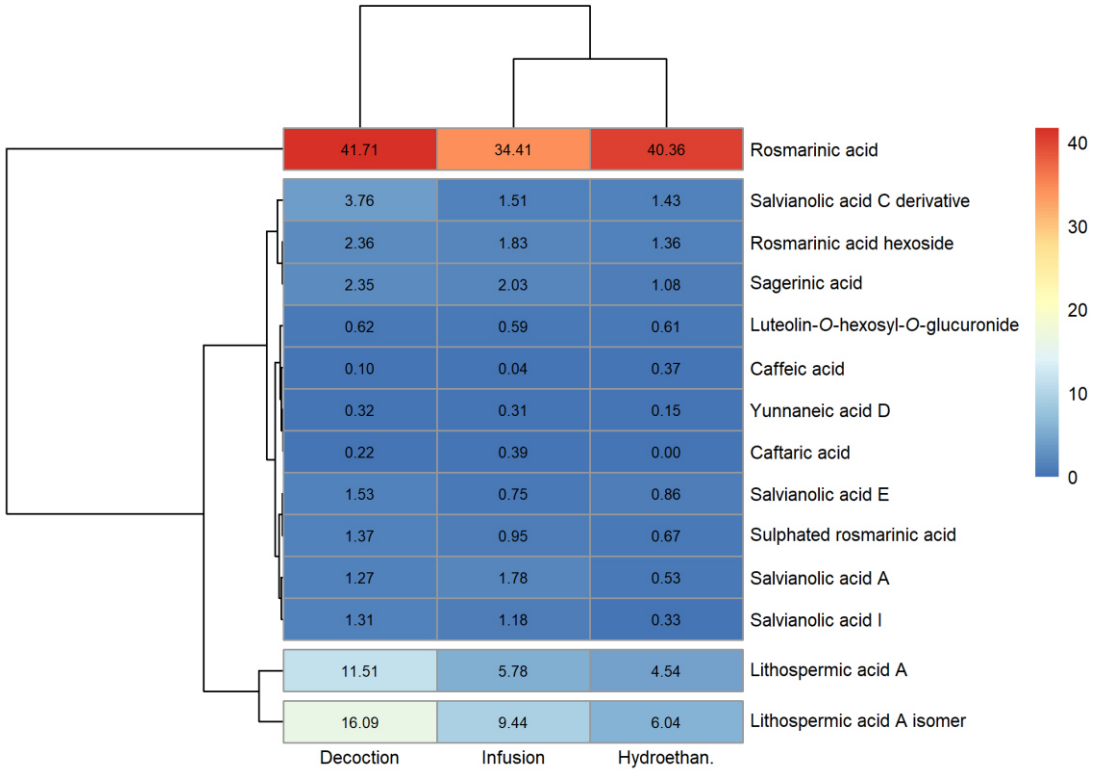

Figure 10. Clustered heatmap visualisation of phenolic compounds detected in lemon balm infusion, decoction and hydroethanolic extract (units: mg/g).

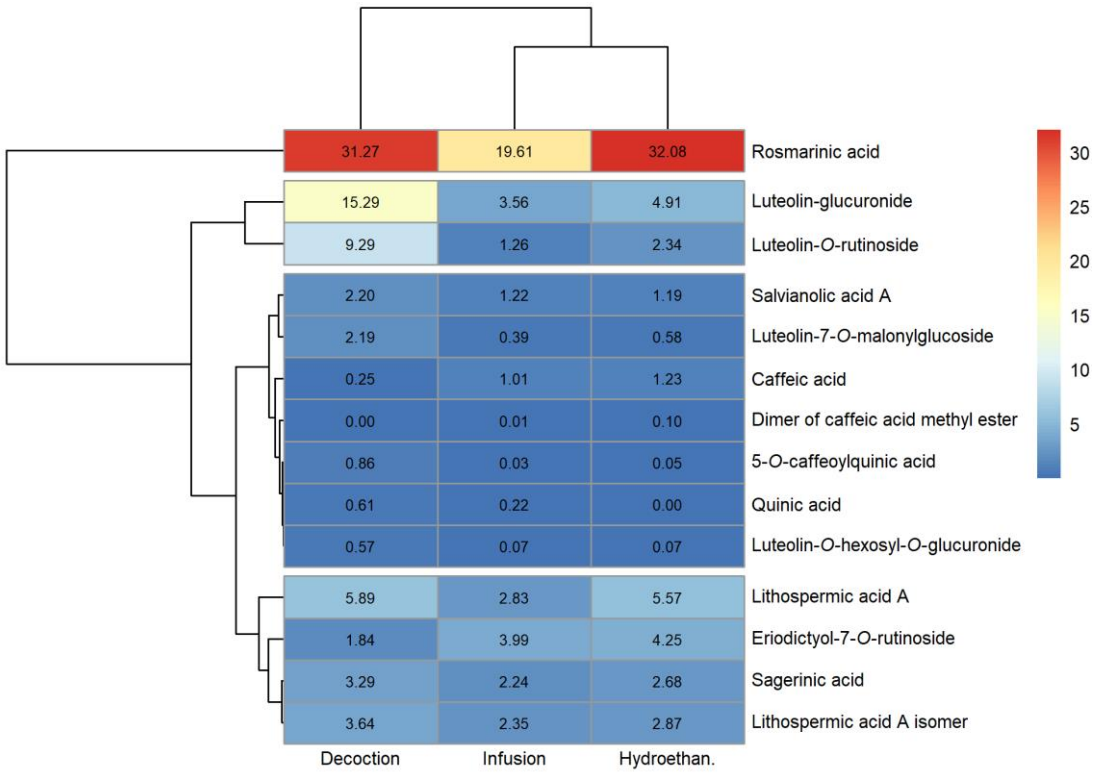

Figure 11. Clustered heatmap visualisation of phenolic compounds detected in spearmint infusion, decoction and hydroethanolic extract (units: mg/g).

Twenty-four phenolic compounds were identified in all sage extracts. From **Figure 9** and **Table 16**, sage decoction and infusion contained higher and similar total phenolic compounds content (84.07 and 77.67 mg/g extract, respectively), compared to the hydroethanolic extract (63.17 mg/g extract). In the case of lemon balm, a maximum of fourteen compounds were identified, depending on the extract type. **Figure 10** and **Table 17** indicate that its infusion and hydroethanolic extract showed comparable total phenolic compounds content (61.00 and 58.35 mg/g extract, respectively); however, lower than that of the decoction (84.51 mg/g extract). As for the spearmint extracts, a maximum of fourteen compounds were identified. **Figure 11** and **Table 18** reveal that spearmint infusion and hydroethanolic extract had closer total phenolic compounds concentration (38.79 and 57.92 mg/g extract, respectively) than spearmint decoction (77.20 mg/g extract). Considering these results, decoctions revealed the highest amount of total phenolic compounds when compared to infusions and hydroethanolic extracts, regardless of the plant (**Table 16** to **Table 18**). Overall, sage and lemon balm decoctions stood out for their higher total phenolic content (84.07 and 84.51 mg/mL, respectively). Oppositely, spearmint infusion yielded the lowest total phenolic content among the nine extracts (38.79 mg/g extract, **Table 18**).

In all cases, the plant extracts revealed a higher content of total phenolic acids compared with total flavonoids (**Table 16** to **Table 18**). This was particularly noticeable in lemon balm extracts, which presented total flavonoid concentrations lower than 0.7 mg/g extract, in comparison with the total phenolic acids content, which ranged between 57.74 and 83.90 mg/g extract. In terms of qualitative profile, sage showed the highest variety of phenolic acids, with a total of fifteen different acids regardless of the type of extract. In comparison, in lemon balm, twelve or thirteen distinct phenolic acids were identified, depending on the extract type, whereas nine or ten acids were identified in spearmint extracts.

Some phenolic acids were found across all the evaluated extracts, namely, rosmarinic and salvianolic acids, as well as lithospermic acid A. Among these, the major compound in all the sage extracts was *cis*-rosmarinic acid (22.72 to 28.40 mg/g extract; **Figure 9** and **Table 16**), followed by a derivative of luteolin, luteolin-7-*O*-glucuronide (12.06 to 18.07 mg/g extract; Figure 1 and Table S2). In lemon balm, rosmarinic acid was found in the greatest amount, irrespective of the type of extract, with concentrations between 34.40 and 41.71 mg/g extract (**Figure 10** and **Table 17**). Similarly, the major phenolic compound in spearmint extracts was rosmarinic acid (19.61 to 32.08 mg/g extract; **Figure 11**, **Table 18**). Rosmarinic acid is known to possess extraordinary therapeutic potential, which includes antiviral, antibacterial, anticarcinogenic, antioxidant, anti-

aging, antidiabetic, cardioprotective, hepatoprotective, nephroprotective, antidepressant, antiallergic and anti-inflammatory activities [27].

Sage extracts presented the highest number of different flavonoids (nine in total). These were derivates of apigenin and luteolin, with the most abundant compound being luteolin-*7-O*-glucuronide. Flavonoids were also detected in lemon balm and spearmint extracts, although in lesser variety (one and four in total, respectively), and these were also luteolin derivatives.

Given these results, sage, lemon balm and spearmint extracts appear to be valuable sources of valuable bioactive compounds, particularly of phenolic acids. Previous studies also investigated the phenolic profile of the plant materials used in this work. In sage and lemon balm hydroethanolic extracts, Spréa et al. [6] identified twenty-one and twelve phenolic compounds, respectively, several of which were also detected in the present study. Maliki et al. [28] studied the polyphenolic profile of a sage aqueous extract, identifying eighteen compounds, most of which belonged to hydroxycinnamic acid, rosmarinic acid and luteolin derivatives. Both the studies of Spréa et al. and Maliki et al. [6,28] found rosmarinic acid (51.00 mg/g and 2.192 mg/g, respectively) and luteolin-7-*O*-glucuronide (27.00 and 1.877 mg/g, respectively) to be the compounds of the highest concentrations in sage extracts, thus supporting the findings of our study. Also, in agreement with our results, Cirlini et al. [12] identified rosmarinic acid and its derivatives as the most prevalent polyphenolic compounds in an aqueous spearmint extract (230.5 mg/g), followed by salvianolic acids (14.70 mg/g) and caffeoylquinic acids (3.06 mg/g). Silva et al. [29] identified rosmarinic acid as the main compound in aqueous (204 mg/L) and hydroethanolic (333 mg/L) spearmint extracts; however, in lemon balm hydroethanolic extract, naringin was the principal compound (116 mg/L), and in sage aqueous and hydroethanolic extracts, hesperidin was present in the greatest amount (279 and 805 mg/L, respectively). This and other studies may have reported different phytochemical compositions [30,31], which however does not conflict with our results, since variations can be caused by different environmental factors during plant development, including soil type, change in season, salinity, light, altitude and humidity, as well as plant growth stage and extraction procedure [12,32]. Since the health-promoting properties of plants have been largely attributed to their phenolic compounds (among other secondary metabolites) [33,34], it is intuitive that differences in phenolic profile among extracts produced from the same plant matrix will also originate variations in their bioactivities (antimicrobial, antioxidant and anti-inflammatory, for example).

5.3.2 Antibacterial and Antifungal Activity

The results of the antibacterial and fungicidal activity are shown in **Table 19** and **Table 20**, respectively.

Table 19. Antibacterial activity of plant extracts expressed as minimum inhibitory concentration and minimum bactericidal concentration, MIC/MBC, respectively (mg/mL; mean ± SD, n = 3).

Extraction	Plant	SA [1]	BC [2]	LM [3]	EC [4]	ST [5]	EntC [6]
Infusion	Lemon balm	0.5/1	1/2	0.5/1	0.5/1	0.5/1	1/2
	Spearmint	0.5/1	0.5/1	0.5/1	0.5/1	0.5/1	1/2
	Sage	0.25/0.5	0.25/0.5	1/2	1/2	0.5/1	1/2
Decoction	Lemon balm	0.5/1	0.5/1	2/4	1/2	0.5/1	1/2
	Spearmint	0.5/1	0.5/1	1/2	0.5/1	0.5/1	1/2
	Sage	0.5/1	0.5/1	1/2	0.5/1	0.5/1	1/2
Hydroethanolic extraction	Lemon balm	0.5/1	0.5/1	1/2	0.5/1	0.5/1	0.5/1
	Spearmint	0.5/1	1/2	0.5/1	0.5/1	0.5/1	0.5/1
	Sage	0.5/1	1/2	0.5/1	0.5/1	0.5/1	0.5/1
E211 [7]		4/4	0.5/0.5	1/2	1/2	1/2	2/4
E224 [8]		1/1	2/4	0.5/1	0.5/1	1/1	0.5/0.5

Legend: [1] *S. aureus*, [2] *B. cereus*, [3] *L. monocytogenes*, [4] *E. coli*, [5] *Salmonella enterica* ser. Typhimurium, [6] *E. cloacae*, [7] Sodium benzoate, [8] Potassium metabisulfite.

Table 20. Antifungal activity of plant extracts expressed as minimum inhibitory and minimum fungicidal concentration, MIC/MFC, respectively (mg/mL; mean ± SD, n = 3).

Extraction	Plant	AF [1]	AN [2]	AV [3]	PF [4]	PVC [5]	TV [6]
Infusion	Lemon balm	0.125/0.25	0.125/0.25	0.25/0.5	0.5/1	0.5/1	0.25/0.5
	Spearmint	0.125/0.25	0.25/0.5	0.25/0.5	0.5/1	1/2	0.25/0.5
	Sage	0.25/0.5	0.25/0.5	0.25/0.5	0.25/0.5	0.25/0.5	0.125/0.25
Decoction	Lemon balm	0.25/0.5	>4/>4	0.25/0.5	0.5/1	0.25/0.5	0.125/0.25
	Spearmint	0.25/0.5	>4/>4	0.25/0.5	0.5/1	0.25/0.5	0.25/0.5
	Sage	0.25/0.5	0.5/1	0.5/1	0.5/1	0.5/1	0.25/0.5
Hydroethanolic extraction	Lemon balm	0.5/1	0.5/1	0.25/0.5	0.25/0.5	0.25/0.5	0.25/0.5
	Spearmint	0.25/0.5	0.25/0.5	0.25/0.5	0.25/0.5	0.25/0.5	0.125/0.25
	Sage	0.5/1	0.5/1	0.25/0.5	0.25/0.5	0.125/0.25	0.125/0.25
E211 [7]		1/2	1/2	2/2	1/2	2/4	1/2
E224 [8]		1/1	1/1	1/1	0.5/0.5	1/1	0.5/0.5

Legend: [1] *A. fumigatus*, [2] *A. niger*, [3] *A. versicolor*, [4] *P. funiculosum*, [5] *P. verrucosum* var. *cyclopium*, [6] *T. viride*, [7] Sodium benzoate, [8] Potassium metabisulfite.

Overall, the extracts revealed antimicrobial activity against all foodborne pathogens tested, namely *S. aureus*, *B. cereus*, *L. monocytogenes*, *E. coli*, *S.* Typhimurium and *E. cloacae* (MIC ≤ 2 mg/mL; MBC ≤ 4 mg/mL). Sage infusion presented the lowest MIC and MBC values of all extracts (i.e., the greatest antimicrobial potential), particularly against *S. aureus* and *B. cereus* (MIC = 0.25 and MBC = 0.5 mg/mL in both cases). On the other hand, lemon balm decoction displayed the highest MIC and MBC values, specifically against *L. monocytogenes* (MIC = 2 and MBC = 4 mg/mL). With a few exceptions, hydroethanolic extracts showed uniform activity (MIC = 0.5 and 1 mg/mL) for all tested bacteria.

In terms of antifungal capacity, all the infusions and hydroethanolic extracts were effective in inhibiting the six fungi tested, *A. fumigatus*, *A. niger*, *A. versicolor*, *P. funiculosum*, *P. verrucosum* and *T. viride* (MIC ≤ 1 mg/mL; MFC ≤ 2 mg/mL). Infusions demonstrated inhibition activity against the tested fungi with MIC values between 0.125 and 0.5 mg/mL, except for spearmint infusion against *P. verrucosum* var. *cyclopium* (MIC = 1 mg/mL*)*. Hydroethanolic extracts stood out for inhibiting *T. viride* at a low concentration (MIC = 0.125 mg/mL for spearmint and sage extracts; MIC = 0.25 mg/mL for lemon balm extract), which demonstrates the susceptibility of this microorganism to such extracts. The three decoctions were also effective against all fungi (MIC ≤ 0.5 mg/mL; MFC ≤ 1 mg/mL) except *A. niger* (MIC > 4 mg/mL for lemon balm and spearmint).

In general, the infusions, decoctions and hydroethanolic extracts showed comparable or higher antimicrobial and fungicidal activities than those of the artificial food preservatives E211 and E224. In particular, the results of E211 against *S. aureus* (MIC and MBC = 4 mg/mL) and *P. verrucosum* (MIC = 2 and MFC = 4 mg/mL), and those of E224 against *B. cereus* (MIC = 2 and MBC = 4 mg/mL) differ noticeably from the lower concentration of plant extracts needed to prevent the growth of such microorganisms. These findings point out the potential of the extracts tested in this study as good candidates for applications in food and possible alternatives for replacing synthetic preservatives, aiming to delay the proliferation of food spoilage and pathogenic bacteria and fungi.

In line with our research, some previous studies have also reported on the antimicrobial and antifungal effects of these plants. The sage infusions of Abdel-Wahab et al. [35] showed MIC values of 50 mg/mL for *E. coli*, and 75 mg/mL for *Salmonella* spp., *S. aureus* and *B. cereus*. Hydroethanolic sage extracts produced by Hemeg et al. [36] revealed MIC values of 5 mg/mL for *S. aureus*, 0.625 mg/mL for *B. cereus* and 2.5 mg/mL for *E. coli* and *S.* Enteritidis. Silva et al. [29] hydroethanolic sage extracts revealed MIC values of 2.5–5 mg/mL for *L. monocytogenes*, 0.625 mg/mL for *S.*

aureus, 10 mg/mL for *S.* Typhimurium and 1.25 mg/mL for *E. coli*. In turn, Ueda et al. [37] investigated hydroethanolic sage extracts obtained through ultrasound-assisted extraction, and MIC values were 1 mg/mL for *S. aureus*, *B. cereus*, *L. monocytogenes*, *E. coli*, *S.* Typhimurium and *E. cloacae*, 0.25 mg/mL for *A. fumigatus*, *A. versicolor*, *P. funiculosum* and *P. verrucosum* and 0.5 mg/mL for *A. niger* and *T. viride*.

Silva et al. [29] also tested the hydroethanolic extracts of spearmint and lemon balm, which revealed MIC values of 2.5 mg/mL for *L. monocytogenes*, 1.25 mg/mL for *S. aureus*, 20 mg/mL for *S.* Typhimurium and 1.25 mg/mL for *E. coli* for spearmint, and 5 mg/mL for *L. monocytogenes*, 2.5 mg/mL for *S. aureus*, 20 mg/mL for *S.* Typhimurium and 2.5 mg/mL for *E. coli* for lemon balm. Caleja et al. [38] analysed the antimicrobial activity of spearmint infusions, reporting MIC values of 0.5 mg/mL for *L. monocytogenes*, *B. cereus* and *E. coli* and 0.25 mg/mL for *S.* Typhimurium. The same study also determined the MIC of lemon balm infusions, which revealed values of 1 mg/mL for all bacteria mentioned before [38]. Furthermore, Caleja et al. [38] evaluated the MIC of said infusions against *A. niger*, *A. versicolor*, *P. funiculosum* and *P. verrucosum*, and the values ranged between 0.25 and 1 mg/mL.

5.3.3 Antioxidant Activity

The results of the TBARS and OxHLIA essays, which assess the ability of the plant extracts to inhibit lipid peroxidation and oxidative haemolysis in vitro, are presented in **Table 21**. The results are expressed as IC_{50} values, meaning that lower values correspond to greater antioxidant potential.

In both TBARS and OxHLIA essays, the antioxidant capacity of each plant infusion was significantly different from that of the other two ($p < 0.05$). Differences were also found among the decoctions, in both essays, depending on the plant species ($p < 0.05$). The antioxidant power of the hydroethanolic extracts also displayed differences depending on the plant used ($p < 0.05$), although not all of them were significant in the case of the OxHLIA essay. Moreover, in both essays, for each plant, different extraction methods yielded distinct antioxidant activities ($p < 0.05$). The exception was the decoction and hydroethanolic extract of lemon balm, which presented similar antioxidant potential in the TBARS essay ($p > 0.05$).

Table 21. Antioxidant activity of plant extracts expressed as half-maximal inhibitory concentration (IC_{50}, μg/mL) measured by the TBARS (mean ± SD, n = 9) and OxHLIA (mean ± SD, n = 3) essays.

Essay	Plant	Infusion	Decoction	Hydroethanolic Extract
TBARS	Lemon balm	125 ± 2.08 [a]	204 ± 2.66 [b]	206 ± 8.99 [b]
	Spearmint	255 ± 11.0 [c]	197 ± 5.68 [a]	295 ± 9.77 [c]
	Sage	235 ± 6.43 [b]	196 ± 5.04 [a]	132 ± 5.07 [a]
OxHLIA Δt = 60 min	Lemon balm	61.4 ± 1.31 [b]	27.0 ± 0.43 [b]	13.5 ± 0.38 [a]
	Spearmint	83.5 ± 1.84 [c]	42.2 ± 0.62 [c]	12.5 ± 0.17 [a]
	Sage	21.9 ± 0.77 [a]	8.93 ± 0.44 [a]	23.9 ± 0.94 [b]
OxHLIA Δt = 120 min	Lemon balm	95.5 ± 2.16 [b]	41.6 ± 0.63 [b]	27.4 ± 0.85 [a]
	Spearmint	120 ± 1.84 [c]	66.8 ± 0.92 [c]	27.6 ± 1.28 [a]
	Sage	38.4 ± 0.89 [a]	23.5 ± 0.67 [a]	56.4 ± 1.51 [b]

TROLOX IC_{50} value: 5.4 ± 0.3 μg/mL (TBARS), 21.8 ± 0.25 μg/mL (OxHLIA Δt = 60 min), 43.5 ± 1.00 μg/mL (OxHLIA Δt = 120 min). For each essay, values with different superscript letters in a column mean significant differences (ANOVA, $p < 0.05$).

Overall, according to the statistical analysis, lemon balm infusion and sage hydroethanolic extract (125 μg/mL and 132 μg/mL, respectively) showed the best capacities to inhibit the formation of malondialdehyde and other reactive substances that are the result of the ex vivo decomposition of lipid peroxidation products (in the TBARS essay).

The results of the OxHLIA essay showed that the sage decoction (8.93 μg/mL and 23.5 μg/mL, for Δt = 60 min and 120 min) and the hydroethanolic extracts of spearmint (12.5 μg/mL and 27.6 μg/mL, for Δt = 60 min and 120 min) and of lemon balm (13.5 μg/mL and 27.4 μg/mL, for Δt = 60 min and 120 min) exhibited the greatest antioxidant protection for the erythrocyte membrane, even compared to the pure antioxidant compound used as a positive control, TROLOX (21.8 μg/mL and 43.5 μg/mL, for Δt = 60 min and 120 min). These results suggest the potential of such extracts to be used against free radical-induced oxidative damage of biological membranes.

Furthermore, the OxHLIA essays allows us to distinguish between short-term and long-term antioxidant protection, as the antioxidant behaviour is monitored over time and the oxidative haemolysis assessed at two Δt. It was observed that all the infusions had anti-haemolytic activity for longer exposure times, as the concentration necessary to protect 50% of the red blood cells for 120 min was less than double the concentration necessary for this protection for 60 min. This also occurred in the case of spearmint and lemon balm decoctions, but not for the remaining extracts.

Our findings agree with other researchers that have also reported on the antioxidant capacities of lemon balm, spearmint and sage. Groupwise summary statistics calculated by Silva et al. [29] showed the high antioxidant power of these three plants, determined by the free radical scavenging (DPPH), radical cation decolorization (ABTS) and ferric reducing antioxidant power (FRAP) essays: the results were between 259 and 507 μmol TROLOX Equivalent/g dry plant, for the DPPH and ABTS essays, and between 722 and 1013 μmol Fe^{2+}/g dry plant for the FRAP essay. Abdel-Wahab et al. [35] also evaluated a sage extract, using the DPPH method, and reported an IC_{50} of 13.34 μg/mL. Ueda et al. [37] reported an IC_{50} of 2.6 mg/g of sage extract, determined by the OxHLIA method, for the time period of 120 min. Caleja et al. [38] used two methods to assess the antioxidant power, reporting IC_{50} values of 6.6 μg/mL and 4.2 μg/mL for lemon balm and spearmint extracts, respectively (using the TBARS essay), and IC_{50} values of 24.8 μg/mL and 38.3 μg/mL for lemon balm and spearmint extracts, respectively (using the OxHLIA method for Δt = 60 min).

5.3.4 Anti-Inflammatory Activity

Table 22 presents the anti-inflammatory activity essay results. These are expressed as IC_{50} values, so lower values correspond to greater anti-inflammatory potential.

Table 22. Anti-inflammatory activity of plant extracts expressed as half-maximal inhibitory concentration (IC_{50}, μg/mL) measured by nitric oxide production inhibitory capacity (mean ± SD, n = 2).

Plant	Infusion	Decoction	Hydroethanolic Extract
Lemon balm	>400 ^{b}	>400 ^{b}	>400 ^{b}
Spearmint	44.4 ± 0.66 ^{a}	43.9 ± 4.26 ^{a}	26.6 ± 1.65 ^{a}
Sage	>400 ^{b}	>400 ^{b}	>400 ^{b}

Dexamethasone IC_{50} value: 6 ± 1 μg/mL. Values with different superscript letters in a column mean significant differences (ANOVA, $p < 0.05$).

The outcomes shown in **Table 22** indicate that most extracts did not reveal anti-inflammatory action at the tested concentrations (IC_{50} > 400 μg/mL). Only those of spearmint showed this capability, regardless of the extraction method. Spearmint hydroethanolic extract showed the greatest anti-inflammatory capacity, considering its IC_{50} of 26.6 μg/mL.

In agreement with our results, the spearmint infusions of Caleja et al. [38] also displayed anti-inflammatory activity against the RAW 246.7 cell line (IC_{50} = 324 μg/mL), whereas those of lemon balm did not (IC_{50} > 400 μg/mL).

Nonetheless, and despite our results, some researchers have reported anti-inflammatory effects of sage and lemon balm extracts, meaning that these plants may be capable of offering such beneficial capacity under different circumstances [39,40].

It could be expected that extracts with high rosmarinic acid concentrations and promising antioxidant activity (low IC_{50} values in **Table 21**), such as sage or lemon balm infusions, for example, would also show anti-inflammatory potential, as antioxidants can reduce the inflammatory process caused by the overproduction of free radicals [25]. However, from the results in **Table 22**, it is noticeable that extracts presenting anti-inflammatory activity were not always the ones with the highest antioxidant capacity (except for spearmint hydroethanolic extract, which presented the lowest IC_{50} = 12.5 μg/mL in the OxHLIA essay among that type of extract). In this sense, it is important, when conducting analyses, to evaluate all bioactivities, and not to infer the results of one essay from the outcomes of another, to avoid arriving at wrongful conclusions, or even discarding plant extracts with substantial potential in terms of one particular bioactivity.

5.3.5 Cytotoxic Activity

The cytotoxicity essay results are shown in **Table 23**. These are expressed as GI_{50}, meaning that lower outcomes correspond to greater cytotoxic capacity.

All nine extracts produced revealed inhibitory potential (GI_{50} < 400 μg/mL) against at least one tumour cell line. Overall, the extracts were more active in tumour cells AGS, CaCo-2, HeLa and MCF-7 than NCI-H460. In fact, the cytotoxic capacity of the infusions and decoctions in the NCI-H460 tumour line was non-existent (GI_{50} > 400 μg/mL); however, some hydroethanolic extracts revealed activity.

The absence of toxicity (GI_{50} > 400 μg/mL) against non-tumour human foetal osteoblast cells, hFOB, was evident in the case of infusions and two decoctions (the exception was that of sage), which is a desirable outcome as extracts to be used in food products must be safe for consumption and cannot display toxicity against healthy cells. In contrast, the majority of hydroethanolic extracts (except that of sage, curiously) showed a cytotoxic effect towards hFOB cells, suggesting that this methodology may induce toxicity to the extracts, thus compromising their applicability as food additives.

Table 23. Cytotoxic activity of plant extracts expressed as half-maximal cell growth inhibitory concentration (GI_{50}, μg/mL) measured by the sulforhodamine B essay (mean ± SD, n = 3).

Extraction	Plant	AGS [1]	CaCo-2 [2]	HeLa [3]	MCF-7 [4]	NCI-H460 [5]	hFOB [6]
Infusion	Lemon balm	215 ± 6.22 [a]	290 ± 0.19 [b]	249 ± 11.5 [a]	239 ± 0.99 [b]	>400	>400
	Spearmint	196 ± 7.44 [a]	304 ± 0.55 [c]	229 ± 21.2 [a]	203 ± 1.50 [a]	>400	>400
	Sage	249 ± 8.68 [b]	242 ± 0.40 [a]	248 ± 25.6 [a]	198 ± 0.97 [a]	>400	>400
Decoction	Lemon balm	255 ± 7.45 [b]	>400 [c]	301 ± 10.9 [b]	>400	>400	>400
	Spearmint	258 ± 5.49 [b]	396 ± 0.05 [b]	289 ± 1.49 [b]	>400	>400	>400
	Sage	215 ± 6.25 [a]	269 ± 0.31 [a]	111 ± 2.14 [a]	320 ± 1.05 [a]	>400	350 ± 4.25 [a]
Hydroethanolic extract	Lemon balm	231 ± 2.75 [b]	351 ± 3.30 [c]	266 ± 11.5 [b]	180 ± 4.43 [a]	369 ± 3.37 [a]	271 ± 2.52 [a]
	Spearmint	162 ± 8.05 [a]	285 ± 0.43 [b]	215 ± 2.21 [a]	210 ± 2.20 [b]	381 ± 0.63 [b]	264 ± 2.29 [a]
	Sage	361 ± 3.74 [c]	272 ± 0.06 [a]	257 ± 1.17 [b]	206 ± 2.34 [b]	>400 [c]	>400 [b]

Legend: [1] Gastric adenocarcinoma, [2] Colorectal adenocarcinoma, [3] Cervical carcinoma, [4] Breast adenocarcinoma, [5] Large cell lung carcinoma, [6] non-tumour hFOB (human foetal osteoblasts). Ellipticine GI_{50} values: 1.23 ± 0.03 μg/mL (AGS), 1.21 ± 0.02 μg/mL (CaCo-2), 1.91 ± 0.12 μg/mL (HeLa), 1.02 ±0.02 μg/mL (MCF-7), 1.01 ± 0.01 μg/mL (NCI-H460) and 1.21 ± 0.08 μg/mL (hFOB).

From all the extracts, those that are non-toxic against hFOB and simultaneously present inhibitory potential against AGS, CaCo-2, HeLa and MCF-7 cells are: sage hydroethanolic extract and the infusions of lemon balm, spearmint and sage. These results point out the cytotoxic potential of the infusions produced in comparison to other extraction methods. The infusion of spearmint, specifically, showed overall greater antiproliferative capacity, with GI_{50} values of 196 μg/mL for the AGS cell line, 304 μg/mL for the CaCo-2 cell line, 229 μg/mL for the HeLa cell line and 203 μg/mL for the MCF-7 cell line.

The results obtained in this study agree, to some extent, with those of other researchers. Sage hydroethanolic extracts produced by Ueda et al. [37] did not show hepatotoxicity in PLP2 cells (non-tumour) at the maximum tested concentration of 400 μg/mL. Lemon balm and spearmint infusions of Caleja et al. [38] did not show toxicity for non-tumour cells PLP2 (GI_{50} > 400 μg/mL) and inhibited the growth of the HeLa cell line (GI_{50} = 241 μg/mL and GI_{50} = 251 μg/mL, respectively), in agreement with our results. Their spearmint infusion also inhibited MCF-7 growth (GI_{50} = 283 μg/mL), as found in our study. However, in contrast to our findings, lemon balm and spearmint infusions were able to inhibit NCI-H460 (GI_{50} = 290 μg/mL and GI_{50} = 322 μg/mL,

respectively), and lemon balm infusion was incapable of affecting MCF-7 viability (GI_{50} > 400 µg/mL) [38].

Overall, these results indicate that extracts originating from any of the plants examined are potentially valuable for their cytotoxic impact on various tumour cell lines. However, it is crucial to further evaluate potential undesired effects against healthy cell lines, as even reduced concentrations may result in dangerous consequences for human health.

5.4 CONCLUSIONS

This work revealed the biological capacities of sage, spearmint and lemon balm extracts. Although only spearmint extracts showed anti-inflammatory potential, all infusions, decoctions and hydroethanolic extracts presented encouraging results in terms of antibacterial, antifungal and antioxidant capacities. Infusions revealed the most promising results, compared to decoctions and hydroethanolic extracts, as they yielded the best outcomes in each of the essays conducted (antimicrobial, antioxidant, anti-inflammatory and antiproliferative tests), while displaying an absence of toxicity against non-tumour cells, and even though infusions did not contain the highest total phenolic contents. Extracts from sage stood out from the remainder as they were often among those presenting the best capacities, both in terms of inhibiting the oxidation and growth of pathogenic bacteria and fungi, as well as impairing the viability of tumour cells. Nonetheless, no anti-inflammatory action was detected.

Overall, the results of this study emphasise the potential value of sage, spearmint and lemon balm extracts as natural food ingredients to prevent spoilage, provide beneficial health effects and potentially replace artificial additives, hence aligning with current trends in the food industry. However, further in vitro and in vivo studies must be conducted to verify the functionality of these extracts: for example, evaluating their pharmacokinetic parameters (bioavailability and bioaccessibility). It is also expected that the food matrix has some impact on the bioactivities of plant extracts, causing differences between the results observed in vitro and in vivo, which may limit the bio-functionalities of such extracts in food products. Another obstacle that must be investigated and that herbal extracts may face is related to their effect on the sensory characteristics of foods, since the concentrations necessary to provide the desired biological capacities can be very high and, therefore, negatively affect the aroma and taste of the products. In this sense, further research must be conducted to complement in vitro studies and address these and other limitations.

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CHAPTER 6

LAVANDULA STOECHAS L., *ARTEMISIA DRACUNCULUS* L. AND *OCIMUM BASILICUM* L. POLYPHENOLIC EXTRACTS AS FUNCTIONAL FOOD INGREDIENTS

This chapter was based on the following submitted paper:

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6.1 INTRODUCTION

For centuries, and around the world, plants have been relied upon as traditional medicines to support and promote human health. Nowadays, they continue to be used to treat multiple conditions and complaints, including digestive or intestinal diseases, sickle-cell anaemia, hypertension, high cholesterol, headaches, insomnia, diarrhoea, microbial infections, bronchitis, diabetes, burns, rashes, and menopause [1].

In the particular case of French lavender (*Lavandula stoechas* L.), basil (*Ocimum basilicum* L.) and tarragon (*Artemisia dracunculus* L.), the first has been used for its anti-inflammatory, antispasmodic, sedative and carminative properties, as well as to treat rheumatic diseases and nephrotic syndromes (kidney-related disorders) [2,3]; basil has a history of being used for the treatment of headaches, cough, constipation, skin warts, parasites and renal malfunctions, and its reported properties include antimicrobial, anticonvulsant, antioxidant and anticarcinogenic effects [4,5]; tarragon, in its turn, possesses anti-inflammatory, antipyretic, antiseptic, eupeptic, laxative, carminative, stomachic, antispasmodic, antiparasitic, antimicrobial, vermifuge and emmenagogue effects, and some of its popular uses include the treatment of skin conditions (wounds, irritations, allergic rashes, dermatitis) and gastritis, for example [6-8].

In the past years, consumers have shown increased concern to take on healthier lifestyles, which include consumption of foods with health-promoting effects beyond basic nutrition. Additionally, the increasing number of research focusing on medicinal plants and spices has shown them as good sources of phytochemicals, with interesting biological capacities and therapeutic effects [9].

Considering the demand from consumers and the scientific knowledge available, the food industry is now aiming to include natural extracts into products as a strategy to limit the use of synthetic additives and to produce functional foods. This, however, is not always straightforward, as natural extracts can be unstable and have a negative impact on the organoleptic characteristics of foods due to their high concentration in terpenoids and phenolic compounds, among other challenges [10,11].

As a result of the interest in natural plant extracts with bioactive molecules as food additives, the objective of this study was to contribute with the analysis of the phenolic composition and biological properties of extracts obtained through green solvent extractions, i.e., using non-toxic solvents, and from easily accessible Mediterranean plants, namely, tarragon, basil and French

lavender. More specifically, the antimicrobial, antifungal, antioxidant, anti-inflammatory, and antiproliferative activities of the extracts were evaluated to assess their preservative action, health-promoting effects and safety.

6.2 MATERIALS AND METHODS

6.2.1 Plant Material and Extraction Procedures

French lavender, tarragon and basil dry aerial parts were kindly provided by *Pragmático Aroma, Lda.* company ("Mais Ervas", Trás-os-Montes, Portugal) and mechanically ground.

To prepare infusions, two g of each plant material were added to 200 mL of boiling distilled water and left to stand at room temperature for 5 min. For the decoctions, two g of plant material were added to 200 mL of distilled water and boiled for 5 min. The aqueous mixtures were then filtered (7–10 μm), frozen, and lyophilised (FreeZone 4.5, Labconco, Kansas City, MO, USA). Hydroethanolic extracts were also produced through dynamic macerations, where one g of plant material was added to 30 mL of ethanol at 80% (v/v) and stirred for 1 h at room temperature. The supernatants were filtered (7–10 μm), additional 30 mL of ethanol 80% (v/v) were mixed with the extraction residues, and the maceration was repeated for 1 h. The ethanolic fraction was then evaporated (Büchi R-210, Germany), and the extracts frozen and lyophilised. Extractions were performed in triplicate (n = 3).

6.2.2 HPLC-DAD-ESI/MS analysis of phenolic compounds

The lyophilised extracts were redissolved in ethanol 20% (v/v) up to a final concentration of 10 mg/mL and filtered (0.22 μm). Individual phenolic compounds were analysed by Dionex Ultimate 3000 UPLC (Thermo Scientific, San Jose, CA, USA) equipped with a diode array detector (280, 330, and 370 nm) and an electrospray ionization mass detector (Linear Ion Trap LTQ XL, Thermo Finnigan, San Jose, CA, USA), working in negative ion mode (100-1500) *m/z*. Chromatographic separation was performed using a Waters Spherisorb S3 ODS-2 C18 column (4.6 mm × 150 mm, 3 μm, Waters, Milford, MA, USA) at 35 °C. The solvents used were water/formic acid 0.1% (A) and acetonitrile (B), with a flow rate of 0.5 mL/min. The elution gradient for solvent B was as follows: 10-15% eluent B up to 5 min, 15-20% B up to 5 min, 20-25% B 10 min, 25-35% B 10 min, 35-50% B 10 min and column re-equilibration for 10 min. Phenolic compounds were identified by comparing their retention time, UV-VIS and mass spectra with those

of corresponding standard solutions, when available. Otherwise, peaks were tentatively identified comparing the obtained information with available data from literature. Quantification was performed using calibration curves prepared with appropriate standards (between 100-2.5 mg/L). Limits of detection (LOD) and quantification (LOQ) were determined, and in all cases, the coefficient of linear correlation was $R^2 > 0.99$ (**Table 24**). The results are expressed in mg per g of dry extract (mg/g). All analyses were made in triplicate (n=3).

Table 24. Limit of detection (LOD), limit of quantification (LOQ) and coefficient of linear correlation (R^2) of the different standards used to obtain the calibration curves required for phenolic compound quantification.

Compound Standard	LOD (μg/mL)	LOQ (μg/mL)	R^2
Ferulic acid	0.20	1.01	0.999
p-Coumaric acid	0.68	1.61	0.999
Apigenin-7-*O*-glucoside	0.10	0.53	0.999
Caffeic acid	0.78	1.97	0.994
Chlorogenic acid	0.20	0.68	0.9999
Naringenin	0.20	0.64	0.9998
Rosmarinic acid	0.15	0.68	0.999
Quercetin-3-*O*-glucoside	0.21	0.71	0.9998
Quercetin-3-*O*-rutinoside	0.18	0.65	0.9998

6.2.3 Biological Evaluation

6.2.3.1 Antibacterial and Antifungal Activity

The minimum inhibitory, bactericidal and fungicidal concentrations (MIC, MBC and MFC) were determined using a previously described broth microdilution method [12]. To test the potential antimicrobial activity of the samples, *Escherichia coli* (ATCC 25922), *Salmonella enterica* ser. Typhimurium (ATCC 13311), *Enterobacter cloacae* (clinical isolate), *Staphylococcus aureus* (ATCC 11632), *Bacillus cereus* (food isolate), and *Listeria monocytogenes* (NCTC 7973) were used. For antifungal essays, *Aspergillus fumigatus* (human isolate), *Aspergillus niger* (ATCC 6275), *Aspergillus versicolor* (ATCC 11730), *Penicillium funiculosum* (ATCC 36839), *Penicillium verrucosum* var. *cyclopium* (food isolate), and *Trichoderma viride* (IAM 5061) were used. As

positive controls, two food grade antioxidant chemical compounds were used, namely, sodium benzoate (E211) and potassium metabisulfite (E224). The results were expressed as mg/mL of the resuspended lyophilised extracts.

6.2.3.2 Antioxidant Activity

The cell-based essays of formation of thiobarbituric acid reactive substances (TBARS) and inhibition of oxidative haemolysis (OxHLIA) were performed using previously described methodologies [13,14]. The lyophilised extracts were initially redissolved in distilled water (for TBARS) or phosphate-buffered saline (PBS, pH 7.4) (for OxHLIA) to different concentrations and TROLOX was used as a positive control in both essays.

For the TBARS essay, porcine brain cell homogenates were used, and lipid peroxidation inhibition was evaluated by measuring the colour intensity (at 532 nm) of the malondialdehyde-thiobarbituric acid (MDA-TBA) complexes formed in the system. The results were expressed as the extract concentration (μg/mL) required to inhibit 50% of the TBARS formation (IC_{50}).

For the OxHLIA essay, an erythrocyte solution (2.8%, v/v; 200 μL) prepared in PBS was added to 400 μL of either: i) extract solution (13–800 μg/mL in PBS), ii) PBS solution (negative control), iii) distilled water (for complete haemolysis), or iv) TROLOX (7.81–250 μg/mL). After pre-incubation at 37 °C for 10 min with shaking, 200 μL of 2,2′-azobis(2-methylpropionamidine) dihydrochloride (AAPH, 160 mM in PBS; from Sigma-Aldrich) were added and the optical density was measured at 690 nm every ~10 min in a microplate reader (Bio-Tek Instruments, ELx800, Winooski, VT, USA) until complete haemolysis. The results were expressed as IC_{50} values (μg/mL) for a Δt of 60 min and 120 min, which indicate the sample concentration required to protect 50% of the red blood cells from the haemolytic action of AAPH for 60 and 120 min, respectively.

6.2.3.3 Anti-Inflammatory Activity

The anti-inflammatory activity was evaluated as described by Jabeur et al [15]. After culture in supplemented DMEM medium, the mouse macrophage-like cell line RAW264.7 was seeded in 96-well plates at 150.000 cells/well and their attachment to the plate allowed overnight. Subsequently, cells were treated with different concentrations of the extracts (6.25–400 μg/mL) for 1 h, followed by stimulation with lipopolysaccharides (1 μg/mL) for 18 h. This procedure allowed to observe the occurrence of induced changes in nitric oxide (NO) basal levels. For that, the tested sample and lipopolysaccharides were dissolved in supplemented DMEM, and

determination of nitric oxide content was performed using a Griess Reagent System kit (Promega, Madison, WI, USA). Nitrite level produced was determined by optical density measurement at 540 nm, in a microplate reader (Bio-Tek Instruments, ELx800, Winooski, VT, USA), and compared with the standard calibration curve. Dexamethasone (50 μM) was used as positive control. The results are expressed as the sample concentration (μg/mL) required to inhibit 50% of NO production (IC_{50}).

6.2.3.4 Antiproliferative Activity

The lyophilised extracts were redissolved in water and successively diluted to obtain various concentrations to be submitted to in vitro antiproliferative activity evaluation, using the sulforhodamine B essay [16]. Extracts were incubated with the tested cell lines (190 μL, 10 000 cells/mL), and final concentrations ranged between 6.25–400 μg/mL. Six human tumour cell lines were tested: AGS (gastric adenocarcinoma), CaCo-2 (colorectal adenocarcinoma), HeLa (cervical carcinoma), MCF-7 (breast adenocarcinoma), NCI-H460 (non-small cell lung cancer), and non-tumour hFOB (human fetal osteoblasts). Ellipticine was used as a positive control. The results were expressed as the extract concentration required to inhibit 50% of the cell growth (GI_{50}).

6.2.4 Statistical Analysis

Data were presented as mean ± standard deviation (SD) values. The statistical differences of the means were obtained through one-way analysis of variance (ANOVA, α=0.05). The pheatmap function from the pheatmap package was used to produce clustered heatmaps [17]. Statistical analysis was conducted in R software (version 4.1.0, R Foundation for Statistical Computing, Vienna, Austria).

6.3 RESULTS AND DISCUSSION

6.3.1 Phytochemical Composition

The phenolic composition of tarragon, basil and French lavender extracts is reported in **Table 25**, **Table 26** and **Table 27**, respectively. The phenolic compounds identified, and their concentrations are also displayed in the heatmaps of **Figure 12**, **Figure 13** and **Figure 14** (tarragon, basil and French lavender, respectively).

Table 25. Phenolic compound content (mg/g dry extract) of tarragon (*Artemisia dracunculus* L.) extracts.

Peak	Rt	λmax	[M-H] *m/z*	MS^2	Tentative Identification	Decoction	Infusion	Hydroethanolic
1	4.42	322	353	191(100)	3-*O*Caffeoylquinic acid [5]	0.65±0.03	0.61±0.03	0.63±0.03
2	4.68	323	515	353(15), 341(8), 323(100), 191(58), 179(6), 161(19)	*cis*-5-*O*Caffeoylquinic acid-hexoside [5]	0.50±0.01	0.42±0.02	0.54±0.02
3	4.97	325	515	353(25), 341(12), 323(100), 191(65), 179(8), 161(29)	*trans*-5-*O*Caffeoylquinic acid-hexoside [5]	0.46±0.03	0.44±0.01	0.36±0.02
4	6.05	324	353	191(20), 179(61), 173(100). 135(12)	4-*O*Caffeoylquinic acid [5]	1.14±0.01	0.76±0.06	0.64±0.03
5	6.44	326	353	191(100), 179(11), 173(6), 135(4)	5-*O*Caffeoylquinic acid [5]	8.56±0.18	6.69±0.46	7.61±0.16
6	7.21	306	355	193(100)	Ferulic acid hexoside [1]	0.65±0.03	0.43±0.02	0.56±0.02
7	8.88	324	311	179(88), 149(100), 135(9)	Caffeoyltartaric acid [5]	1.26±0.05	0.89±0.02	1.16±0.05
8	9.26	322	311	179(81), 149(100), 135(19)	Caffeoyltartaric acid isomer [5]	0.40±0.04	0.28±0.02	0.32±0.02
9	9.83	277sh315	357	313(27),269(100),203(52),159(42),109(12)	Prolithospermic acid [7]	0.42±0.02	0.33±0.02	0.31±0.01
10	11.13	326	433	271(100)	Naringenin-*O*-hexoside [6]	0.21±0.00	0.01±0.00	nd
11	12.67	277sh320	397	191(100)	*p*-Coumaroylquinic acid [2]	0.99±0.07	0.60±0.01	0.53±0.01
12	13.43	320	367	193(8), 191(100), 173(6), 134(10)	Feruloylquinic acid [1]	1.25±0.06	0.90±0.03	0.90±0.01
13	15.44	281sh324	549	387(100)	Medioresinol-*O*-hexoside [6]	0.34±0.03	0.10±0.01	0.11±0.00
14	16.57	354	609	301(100)	Quercetin-3-*O*-rutinoside [9]	9.88±0.54	3.55±0.16	6.36±0.10
15	17.99	298sh337	711	549(5), 355(26), 193(100), 149(81)	Ferulic acid hexoside dimer [1]	0.38±0.02	0.27±0.01	0.24±0.00
16	19.23	327	515	353(100), 335(25),191(36),179(51),173(62),161(8)	3,4-*O*Dicaffeoylquinic acid [5]	2.75±0.14	0.56±0.01	1.89±0.07
17	19.70	335	593	285(100)	Luteolin-*O*-rutinoside [3]	1.09±0.06	0.80±0.01	0.80±0.00
18	20.73	355	623	315(100)	Isorhamnetin-3-*O*-rutinoside [8]	0.11±0.00	nd	nd
19	22.16	292sh328	515	353(81),191(18),179(45),173(100),135(54)	4,5-*O*Dicaffeoylquinic acid [5]	3.39±0.17	1.52±0.06	2.07±0.09
20	29.37	323	677	515(12),497(15),353(60), 335(20),191(12),179(8)	1,3,5-*O*Tricaffeoylquinic acid [5]	7.86±0.01	5.87±0.40	7.30±0.28
					Total Phenolic Acids	31.0±0.88	20.7±0.37	25.2±0.34
					Total Flavonoids	11.3±0.60	4.37±0.17	7.16±0.09
					Total Phenolic Compounds	42.3±1.48	25.1±0.55	32.3±0.44

Rt: Retention time (min), λ_{max}: wavelengths of maximum absorption in the visible region (nm); MS^2: second stage of mass spectrometry. Superscript numbers indicate the compound standard used for the quantification: (1) Ferulic acid; (2) *p*-Coumaric acid; (3) Apigenin-7-*O*-glucoside; (4) Caffeic acid; (5) Chlorogenic acid; (6) Naringenin; (7) Rosmarinic acid; (8) Quercetin-3-*O*-glucoside; (9) Quercetin-3-*O*-rutinoside. Content values expressed as mean ± SD (n= 3).

Table 26. Phenolic compound content (mg/g dry extract) of basil (*Ocimum basilicum* L.) extracts.

Peak	Rt	λmax	[M-H] *m/z*	MS^2	Tentative Identification	Decoction	Infusion	Hydroethanolic
1	4.67	328	311	179(61),149(100),135(8)	Caftaric acid [4]	1.52±0.03	4.71±0.24	0.44±0.02
2	5.74	287sh325	377	191(90),173(5),163(100),155(3),137(5),119(4)	3-*p*-Coumarouylquinic acid [2]	0.43±0.01	1.97±0.08	0.45±0.01
3	7.17	289sh324	341	179(100),149(9),135(28)	Caffeic acid hexoside [4]	0.36±0.02	0.48±0.03	0.10±0.00
4	8.80	288sh324	179	135(100)	Caffeic acid [4]	0.17±0.01	1.00±0.01	0.28±0.01
5	9.44	279sh321	597	359(31),295(27),197(16),179(10),135(12)	Yunnaneic acid F [7]	0.57±0.02	0.83±0.05	nd
6	12.83	279sh326	537	493(36),339(100),313(5),295(7),179(8)	Salvianolic acid H/I [7]	0.29±0.03	1.78±0.05	0.28±0.00
7	14.89	346	595	301(100)	Quercetin-*O*-pentosyl-hexoside [8]	0.58±0.01	2.33±0.11	0.65±0.01
8	15.97	328	473	313(20),293(28),179(86),149(100),135(34)	*cis*-Chicoric acid [4]	1.21±0.03	3.38±0.04	0.17±0.00
9	16.65	330	473	313(20),293(28),179(86),149(100),135(34)	*trans*-Chicoric acid [4]	0.48±0.03	1.38±0.02	0.59±0.03
10	17.77	288sh329	717	537(21),519(54),493(21),339(24),321(27),313(9),295(100),277(18)	Salvianolic acid E [7]	0.89±0.07	0.89±0.03	0.83±0.05
11	18.67	284sh324	719	359(100),197(8),179(18),161(50),135(7)	Sagerinic acid [7]	0.40±0.03	1.46±0.04	0.26±0.00
12	19.22	337	549	505(100), 301(69)	Quercetin-7-*O*-malonylhexoside [8]	0.73±0.00	2.04±0.13	0.88±0.03
13	20.83	329	359	197(100),179(94),161(87),135(68)	Rosmarinic acid [7]	5.57±0.07	17.5±0.41	10.2±0.33
14	21.65	324	493	313(11), 295(100), 185(7)	Salvianolic acid A [7]	0.98±0.01	0.86±0.02	1.65±0.07
15	23.31	335	537	493(100),359(12),295(9),179(4)	Lithospermic acid A [7]	0.29±0.00	0.99±0.02	0.38±0.00
16	25.41	338	717	537(13),519(100),493(8),339(39),321(92),295(23),279(7),197(3)	Salvianolic acid B [7]	0.50±0.00	0.71±0.03	0.75±0.01
17	28.41	282sh331	355	193(100), 179(13), 149(80)	Ferulic acid hexoside [1]	0.33±0.00	1.05±0.05	0.40±0.01
					Total Phenolic Acids	14.0±0.07	39.0±1.01	16.7±0.51
					Total Flavonoids	1.31±0.01	4.37±0.24	1.53±0.04
					Total Phenolic Compounds	15.3±0.05	43.4±1.25	18.3±0.55

Rt: Retention time (min), λ_{max}: wavelengths of maximum absorption in the visible region (nm); MS^2: second stage of mass spectrometry. Superscript numbers indicate the compound standard used for the quantification: (1) Ferulic acid; (2) *p*-Coumaric acid; (3) Apigenin-7-*O*-glucoside; (4) Caffeic acid; (5) Chlorogenic acid; (6) Naringenin; (7) Rosmarinic acid; (8) Quercetin-3-*O*-glucoside; (9) Quercetin-3-*O*-rutinoside. Content values expressed as mean ± SD (n= 3); nd: not detected.

Table 27. Phenolic compound content (mg/g dry extract) of French lavender (*Lavandula stoechas* L.) extracts

Peak	Rt	λmax	[M-H] *m/z*	MS^2	Tentative Identification	Decoction	Infusion	Hydroethanolic
1	4.92	296sh322	311	179(61),149(100),135(8)	Caftaric acid [4]	0.08±0.01	0.05±0.00	0.03±0.00
2	5.15	297sh321	311	179(68),149(100),135(4)	Caftaric acid isomer [4]	0.63±0.04	0.26±0.02	0.28±0.01
3	5.73	289sh315	341	179(100)	Caffeic acid hexoside [4]	nd	0.02±0.00	0.05±0.00
4	6.09	313	377	207(100),191(5),163(38),137(7)	3-*p*-Coumarouylquinic acid [2]	0.79±0.04	0.58±0.03	0.59±0.01
5	7.42	288sh323	387	369(26), 207(100), 163(47)	Caffeic acid acetylhexoside [4]	0.82±0.03	0.73±0.05	0.54±0.02
6	8.02	284sh310	325	163(100)	*p*-Coumaric hexoside [2]	0.22±0.01	0.20±0.01	0.25±0.00
7	8.44	292sh318	325	163(100)	*p*-Coumaric hexoside isomer [2]	0.24±0.00	0.21±0.00	0.23±0.02
8	8.84	296sh321	179	135(100)	Caffeic acid [4]	0.30±0.01	0.19±0.01	0.33±0.00
9	10.25	283sh325	571	527(16),483(100),439(49),329(21),259(21),241(3),197(25)	Yunnaneic acid E [7]	0.31±0.00	0.31±0.00	0.36±0.01
10	12.87	271sh326	623	461(100),285(31)	Luteolin-*O*-hexoside-*O*-glucuronide [3]	1.29±0.01	1.43±0.08	0.65±0.00
11	14.3	341	477	301(100)	6-Hydroxyluteolin-7-*O*-glucuronide [3]	0.81±0.02	0.90±0.04	0.62±0.01
12	15.28	284sh322	463	287(100)	Eriodictyol-*O*-glucuronide [6]	3.93±0.13	2.93±0.11	2.75±0.07
13	16.36	286sh326	521	359(50), 197(20),179(37),161(100)	Rosmarinic acid hexoside [7]	0.64±0.01	0.61±0.04	0.44±0.01
14	17.99	346	461	285(100)	Luteolin-glucuronide [3]	7.79±0.08	8.32±0.55	5.83±0.01
15	20.75	329	359	197(100),179(93),161(82),135(61)	Rosmarinic acid [7]	13.6±0.20	15.0±0.41	16.9±0.11
16	22.19	327	537	493(100),359(35),313(4),295(2)	Lithospermic acid A [7]	2.36±0.06	1.91±0.13	1.90±0.10
17	24.84	287sh327	717	537(13),519(100),493(8),339(39),321(92),295(23),279(7),197(3)	Salvianolic acid B [7]	7.62±0.15	9.12±0.64	4.78±0.03
18	27.89	287sh330	717	537(11),519(100),493(10),339(29),321(82),295(13),279(7)	Salvianolic acid B isomer [7]	0.58±0.00	0.70±0.03	0.51±0.05
					Total Phenolic Acids	28.2±0.43	29.9±1.29	27.2±0.13
					Total Flavonoids	13.8±0.24	13.6±0.77	9.85±0.10
					Total Phenolic Compounds	42.0±0.67	43.5±2.07	37.1±0.23

Rt: Retention time (min), λ_{max}: wavelengths of maximum absorption in the visible region (nm); MS^2: second stage of mass spectrometry. Superscript numbers indicate the compound standard used for the quantification: (1) Ferulic acid; (2) *p*-Coumaric acid; (3) Apigenin-7-*O*-glucoside; (4) Caffeic acid; (5) Chlorogenic acid; (6) Naringenin; (7) Rosmarinic acid; (8) Quercetin-3-*O*-glucoside; (9) Quercetin-3-*O*-rutinoside. Content values expressed as mean ± SD (n= 3).

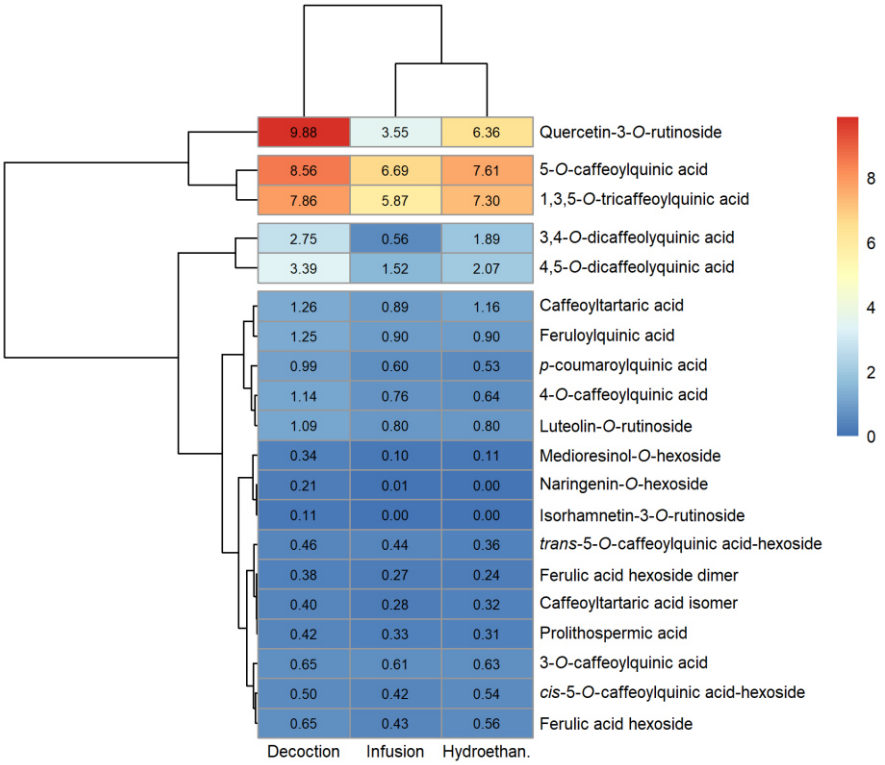

Figure 12. Clustered heatmap visualisation of phenolic compounds detected in tarragon infusion, decoction and hydroethanolic extract (units: mg/g).

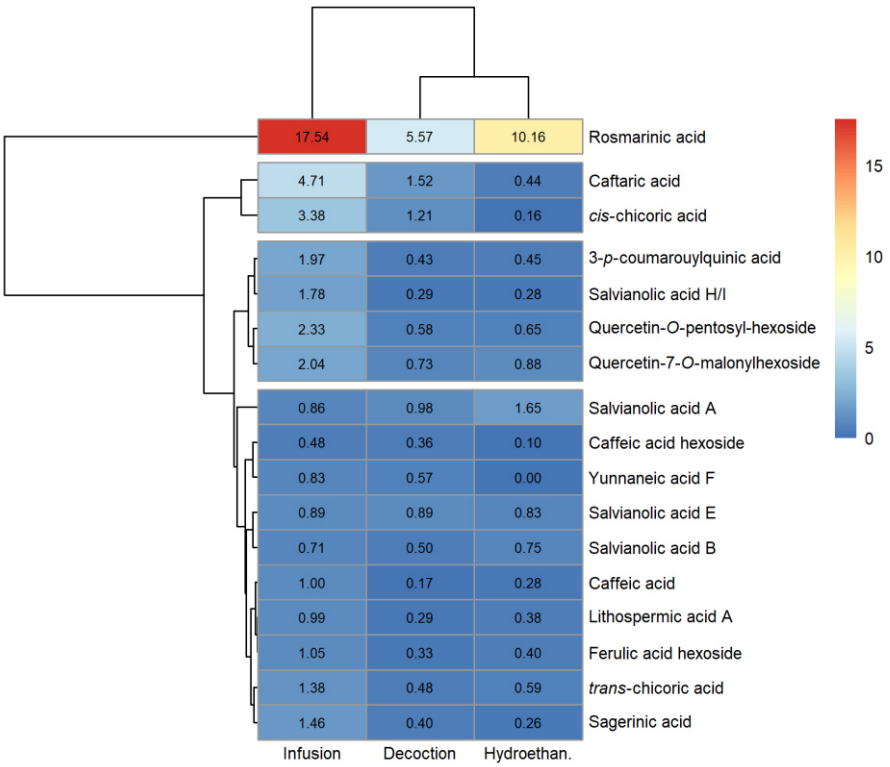

Figure 13. Clustered heatmap visualisation of phenolic compounds detected in basil infusion, decoction and hydroethanolic extract (units: mg/g).

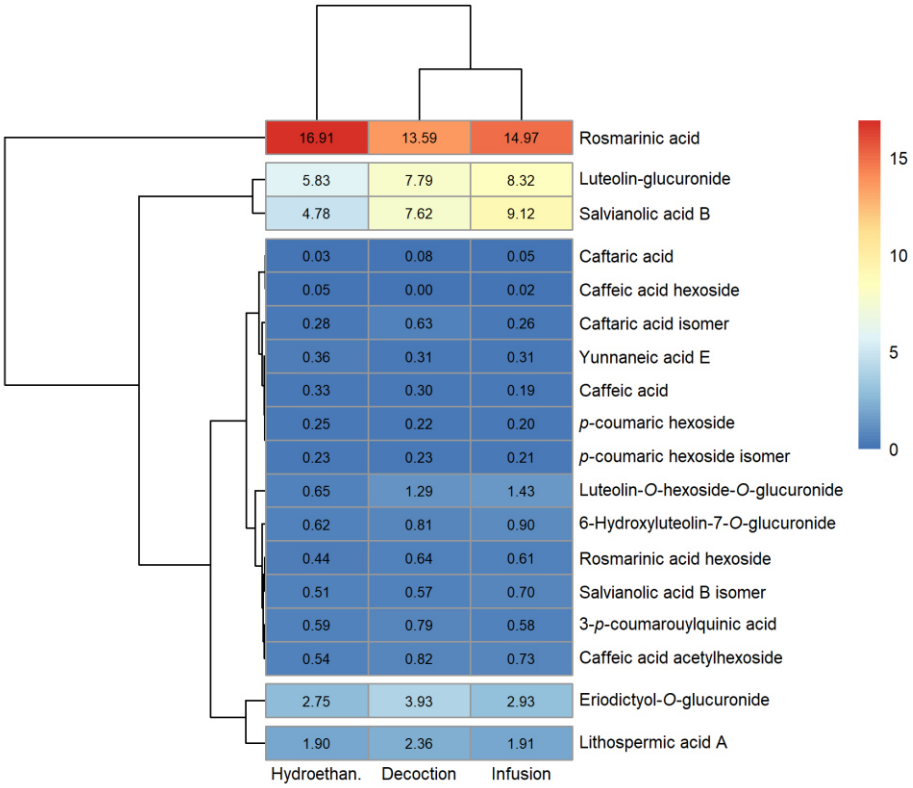

Figure 14. Clustered heatmap visualisation of phenolic compounds detected in French lavender infusion, decoction and hydroethanolic extract (units: mg/g).

The heatmaps display dendrograms that arrange the information in terms of degrees of similarity: the greater the height at which any two objects are joined, the smaller the similarity. In this sense, the dendrogram on the left organises compounds detected in similar concentrations across different extraction methods (infusion, decoction and hydroethanolic maceration); and the upper dendrogram informs about comparable total phenolic compound content across the three extracts produced, for each plant.

In tarragon extracts, twenty phenolic compounds were identified. **Figure 12** and **Table 25** suggest that tarragon infusion and hydroethanolic extract have a more similar profile and total phenolic content (25.05 and 32.33 mg/g extract, respectively), compared to the decoction (42.27 mg/g extract). As far as basil extracts, seventeen compounds were classified, and in this case, the decoction and hydroethanolic extract appear to be more closely related with each other than each of them with basil infusion, as suggested by **Figure 13.** This was evident by the much higher total phenolic content of the infusion (43.41 mg/g extract) in comparison to the decoction and hydroethanolic extract (15.31 and 18.26 mg/g extract), as shown in **Table 26**. In French lavender extracts, eighteen compounds were identified, and the difference in total phenolic content between

methods was less evident than for basil, but still observable. In this case, the infusion and decoction revealed higher and more similar concentration of compounds (41.99 and 43.45 mg/g extract), compared to the hydroethanolic extract (37.06 mg/g extract), as indicated by the upper dendrogram of **Figure 14.** Overall, the infusions of basil and French lavender stood out for their higher total phenolic content (43.43 and 43.45 mg/g extract), whereas basil decoction revealed the lowest total phenolic content among all extracts tested (15.31 mg/g extract).

Comparing the total amount of phenolic acids with the total flavonoids, the former were superior in quantity and diversity in all extracts produced (**Table 25**, **Table 26** and **Table 27**). In the case of tarragon extracts, four flavonoids were identified, compared to sixteen phenolic acids, and depending on the extract type, total flavonoids concentration ranged between 17.4% and 26.7% of the total phenolic content (**Table 25**). In both basil and French lavender extracts, only two flavonoids were detected, and the remaining compounds identified were phenolic acids. In basil extracts, flavonoid content ranged from 8.37% to 10.1% of the total phenolic content, whereas in those of French lavender, flavonoid content was between 26.6% and 32.9% of all phenolic compounds content.

Basil and French lavender extracts contained six phenolic acids in common, namely, rosmarinic acid, salvianolic acid B, caftaric acid, caffeic acid and caffeic acid hexoside, and 3-*p*-coumarouylquinic acid. Rosmarinic acid was the major compound in these extracts, with concentrations between 5.57 and 17.54 mg/g extract (**Figure 13** and **Table 26**) in the case of basil, and between 13.59 and 16.91 mg/g extract (**Figure 14** and **Table 27**) in the case of French lavender. It is evident that the extraction type influenced the recovery of this compound more in the case of basil than French lavender, as suggested by the wider range of concentrations observed the first case. The biological properties of rosmarinic acid that have been reported include anticarcinogenic, antioxidant, antiviral, antibacterial, anti-aging, antidiabetic, cardio-, hepato- and nephroprotective, antidepressant, antiallergic, and anti-inflammatory activity [18].

Tarragon extracts did not show any compounds in common with those detected in the other plant extracts. In this case, quercetin-3-*O*-rutinoside, 5-*O*-caffeoylquinic acid and 1,3,5-*O*-tricaffeoylquinic acid were the most abundant compounds. While the two caffeoylquinic acids were detected in comparable concentrations for the three extraction methods, quercetin-3-*O*-rutinoside content was clearly very dependent on the type of extraction, as its quantification ranged from 3.55 to 9.88 mg/g extract. In terms of potential benefits with therapeutic applications, caffeoylquinic acids have been reported to exhibit anti-inflammatory, antioxidant, antibacterial, cancer-related,

antiviral, anti-Alzheimer, and neuroprotective activities, as well as potential to improve cognitive decline and lifestyle-related diseases, such as diabetes [19]. In its turn, quercetin-3-*O*-rutinoside was reported to have anti-inflammatory, antidiabetic, antioxidant, anticarcinogenic, cytoprotective, antiplatelet, antithrombic, vasoprotective, and cardioprotective activities, to name a few [20-22].

Considering the phenolic profiles presented, tarragon, basil and French lavender can be considered for the production of extracts containing bioactive compounds. Moreover, other researchers have also investigated the phenolic profile of the plant materials tested in this work, thus attesting our results. The presence of quercetin-3-*O*-rutinoside, hydroxycinnamates (particularly di- and tricaffeoylquinic acids and ferulic acids) and/or other compounds of the same class as those detected in our tarragon extracts was also reported by Ribeiro et al., Lin and Harnly, Miron et al., and Silva et al. [23-26].

In the case of basil extracts, Kwee and Niemeyer [5] identified rosmarinic and chicoric acids as the dominant phenolic acids, and caftaric and caffeic acids at lower concentrations; Spréa et al. and Carocho et al. [27,28] also reported the presence of the same phenolic acids, plus sagerinic, salvianolic and yunnaneic acids. These results mostly agree with those of our work.

The phenolic compounds detected by Ceylan et al. and Contretas et al. [29,30] in French lavender extracts included caffeic acid, *p*-coumaric acid, rosmarinic acid, ferulic acid, eriodictyol, quercetin, luteolin-7-*O*-glucuronide, apigenin-7-*O*-glucoside, and apigenin-7-*O*-glucuronide, which are also generally in agreement with our results.

6.3.2 Antibacterial and Antifungal Activity

The minimum inhibitory, bactericidal and fungicidal concentrations determined for each extract are available in **Table 28** and **Table 29**, respectively.

All extracts revealed antimicrobial activity against *E. coli*, *S.* Typhimurium, *E. cloacae*, *S. aureus*, *B. cereus* and *L. monocytogenes*. However, the various pathogens tested showed varying susceptibility to different extracts, depending on the extraction method and plant used, as suggested by the MIC values of 0.5 or 1 mg/mL; and MBC of 1 or 2 mg/mL. Only *B. cereus* was equally affected by the nine extracts produced, as the MIC and MBC values were the same in all cases (MIC = 0.5 mg/mL; MBC = 1 mg/mL).

Table 28. Minimum inhibitory and minimum bactericidal concentration of plant extracts, MIC/MBC, respectively (mg/mL; mean ± SD, n = 3)

Extraction	Plant	SA [1]	BC [2]	LM [3]	EC [4]	ST [5]	EntC [6]
Infusion	Tarragon	0.5/1	0.5/1	0.5/1	0.5/1	0.5/1	1/2
	French lavender	1/2	0.5/1	1/2	0.5/1	0.5/1	1/2
	Basil	1/2	0.5/1	1/2	1/2	0.5/1	1/2
Decoction	Tarragon	0.5/1	0.5/1	1/2	0.5/1	0.5/1	1/2
	French lavender	1/2	0.5/1	1/2	0.5/1	0.5/1	0.5/1
	Basil	0.5/1	0.5/1	1/2	0.5/1	0.5/1	1/2
Hydroethanolic extraction	Tarragon	0.5/1	0.5/1	0.5/1	1/2	1/2	0.5/1
	French lavender	0.5/1	0.5/1	0.5/1	0.5/1	0.5/1	0.5/1
	Basil	0.5/1	0.5/1	0.5/1	0.5/1	1/2	0.5/1
E211		4/4	0.5/0.5	1/2	1/2	1/2	2/4
E224		1/1	2/4	0.5/1	0.5/1	1/1	0.5/0.5

Legend: [1] *S. aureus*, [2] *B. cereus*, [3] *L. monocytogenes*, [4] *E. coli*, [5] *Salmonella enterica* ser. Typhimurium, [6] *E. cloacae*, [7] Sodium benzoate, [8] Potassium metabisulfite

Table 29. Minimum inhibitory and minimum fungicidal concentration of plant extracts, MIC/MFC, respectively (mg/mL; mean ± SD, n = 3)

Extraction	Plant	AF [1]	AN [2]	AV [3]	PF [4]	PVC [5]	TV [6]
Infusion	Tarragon	0.25/0.5	0.25/0.5	0.25/0.5	0.25/0.5	0.25/0.5	0.25/0.5
	French lavender	0.25/0.5	0.25/0.5	0.25/0.5	0.25/0.5	0.5/1	0.25/0.5
	Basil	0.25/0.5	0.5/1	0.25/0.5	0.25/0.5	0.25/0.5	0.25/0.5
Decoction	Tarragon	0.25/0.5	0.25/0.5	0.25/0.5	0.25/0.5	0.25/0.5	0.25/0.5
	French lavender	0.25/0.5	0.25/0.5	0.25/0.5	0.5/1	0.5/1	0.25/0.5
	Basil	0.25/0.5	0.5/1	0.25/0.5	0.25/0.5	0.5/1	0.25/0.5
Hydroethanolic extraction	Tarragon	0.25/0.5	0.5/1	0.25/0.5	0.25/0.5	0.25/0.5	0.125/0.25
	French lavender	0.5/1	0.5/1	0.25/0.5	0.25/0.5	0.25/0.5	0.125/0.25
	Basil	0.25/0.5	0.5/1	0.25/0.5	0.25/0.5	0.25/0.5	0.125/0.25
E211		1/2	1/2	2/2	1/2	2/4	1/2
E224		1/1	1/1	1/1	0.5/0.5	1/1	0.5/0.5

Legend: [1] *A. fumigatus*, [2] *A. niger*, [3] *A. versicolor*, [4] *P. funiculosum*, [5] *P. verrucosum* var. *cyclopium*, [6] *T. viride*, [7] Sodium benzoate, [8] Potassium metabisulfite

Regarding fungi, all tested species were susceptible to all extracts (MIC ≤ 0.5 mg/mL; MFC ≤ 1 mg/mL), but especially *T. viride* in the presence of hydroethanolic extracts (MIC ≤ 0.125 mg/mL; MFC ≤ 0.25 mg/mL). With some exceptions, infusions and decoctions showed uniform activity (MIC = 0.25 and MFC = 0.5 mg/mL) for all tested bacteria.

Overall, most infusions, decoctions and hydroethanolic extracts showed equivalent or superior bactericidal and fungicidal activities against microorganisms with relevance for public health and food contamination when compared to those of sodium benzoate (E211) and potassium metabisulfite (E224). Moreover, the concentration of synthetic additives needed to inhibit each type of bacteria varies widely (from 0.5 to 4 mg/mL, in the case of E211), thus indicating species selectivity. Plant extracts, on the other hand, appeared to inhibit all bacteria at a reduced range of concentrations (0.5 to 1 mg/mL).

Other researchers have also reported on the antimicrobial and antifungal effects of these plants. Ueda et al. [31] determined MICs of hydroethanolic basil extracts of 2 mg/mL for *S. aureus*, *L. monocytogenes*, *E. coli*, *S.* Typhimurium, and *E. cloacae*, 1 mg/mL for *B. cereus*, 0.25 mg/mL for *A. fumigatus, P. funiculosum*, and *P. verrucosum*, and 0.5 mg/mL for *A. niger*, *A. versicolor* and *T. viride*. Ribeiro et al. [23] evaluated the antimicrobial and antifungal activity of hydroethanolic (80% (v/v)) tarragon extracts, and reported MIC values of 0.12 mg/mL for *S.* Typhimurium, 0.08 mg/mL for *S. aureus*, *L. monocytogenes* and *A. niger*, 0.06 mg/mL for *E. coli* and *A. versicolor*, 0.04 mg/mL for *E. cloacae*, *A. fumigatus*, *T. viride*, *P. funiculosum* and *P. verrucosum*, and 0.02 mg/mL for *B. cereus*. For hydroethanolic extracts obtained with ethanol 96% (v/v), Behbahani et al. [32] described higher MIC values: 8 mg/mL for *E. coli*, 4 mg/mL for *B. cereus*, and 2 mg/mL for *S. aureus*, *C. albicans* and *A. fumigatus*. Regarding ethanolic extracts of French lavender, Canlı et al. [33] reported considerably reduced MIC values: 0.0359 mg/mL for *C. albicans*, *L. monocytogenes*, *S. enteritidis* and *S.* Typhimurium, and 0.01795 mg/mL for two *S. aureus* strains.

6.3.3 Antioxidant Activity

In the present work, two in vitro essays were employed to evaluate the antioxidant capacity of the extract: TBARS and OxHLIA. The results are expressed in **Table 30** as IC_{50} values, meaning that lower values imply greater antioxidant potential.

Table 30. Antioxidant activity of plant extracts expressed as half-maximal inhibitory concentration (IC_{50}, μg/mL) measured by the TBARS (mean ± SD, n = 9) and OxHLIA (mean ± SD, n = 3) essays.

Essay	Plant	Infusion	Decoction	Hydroethanolic Extract
TBARS	Tarragon	392 ± 16.8^{c}	549 ± 23.4^{c}	177 ± 4.16^{a}
	French lavender	182 ± 3.67^{a}	186 ± 4.67^{a}	239 ± 6.99^{c}
	Basil	210 ± 2.98^{b}	213 ± 7.47^{b}	206 ± 4.53^{b}
OxHLIA Δt = 60 min	Tarragon	170 ± 2.24^{c}	91.8 ± 1.61^{c}	48.5 ± 1.64^{b}
	French lavender	48.8 ± 1.64^{a}	28.6 ± 1.01^{a}	15.4 ± 0.44^{a}
	Basil	97.2 ± 1.40^{b}	49.2 ± 1.21^{b}	89.4 ± 2.89^{c}
OxHLIA Δt = 120 min	Tarragon	262 ± 3.75^{c}	141 ± 2.34^{c}	117 ± 2.92^{b}
	French lavender	94.6 ± 1.44^{a}	45.3 ± 1.00^{a}	32.5 ± 0.53^{a}
	Basil	151 ± 2.05^{b}	93.4 ± 2.54^{b}	160 ± 4.43^{c}

TROLOX IC_{50} value: 5.4 ± 0.3 μg/mL (TBARS), 21.8 ± 0.25 μg/mL (OxHLIA Δt = 60 min), 43.5 ± 1.00 μg/mL (OxHLIA Δt = 120 min). For each essay, values with different superscript letters in a column mean significant differences (ANOVA, $p < 0.05$).

The results show that all extracts have antioxidant activity, but in different degrees. In fact, from both essays, for each extraction method, different plants yield different results ($p < 0.05$). In the TBARS essay, French lavender infusion and decoction, as well as tarragon hydroethanolic extract presented the highest antioxidant capacities. In the OxHLIA essay, the three French lavender extracts presented the greatest protective capacity, with the hydroethanolic extract standing out for its lowest IC_{50} values (15.4 ± 0.44 μg/mL and 32.5 ± 0.53 μg/mL for 60 min and 120 min, respectively), even better than TROLOX.

In OxHLIA, the antioxidant behaviour was monitored over time, as the action of the antioxidants depends on several factors, including short-term and long-term reaction kinetics and the rate at which antioxidants react with specific free radicals [34]. In this sense, while some antioxidants may react more quickly and become depleted in the system, others may offer prolonged antioxidant protection over time. Therefore, a distinction between short-term and long-term antioxidant protection was made by assessing oxidative haemolysis at two Δt. With two exceptions (tarragon and French lavender hydroethanolic extracts), the concentration necessary to protect 50% of the red blood cells from the haemolytic action of AAPH for 120 min was less than double the concentration necessary for this protection for 60 min. This means that most extracts had anti-haemolytic activity for longer exposure times, while the hydroethanolic extracts of tarragon and French lavender were not as efficient for 120 min compared to 60 min.

The antioxidant activity of tarragon, French lavender and basil extracts has been determined previously; however, comparison of results is not straightforward as in some cases, besides different extraction methods and solvents being used, distinct antioxidant essays (other than TBARS and OxHLIA) are also employed [23,25,31,32,35].

6.3.4 Anti-Inflammatory Activity

The anti-inflammatory capacity of the extracts produced is presented in **Table 31** as IC_{50} values, from which lower values correspond to greater nitric oxide production inhibition.

Table 31. Anti-inflammatory activity (IC_{50} values; μg/mL) of the plant extracts measured by NO production inhibitory capacity (mean ± SD, n = 2)

Plant	Infusion	Decoction	Hydroethanolic Extract
Tarragon	> 400 ^{b}	34.6 ± 0.53 ^{a}	44.1 ± 3.96 ^{a}
French lavender	> 400 ^{b}	> 400 ^{c}	> 400 ^{b}
Basil	88.6 ± 0.47 ^{a}	64.5 ± 0.68 ^{b}	54.7 ± 5.37 ^{a}

Dexamethasone IC_{50} value: 6 ± 1 μg/mL. Values with different superscript letters in a column mean significant differences (ANOVA, $p < 0.05$).

None of the French Lavender extracts revealed anti-inflammatory action at the tested concentrations (IC_{50} > 400 μg/mL). Only those of basil and tarragon proved this capability, with basil extracts showing anti-inflammatory action regardless of the extraction method, unlike tarragon, which did not maintain its potential when the infusion method was used. Tarragon decoction showed the highest anti-inflammatory capacity, considering its IC_{50} of 34.6 ± 0.53 μg/mL, followed by tarragon hydroethanolic extract, with IC_{50} = 44.1 ± 3.96 μg/mL.

In line with our results, Takeuchi et al. [36] also observed the anti-inflammatory effects of basil extracts in vitro, on inflammation related to obesity; while Eidi et al. [37] reported on anti-inflammatory capacity of ethanolic tarragon extracts in vivo, in adult mice. To our knowledge, studies on the anti-inflammatory potential of French lavender extracts (not essential oils) are scarce. Only two studies were identified: one by Algieri et al. [38] reporting on the anti-inflammatory effects of hydroalcoholic French lavender both in vitro and in vivo; and one by Ez Zoubi et al. [39], which evaluated the in vivo anti-inflammatory effect.

6.3.5 Antiproliferative Activity

Table 32 details the antiproliferative activity of extracts expressed as GI_{50} values, meaning that lower values correspond to greater antiproliferative capacity.

Table 32. Antiproliferative activity (mean ± SD, n = 2) of the plant extracts produced (GI_{50} values; μg/mL)

Extraction	Plant	AGS [1]	CaCo-2 [2]	HeLa [3]	MCF-7 [4]	NCI-H460 [5]	hFOB [6]
Infusion	Tarragon	> 400^{b}	> 400^{b}	323 ± 7.48^{a}	117 ± 2.01^{a}	> 400	> 400
	French lavender	223 ± 5.39^{a}	259 ± 0.03^{a}	255 ± 36.2^{a}	213 ± 2.34^{b}	> 400	> 400
	Basil	> 400^{b}	> 400^{b}	> 400^{b}	> 400^{c}	> 400	> 400
Decoction	Tarragon	> 400^{b}	> 400^{b}	> 400^{b}	> 400	> 400	> 400
	French lavender	177 ± 7.26^{a}	315 ± 0.14^{a}	342 ± 5.07^{a}	> 400	> 400	> 400
	Basil	> 400^{b}	> 400^{b}	> 400^{b}	> 400	> 400	> 400
Hydroethanolic extract	Tarragon	> 400^{c}	> 400^{c}	> 400^{c}	237 ± 2.23^{b}	> 400^{c}	290 ± 3.05^{c}
	French lavender	234 ± 5.40^{b}	294 ± 0.35^{b}	310 ± 4.60^{b}	190 ± 1.21^{a}	306 ± 4.51^{a}	257 ± 2.06^{a}
	Basil	113 ± 9.55^{a}	264 ± 1.53^{a}	257 ± 9.78^{a}	186 ± 0.04^{a}	366 ± 4.01^{b}	275 ± 0.60^{b}

Legend: [1] Gastric adenocarcinoma, [2] Colorectal adenocarcinoma, [3] Cervical carcinoma, [4] Breast adenocarcinoma, [5] Large cell lung carcinoma, [6] non-tumour hFOB (human foetal osteoblasts). Ellipticine GI_{50} values: 1.23 ± 0.03 μg/mL (AGS), 1.21 ± 0.02 μg/mL (CaCo-2), 1.91 ± 0.12 μg/mL (HeLa), 1.02 ±0.02 μg/mL (MCF-7), 1.01 ± 0.01 μg/mL (NCI-H460) and 1.21 ± 0.08 μg/mL (hFOB).

In terms of antiproliferative capacity against tumour cell lines, tarragon infusion was active against HeLa and MCF-7 cells; but only against MCF-7 cells in the case of tarragon hydroethanolic extracts; French lavender decoction was active against AGS, CaCo-2 and HeLa cells, but also against MCF-7 in the case of its infusion. French lavender and basil hydroethanolic extracts revealed inhibitory potential against all tumour cell lines.

All infusions and decoctions were non-toxic against healthy cells, namely, human foetal osteoblasts cells, hFOB (GI_{50} > 400 μg/mL). However, toxicity for hFOB cells was detected in all hydroethanolic extracts, which compromises the use of such extracts as food additives.

Some literature is available regarding the antiproliferative capacities of extracts obtained from the plants tested in our study. Basil hydroethanolic extract produced by Ueda et al. [31] did not show hepatotoxicity in PLP2 cells (non-tumour) at the maximum tested concentration of 400

µg/mL. Carocho et al. [28] screened basil decoctions, which revealed antiproliferative activity against HeLa cell line (GI_{50} = 254 ± 5 µg/mL), but no effect on MCF-7 and NCI-H460 lines, and no hepatotoxicity for PLP2 non-tumour cells (GI_{50} > 400 µg/mL).

Ribeiro et al. [23] determined the cytotoxic properties of hydroethanolic tarragon extracts and observed their ability to inhibit the growth of MCF-7 (GI_{50} = 272 ± 22 µg/mL) but not of NCI-H460 (GI_{50} > 400 µg/mL), in concordance with our study. However, the authors reported inhibitory effects in HeLa cell line (GI_{50} = 245 ± 14 µg/mL) [23], which we did not observe. Furthermore, their extracts did not show any effect on non-tumour cells (PLP2) [23], contrary to ours.

As for French lavender extracts, Siddiqui et al. and Nunes et al. [40, 41] evaluated their antiproliferative activity using the microculture tetrazolium essay (MTT). Siddiqui et al. [40] prepared an ethanolic fraction from a methanolic extract and reported a major reduction in the survival percentage of tumour cells HEP G2. Similarly, the results obtained by Nunes et al. [41] indicate antiproliferative action on HEP G2 cells, but also some impact was measured in fibroblasts, which suggests toxicity against non-tumour cells. Tayarani-Najaran et al. [42] also investigated the antiproliferative effects of French lavender methanol extract, and the results indicated that pre-treatment of PC12 cells with the extract could significantly decrease 6-OHDA cytotoxicity and cell apoptosis, thus suggesting an important neuroprotective and anti-apoptotic activity.

6.4 CONCLUSIONS

The extracts produced using tarragon, basil and French lavender revealed antimicrobial, antifungal and antioxidant properties. No trend was observed as far as the extraction method (infusion, decoction or hydroethanolic maceration) that would provide the best bioactivities in each essay (antimicrobial, antifungal, antioxidant, anti-inflammatory, and antiproliferative). Instead, higher differences were observed according to the plant material tested. To this, French lavender extracts stood out for having overall high total phenolic content, across the three extraction methods tested, whereas the phenolic content of the other plant extracts was more influenced by the extraction procedure used. Additionally, most French lavender extracts showed the highest oxidation inhibitory capabilities and were able to damage tumour cells. The weaknesses of these extracts were their incapacity to provide an anti-inflammatory response, and, in the case of the hydroethanolic extract, the toxicity against healthy cells.

Considering these results, tarragon, basil and French lavender extracts appear as potential natural additives for the preservation of foods and elaboration of functional foods. Nonetheless,

additional studies should be conducted to evaluate the stability and sensory appreciation of extracts when incorporated into foods, the bioavailability and bioaccessibility of the bioactive compounds of the extracts, as well as the "extract-food matrix" interaction when plant extracts are applied to the enrichment of foods.

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CHAPTER 7

TECHNOLOGICAL POTENTIAL OF LACTIC ACID BACTERIA ISOLATED FROM PORTUGUESE GOAT'S RAW MILK CHEESES

This chapter was based on the following proceedings paper:

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7.1 INTRODUCTION

Lactic acid bacteria (LAB) are responsible for the cheese fermentation process, whether they are naturally present in milk or purposefully added (starter culture), as they produce organic acids (mainly lactic and acetic acids) that cause the rapid acidification of milk and consequently promote coagulation, curd firmness and control of contaminants [1,2]. The metabolic characteristics of LAB, such as the proteolytic capacity, can contribute to the development of pleasurable organoleptic properties (such as texture, aroma and flavour compounds) [3,4], which are particularly important in artisanal cheeses.

Furthermore, LAB can also increase the safety of cheeses and act as biopreservative agents, as a result of the production of antimicrobial metabolites during fermentation, such as organic acids, hydrogen peroxide, diacetyl, fatty acids, reuterin and bacteriocins [5], and thus replace chemical preservatives that are used in dairy products, such as sorbic acid, sodium benzoate, calcium sorbate, potassium sorbate, and natamycin [6,7]. However, not all LAB are suitable to be added to food products. LAB that belong to the genera *Lactococcus*, *Lactobacillus*, *Leuconostoc*, *Pediococcus*, and some *Streptococcus* have Generally Regarded as Safe (GRAS) or Qualified Presumption of Safety (QPS) status, which means that there is reasonable evidence that such microorganisms do not raise safety concerns and that their use in foods is approved by the U.S. Food and Drug Agency (FDA) or by the European Food Safety Authority (EFSA), respectively [8]. On the other hand, *Enterococcus* genus and some *Streptococcus* species can be pathogenic and present virulence factors and resistance to a variety of antibiotics, reason why they are not eligible for GRAS/QPS status and, therefore, may not be used in foods [8].

Considering that artisanal cheeses produced from goat's raw milk may have poor microbial quality [5,9], it was hypothesised that the addition of selected LAB strains with functional properties, as a customised starter culture, could be used as a control measure for the growth of *Staphylococcus aureus*. In that regard, the autochthonous microbiota of raw milk cheeses is complex and diverse, offering a wide range of species with antimicrobial and/or acidifying capacities [10-14]. It is possible that a single LAB strain is not capable of inhibiting a pathogen in milk or cheese. In the case of bacteriocinogenic LAB, for example, there may be various limiting factors, such as the level of bacteriocin expression, the low capacity for bacteriocin production in the food system, the interaction between bacteriocin and food matrix, the antagonism of other

bacteria toward the LAB strain, and the effect of the physicochemical parameters on the bacteriocin activity [6].

For that reason, a mixture of strains can be used to build the starter culture, and to guarantee the desired antagonistic effect, it may be convenient to use strains with distinct capacities: acidifying LAB strains, for instance, also play a key role in inhibiting pathogenic bacteria during cheese ripening by promoting an acidic environment [14], and can be combined with bacteriocinogenic LAB to enhance the antimicrobial power of the starter culture.

In this sense, the first objective of this work was to collect and assess the antimicrobial, acidifying and proteolytic capacities of LAB isolates from the microflora of artisanal Portuguese goat's raw milk cheeses. Then, using statistical analysis, the second objective was to select a subset of LAB isolates with potential to be included in a customised starter culture and used in cheese manufacture, and perform their molecular identification by 16S rRNA sequencing.

7.2 MATERIALS AND METHODS

7.2.1 Bacterial strains and preparation of cell suspensions

Salmonella enterica subsp. *enterica* serovar Typhimurium ATCC 43971, *Listeria monocytogenes* WDCM 00019, and *Staphylococcus aureus* ATCC 6538, obtained from the Polytechnic Institute of Bragança stock collection, were used for the antimicrobial essay. A loop of culture kept on Nutrient Agar slants was inoculated separately in 10 mL of BHI broth. Broth tubes were incubated at 37 °C for 16 h, following two successive inoculations, to achieve a concentration of approximately 10^8 CFU/mL. *L. monocytogenes* required a pre-activation in 5 mL of BHI at 37 °C for 16 h.

7.2.2 Isolation and confirmation of LAB

Samples of four batches of artisanal goat's raw milk cheeses (n = 20) were collected at the end of production from a regional factory located in Mirandela, Portugal, between November 2020 and March 2021. LAB were isolated from cheese samples as described by the ISO standard 15214:1998, with minor modifications [15]. Briefly, after dilution, aliquots were incorporated in MRS agar (selective medium for enumeration and isolation of lactobacilli) and M17 agar (non-selective medium for enumeration and isolation of lactococci), overlayed with 1.2% bacteriological agar, and plates were incubated at 30 °C for 48 h. Then, eight typical colonies on MRS and M17

agar were selected for purification and incubated at 30 °C for another 48 h in the respective media. Lastly, to eliminate non-LAB isolates, catalase (3% hydrogen peroxide) and Gram tests, as well as morphologic observation, were performed. Cultures presumptively identified as LAB were maintained in MRS broth with 25% glycerol at -80 °C.

7.2.3 Screening of LAB for antimicrobial, proteolytic and acidifying capacities

The antimicrobial ability of LAB isolates was determined by the spot-on-lawn essay as described by Campagnollo et al. [16], with some modifications. Briefly, each LAB isolate was reactivated separately in MRS broth overnight (37 °C, 24 h), spotted onto the surface of MRS or M17 agar plates (3 μL for LAB isolated in MRS and 5 μL for M17 agar, respectively), following incubation at 30 °C for 16h. Inoculated plates were covered with 10 mL of BHI soft agar (BHI broth with 0.75% (w/v) bacteriological agar) seeded with 1 mL of each bacterial strain at approximately 8 log CFU/mL (pathogens were tested separately). After incubating plates at 37 °C for 16h (pre-incubation at 4 °C for 2h), the diameter of the inhibition zones of each pathogen were measured with a calliper. LAB isolates that presented antimicrobial capacity at 37 °C according to the following criteria were also tested at 10 °C for 10 days: distance between halo circumference and LAB colony limit greater than 5 mm for *S. aureus*, or 8 mm for *L. monocytogenes* and *S. enterica* ser. Typhimurium – for MRS agar; or greater than 0.5 mm for *S. aureus*, 6 mm for *L. monocytogenes* or 3.5 mm for *S. enterica* ser. Typhimurium – for M17 agar.

Proteolytic activity and acidifying capacity were evaluated according to the protocols of Franciosi et al. [17] and Durlu-Ozkaya et al. [18] respectively, with a few modifications, for the subset of LAB isolates presenting antimicrobial activity at 37 °C. From the cryopreserved stock culture, each LAB isolate was reactivated separately in MRS broth overnight (30 °C, 24 h). Then, a loop of culture was placed in 10 mL of sterile reconstituted skim milk supplemented with yeast extract (0.3% (w/v)) and glucose (0.2% (w/v)) for two successive subcultures (30 °C for 24 h). Sterile reconstituted skim milk (100 mL) was then inoculated with 1 mL of the 24 h activated culture. For the acidification profiling, pH changes were determined using a pH meter (Hanna Instruments, model HI5522, USA) equipped with a HI1131 glass penetration probe during incubation at 30°C during 8 h (t = 0, 2, 4, 6, 8 h), and after 24 h [18]. For every strain, pH data was fitted to a decay curve to characterise acidification capacity [19]. The following descriptors were extracted from the fitted curves: ΔpH_{02}: pH decrease between t = 0 h and t = 2 h; ΔpH_{06}: pH decrease between t = 0 h and t = 6 h; ΔpH_{26}: pH decrease between t = 2 h and t = 6 h; and pH_6:

pH at t = 6 h. LAB isolates were considered good acidifiers when able reduce the pH below 5.3 after 6 h at 30 °C [20].

For the determination of exocellular proteolytic activity, the 24 h activated cultures were spotted (3 μL) onto the surface of milk agar (composed of 10% (w/v) skim milk powder and 2.5% (w/v) agar) and incubated at 35 °C for 4 days [17]. Proteolytic activity was confirmed as clear zones around each LAB colony, whose diameters were measured against the LAB colony diameter.

7.2.4 Molecular identification of LAB isolates by 16S rRNA sequencing

The cryopreserved isolates of a subset of forty isolates with promising antimicrobial and technological properties were reactivated in MRS or M17 agar and incubated at 37 °C for 48 h. Isolated colonies were inoculated in 5 mL of MRS broth (Himedia, Einhausen, Germany) and incubated at 37 °C for 24 h. After incubation, 1.5 mL of culture was transferred to Eppendorf tubes and centrifuged at 10,000 × g for 2 min; the process was repeated two times for each culture. The supernatant was discarded, and the pellet was kept at 4 °C.

Genomic DNA (gDNA) was extracted from samples using a GF-1 Bacterial DNA Extraction Kit (Vivantis, Shah Alam, Malaysia), with the optional RNA removal step. The DNA concentration and purity were analysed using the 260/280 ratio. The primers used for amplification of the 16S rRNA gene were 27f 5′-AGA GTT TGA TCC TGG CTC AG-3′ and 1492r 5′-CTA CGG CTA CCT TGT TAC GA-3′ [21]. The PCR cycle was 94 °C for 2 min, followed by 30 cycles of 94 °C for 10 s, 55 °C for 20 s and 72 °C for 1 min, using DFS-Taq DNA polymerase (Bioron Life Sciences, Römerberg, Germany). PCR products were visualized via electrophoresis on 1% (w/v) agarose gel, stained with ethidium bromide, purified with the GF-1 PCR Clean-up Kit (Vivantis, Shah Alam, Malaysia), and used as template in the sequencing reactions. The quality of amplicons was measured using the 260/280 ratio. For sequencing reactions, a BigDyeTM Terminator v3.1 system was used, and for the purification of samples, a SAM/BigDyeXTerminatorTM bead solution was employed (ThermoFisher Scientific, Oeiras, Portugal). Capillary electrophoresis was carried out using a SeqStudio Genetic Analyzer (Applied Biosystems, Porto, Portugal).

The sequence data obtained were aligned with sequences from the NCBI 16S rRNA database using the basic local alignment search tool (BLAST) algorithm. Finally, sequences with identity higher than 97% were accepted as the best matches for the LAB isolates.

7.2.5 Statistical analysis

7.2.4.1 Principal Component Analysis: MRS- *versus* M17-isolated LAB

Data were separated into two subsets (one for MRS-isolated LAB; another for M17-isolated LAB) and each subset subjected to principal component analysis (PCA) to evaluate the contribution of the antimicrobial, proteolytic and acidifying properties to the discrimination of isolates. From the antimicrobial essays, only the data referring to *L. monocytogenes* and *S. aureus* inhibition was used (N=84), as these are the pathogens of greater concern (among the three tested) in cheese.

The function principal from the psych package was used in R software (version 3.6.2, R Foundation for Statistical Computing, Vienna, Austria), where a varimax-rotated solution for three principal components was obtained. From the three-dimensional PCA, maps of antimicrobial, acidifying and proteolytic characteristics of cheeses were built from the projection of sample scores onto the span of the principal components, using the function prcomp from the factoextra package.

7.2.4.2 Principal Component Analysis: subset of isolates with promising antimicrobial and technological properties

Considering the results of the previous PCA, a subset of forty LAB isolates (N=40; twenty MRS-isolated; twenty M17-isolated) with promising antimicrobial and technological properties was defined and a second PCA was carried out to appraise the relationship between genus and species and the antimicrobial, proteolytic and acidifying properties of the isolates. Again, the function prcomp from the factoextra package was used in R.

7.3 RESULTS AND DISCUSSION

7.3.1 Antimicrobial, acidifying and proteolytic capacities of LAB isolates

In total, 232 LAB isolates (97 isolated in MRS agar and 135 isolated in M17 agar) were isolated. Antimicrobial tests at 37 °C revealed that 98%, 100% and 100% of LAB isolated in MRS agar presented antagonism against *L. monocytogenes*, *S. aureus* and *S. enterica* ser. Typhimurium, respectively. In contrast, only 13.3% and 28.1% of LAB isolated in M17 agar revealed antagonism against *L. monocytogenes* and *S. enterica* ser. Typhimurium, respectively (no antagonism was observed against *S. aureus*).

After selecting isolates with considerable antimicrobial activity at 37 °C, 84 isolates (58 isolated in MRS agar and 26 isolated in M17 agar) were subjected to the spot-on-lawn essay at 10 °C. The results of this assessment revealed that all 84 isolates from this subset maintained their antimicrobial activity even at 10 °C. The microbial inhibition offered by the isolated LAB may be a consequence of competition against pathogens for the available substrate, production of antimicrobial substances (for example, bacteriocins) and/or production of non-proteinaceous compounds such as H_2O_2 [1].

Regarding the acidifying capacity, LAB isolated from M17 agar presented better outcomes than LAB isolated from MRS agar. More specifically, 12 out of the 26 isolates (46%) obtained from M17 agar were able to reduce the pH of milk broth below 5.3 after 6 h at 30 °C. This result indicates the potential of some isolates to contribute to a rapid pH decrease, which is essential in cheese-making to achieve adequate coagulation, curd firmness and control of bacterial pathogens growth, among other contaminants [1,16]. In this sense, these LAB isolates demonstrate potential to be used as starter and/or adjunct cultures to avoid faulty fermentations. On the other hand, no isolates from MRS agar provided such a pH reduction under these conditions. Nevertheless, LAB strains with poor acidifying capacity can still be part of a starter mixture, if they possess other technological properties that may benefit cheese production [1].

Regarding the proteolytic capacity, only two isolates from MRS agar (labelled isolate 16 and isolate 24) demonstrated irrefutable clear (transparent) zones around the colonies. Isolate 16 showed a diameter of proteolytic activity of 1.94 mm, whereas isolate 24 presented a smaller halo, of 1.45 mm in diameter around the LAB colony. The antimicrobial and acidifying properties of these isolates are presented in section 7.3.3, in **Table 35**, along with the corresponding identified genus and species. Although only these two isolates showed transparent halos, other isolates also revealed a zone around the colony with less density than the milk agar, but not completely transparent. The clear zone surrounding the colonies is an indicator that proteolytic bacteria hydrolyse casein to form soluble nitrogenous compounds; more clear zones are seen on milk agar if the bacteria also produce acid from fermentable carbohydrates present in the medium [22]. This may explain the two types of zones observed in this essay. From the cheese-making perspective, casein hydrolysis is crucial for texture development, and the released peptides can also accelerate aroma development [16]. In this sense, the results obtained may suggest the potential, even if limited, of some isolates to contribute to the improvement of cheese texture and aroma.

7.3.2 Principal Component Analysis: subsets of MRS- and M17-isolated LAB

In the subsets of MRS- and M17-isolated LAB, the contribution of the antimicrobial and acidifying attributes to the principal components can be evaluated in **Table 33** and **Table 34**, respectively, by their correlations with the three components extracted. In the subset of MRS-isolated LAB, the contribution of the proteolytic attribute to the principal components could also be evaluated. In the case of M17-isolated LAB, the contribution of such technological property could not be assessed as no isolate presented casein hydrolysis capacity. **Figure 15** and **Figure 16** represent the biplots of variables loadings and observation scores.

Table 33. Coefficients of correlation of the tested technological properties of MRS-isolated LAB, with the three varimax-rotated factors (PC1, PC2, PC3) along with communalities and explained variances.

Variable	PC1	PC2	PC3	Communalities
pH_6	**-0.77**	-0.13	0.10	1.1
ΔpH_{02}	-0.05	**0.96**	-0.09	1.0
ΔpH_{06}	0.50	**0.83**	-0.15	1.7
ΔpH_{26}	**0.89**	0.06	-0.13	1.0
ID *L. monocytogenes* 37 °C	-0.26	-0.13	**0.65**	1.4
ID *S. aureus* 37 °C	**-0.56**	0.43	**0.48**	2.9
ID *L. monocytogenes* 10 °C	0.41	-0.14	**0.64**	1.8
ID *S. aureus* 10 °C	-0.26	0.04	**0.68**	1.3
PAct	0.21	0.06	-0.17	2.2
Proportion Variance	0.26	0.21	0.18	-
Cumulative Variance	**0.26**	**0.47**	**0.65**	-

pH_6: pH value of milk broth after 6h at 30 °C; $\Delta pH_{02, 06}$ and $_{26}$: pH decrease between t= 0h and t= 2h, t= 0h and t= 6h and t= 2h and t= 6h, respectively; ID *L. monocytogenes* 37 °C and 10 °C: diameter of inhibition (mm) of *L. monocytogenes* tested at 37 °C and 10 °C, respectively; ID *S. aureus* 37 °C and 10 °C: diameter of inhibition (mm) of *S. aureus* tested at 37 °C and 10 °C, respectively; PAct: diameter of proteolytic activity (mm).

Table 34. Coefficients of correlation of the tested technological properties of M17-isolated LAB, with the three varimax-rotated factors (PC1, PC2, PC3) along with communalities and explained variances.

Variable	PC1	PC2	PC3	Communalities
pH_6	**-0.82**	-0.14	0.52	1.8
ΔpH_{02}	**0.91**	0.15	-0.26	1.2
ΔpH_{06}	**0.89**	0.10	-0.45	1.5
ΔpH_{26}	**0.86**	0.09	-0.47	1.6
ID *L. monocytogenes* 37 °C	-0.50	-0.06	**0.83**	1.7
ID *S. aureus* 37 °C	0.14	**0.99**	-0.07	1.1
ID *L. monocytogenes* 10 °C	-0.44	-0.04	**0.86**	1.5
ID *S. aureus* 10 °C	-0.29	-0.08	**0.86**	1.2
Proportion Variance	0.45	0.13	0.37	-
Cumulative Variance	**0.45**	**0.58**	**0.95**	-

pH_6: pH value of milk broth after 6h at 30 °C; $\Delta pH_{02,\ 06}$ and $_{26}$: pH decrease between t= 0h and t= 2h, t= 0h and t= 6h and t= 2h and t= 6h, respectively; ID *L. monocytogenes* 37 °C and 10 °C: diameter of inhibition (mm) of *L. monocytogenes* tested at 37 °C and 10 °C, respectively; ID *S. aureus* 37 °C and 10 °C: diameter of inhibition (mm) of *S. aureus* tested at 37 °C and 10 °C, respectively.

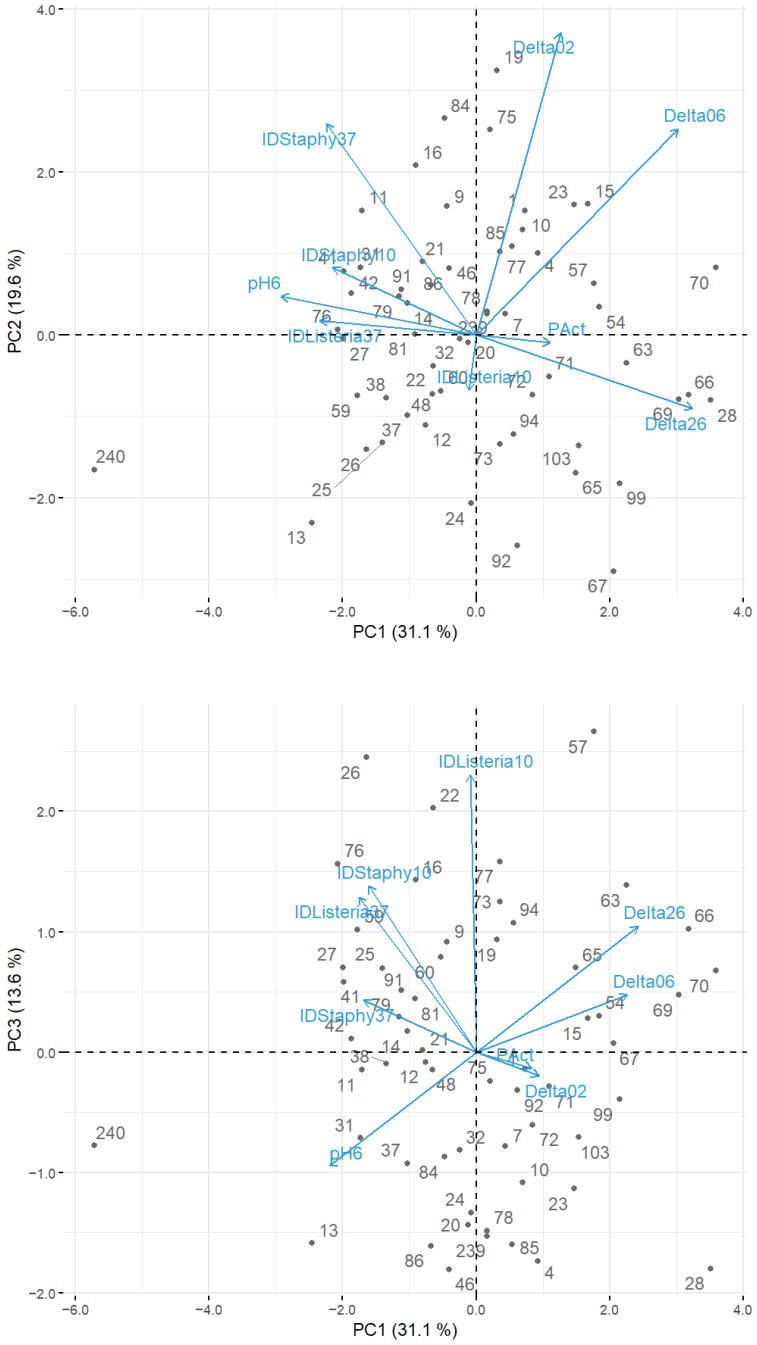

Figure 15. Maps of the first and second principal components (top) and the first and third principal components (bottom) of the tested technological properties of MRS-isolated LAB. Legend: Delta02, Delta06, Delta26: pH decrease between t= 0h and t= 2h, t= 0h and t= 6h and t= 2h and t= 6h, respectively; pH6: pH value of milk broth after 6h at 30 °C; IDListeria37 and IDListeria10: diameter of inhibition (mm) of *L. monocytogenes* tested at 37 °C and 10 °C, respectively; IDStaphy37 and IDStaphy10: diameter of inhibition (mm) of *S. aureus* tested at 37 °C and 10 °C, respectively; PAct: diameter of proteolytic activity (mm).

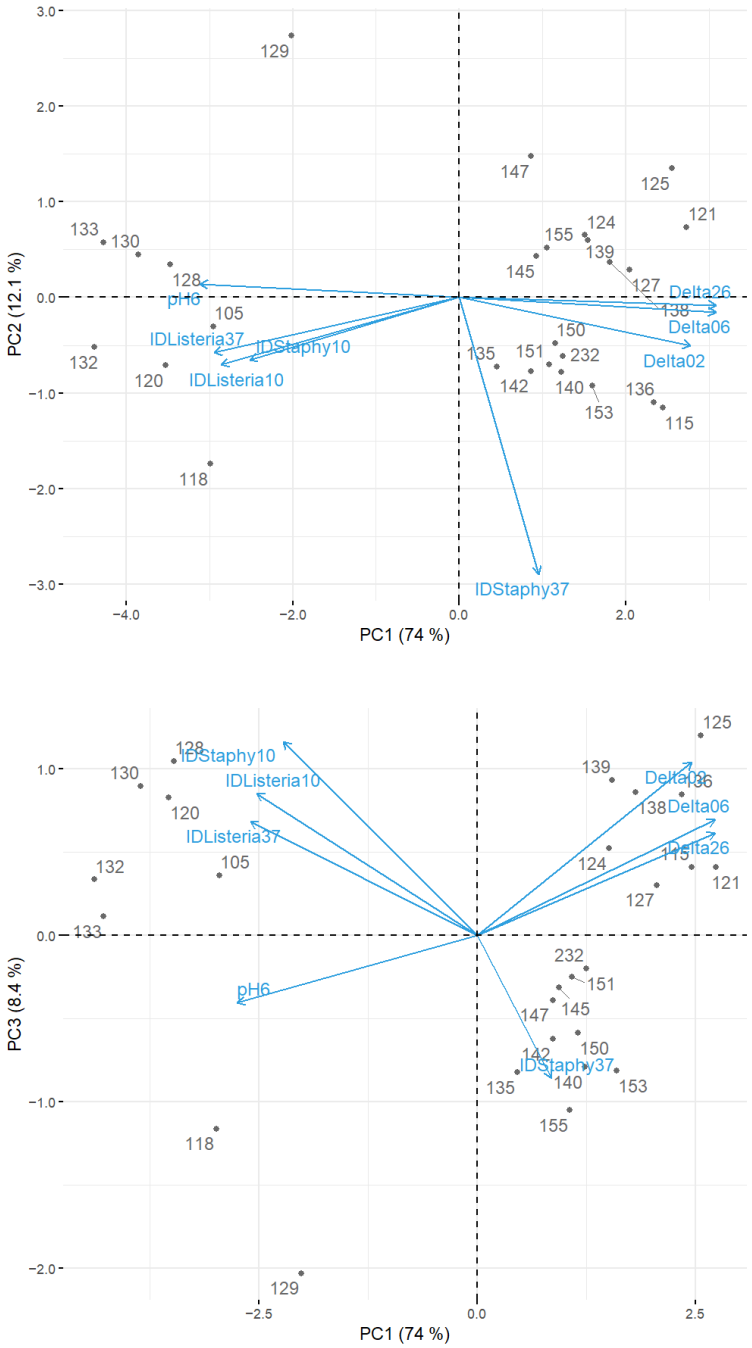

Figure 16. Maps of the first and second principal components (top) and the first and third principal components (bottom) of the tested technological properties of M17-isolated LAB. Legend: Delta02, Delta06, Delta26: pH decrease between t= 0h and t= 2h, t= 0h and t= 6h and t= 2h and t= 6h, respectively; pH6: pH value of milk broth after 6h at 30 °C; IDListeria37 and IDListeria10: diameter of inhibition (mm) of *L. monocytogenes* tested at 37 °C and 10 °C, respectively; IDStaphy37 and IDStaphy10: diameter of inhibition (mm) of *S. aureus* tested at 37 °C and 10 °C, respectively.

From **Table 33** (MRS subset), a total of 65% of the variability in the 9 attributes was jointly explained by the three principal components. The first component (PC1) explained 26% of the total variability and was highly correlated with pH decrease of milk broth between t = 2 h and t = 6 h (ΔpH_{26}, R = 0.89), and highly and inversely correlated with *S. aureus* inhibition at 37 °C (R = -0.56) and with milk broth pH value after 6 h (R = -0.77); in contrast, it was weakly correlated with milk broth pH decrease between 0 h and 2 h (R = -0.05). In this sense, PC1 indicates isolates with different inhibitory capacity against *S. aureus* at 37 °C and distinguishes the ability of isolates to promote a reduced pH value in milk broth after 2 h at 30 °C (**Figure 15**).

The second component (PC2) explained 21% of the data variability and presented high loadings on two pH-related variables: ΔpH_{02} (R = 0.96) and ΔpH_{06} (R = 0.83). For this reason, dissimilarities across the PC2 axis (**Figure 15**) indicate LAB with distinct acidification profiles, specifically between t = 0 h and t = 2 h (ΔpH_{02}), and t = 0 h and t = 6 h (ΔpH_{06}).

Finally, the third component (PC3) explained 18% of the total variability and was highly correlated with inhibition of *L. monocytogenes* at 10 °C and 37 °C (R = 0.64 and R = 0.65, respectively), and *S. aureus* at 10 °C and 37 °C (R = 0.68 and R = 0.48, respectively. Thus, PC3 reveals LAB isolates with distinctive antimicrobial capacities, namely against *L. monocytogenes* and *S. aureus* at 10 °C and 37 °C (**Figure 15**).

The properties of M17 isolates presented stronger relationships between variables than MRS ones, since higher total variability could be explained (95% in **Table 34**). PC1 explained most (45%) of the total variability and was highly correlated with the pH decrease of milk broth (R = -0.82, R = 0.91, R = 0.89 and R = 0.86 for pH_6, ΔpH_{02}, ΔpH_{06} and ΔpH_{26}, respectively). For this reason, PC1 provides insight on the isolates capacity to reduce the pH of milk broth after 6 h and their acidification profile between t = 0 h and t = 6 h (**Figure 16**).

PC2 and PC3 explained 13% and 37% of the total variability, respectively. The first was highly correlated with *S. aureus* inhibition at 37 °C (R = 0.99), whereas the second was well correlated with inhibition of *L. monocytogenes* at 10 °C and 37 °C (R = 0.86 and R = 0.83, respectively), as well as inhibition of *S. aureus* at 10 °C (R = 0.86) (**Figure 16**).

Further analysing the figures produced, from MRS isolates (**Figure 15**), clusters were not easily identified, hence suggesting isolates of similar antimicrobial capacity and technological characteristics. However, from **Figure 16**, two clusters of M17 isolates can be distinguished: one with greater acidification capacity and related to higher *S. aureus* inhibition at 37 °C; and another

with better antimicrobial activity against *S. aureus* (at 10 °C) and *L. monocytogenes* (at 10 °C and 37 °C).

The joint in-vitro information of the LAB isolates can be helpful in selecting a particular set with desirable characteristics to produce cheeses. More specifically, this PCA may assist in the design of a tailored starter culture that can offer antimicrobial protection against pathogens and assist in the development of pleasing aroma and flavour compounds in the product.

7.3.3 Principal Component Analysis: subset of isolates identified by 16S rRNA sequencing with promising antimicrobial and technological properties

Considering the results of the first PCA conducted, another subset of forty LAB isolates (twenty MRS-isolated and twenty M17-isolated) with promising technological properties was defined; and the molecular characterisation of such isolates was performed, with the goal of conducting a second PCA to appraise the relationship between genus and species and the antimicrobial, proteolytic and acidifying properties of the isolates.

The results of the pH_6 descriptor obtained from the fitted pH curve, and of the antimicrobial and proteolytic essays of MRS- and M17-isolated LAB, along with the corresponding identified species obtained by 16S rRNA sequencing are presented in **Table 35** and **Table 36**. **Figure 17** shows the variables loadings and observation scores for each pathogen, and the ellipses group strains by species and genus.

Overall, according to the BLAST results from the 16S rRNA sequencing, LAB from the genera *Lactococcus* and *Leuconostoc* were dominant (62.5%) among the subset of isolates with favourable technological capacities. *Lactococcus* and *Leuconostoc* were found in 35% and 27.5% of samples, respectively, whereas other bacteria of the genera *Lacticaseibacillus*, *Enterococcus, Loigolactobacillus* and *Lactobacillus* were less frequent (17.5%, 15%, 2.5% and 2.5% of the samples, respectively). At the species level, *Leuconostoc mesenteroides* was the most abundant organism (27.5%), followed by *Lactococcus lactis* (25%), *Lacticaseibacillus paracasei* (17.5%), *Enterococcus faecalis* (15%), *Lactococcus cremoris* (10%) and *Lactobacillus plantarum* and *Loigolactobacillus coryniformis* (2.5% each). Despite the promising technological properties observed, isolates confirmed as *Enterococcus faecalis* could not be considered for incorporation into foods, as this species does not have QPS status for being among the leading causes of community- and hospital-acquired (nosocomial) infections [8]. In fact, the detection of this species

in artisanal cheeses confirms its poor microbial quality, which motivated this study, as *Enterococcus faecalis* are present in the gastrointestinal tract of humans and animals.

Contrasting the results from **Table 35** and **Table 36**, and analysing the biplots of **Figure 17**, it is evident that strains belonging to the *Lactococcus* genus (M17-isolated) have the highest acidifying capacity, regardless of the species (*Lactococcus cremoris* and *Lactococcus lactis*), as suggested by the high correlation with the Delta06, Delta26 and Delta26 variables, and the negative correlation with the pH_6 variable. Among the *Lactococcus* strains, isolate 121 (*Lactococcus lactis*) and isolate 125 (*Lactococcus cremoris*) promote the greatest milk broth acidification, as evidenced by the lowest estimated pH value after 6 h among all isolates (5.28).

On the other hand, the genera *Leuconostoc* and *Lacticaseibacillus* (both MRS-isolated; **Table 35** and **Figure 17**) displayed important antimicrobial capacities, regardless of the temperature and pathogen tested, as suggested by the high correlation with the ID_37C and ID_10C variables.

In two of the biplots (those for *S*. Typhimurium and *S. aureus*), the genus *Enterococcus* (M17-isolated) did not reveal an explicit correlation with a particular property, considering the position of its strains close to the plot origin. The biplot for *L. monocytogenes*, however, suggests a greater association with antimicrobial capacities, as strains are clustered in the direction of the ID_37C and ID_10C arrows, yet away from the Delta02, Delta06 and Delta26 variables. This implies that *Enterococcus* strains present higher antimicrobial effect against *L. monocytogenes* than against *S*. Typhimurium or *S. aureus*, which can be confirmed by examining the inhibition diameters in **Table 36**.

Lactobacillus and *Loigolactobacillus* genera (both MRS-isolated) were composed of only one strain each (*Lactobacillus plantarum* and *Loigolactobacillus coryniformis*), so no ellipses could be modelled; and, therefore, no conclusion could be drawn regarding the capacities correlated with each of these genus. Nevertheless, the biplots indicate that both strains were associated with high proteolytic and antimicrobial activities, considering their placement along the horizontal axis. Since these two isolates (number 16 and 24) were the only ones collected that presented proteolytic capacity, as mentioned before in section 7.3.1, one may wonder if these genera are associated with such technological property.

Table 35. Antimicrobial, acidifying and proteolytic capacities of MRS-isolated LAB along with the corresponding identified genus and species.

Isolate	pH_6	IDListeria37	IDSalmo37	IDStaphy37	IDListeria10	IDSalmo10	IDStaphy10	Pact	Genus	Species
11	6.55	9.96	9.79	6.35	15.2	11.9	9.45	0	*Lacticaseibacillus*	*Lacticaseibacillus paracasei*
16	6.48	9.33	12.1	7.88	16.8	10.6	14.5	1.94	*Lactobacillus*	*Lactobacillus plantarum*
19	6.45	10.6	9.83	6.92	14.6	8.40	9.07	0	*Lacticaseibacillus*	*Lacticaseibacillus paracasei*
21	6.49	9.17	10.1	6.77	15.6	7.69	6.85	0	*Lacticaseibacillus*	*Lacticaseibacillus paracasei*
24	6.51	8.56	9.47	4.53	16.5	9.74	0.00	1.45	*Loigolactobacillus*	*Loigolactobacillus coryniformis*
25	6.49	8.95	9.86	5.79	19.6	8.94	7.93	0	*Lacticaseibacillus*	*Lacticaseibacillus paracasei*
28	6.46	4.84	8.91	3.60	12.0	6.82	2.32	0	*Leuconostoc*	*Leuconostoc mesenteroides*
31	6.49	10.9	10.2	6.17	9.28	6.69	10.3	0	*Lacticaseibacillus*	*Lacticaseibacillus paracasei*
57	6.40	9.18	7.72	5.35	25.0	8.98	5.44	0	*Lacticaseibacillus*	*Lacticaseibacillus paracasei*
63	6.36	8.14	7.87	5.25	17.9	10.5	6.26	0	*Leuconostoc*	*Leuconostoc mesenteroides*
65	6.40	8.92	6.66	4.50	18.6	9.83	1.95	0	*Leuconostoc*	*Leuconostoc mesenteroides*
67	6.37	8.63	8.72	2.72	16.2	6.49	2.54	0	*Leuconostoc*	*Leuconostoc mesenteroides*
69	6.36	8.95	8.01	4.07	14.7	6.96	2.36	0	*Leuconostoc*	*Leuconostoc mesenteroides*
70	6.42	8.88	7.81	3.65	17.1	6.14	2.33	0	*Leuconostoc*	*Leuconostoc mesenteroides*
73	6.46	10.5	7.63	4.19	15.9	7.19	9.75	0	*Leuconostoc*	*Leuconostoc mesenteroides*
84	6.46	7.30	7.64	7.45	14.4	6.62	7.49	0	*Leuconostoc*	*Leuconostoc mesenteroides*
92	6.49	7.01	8.25	4.21	17.4	5.60	4.79	0	*Leuconostoc*	*Leuconostoc mesenteroides*
94	6.46	8.98	8.57	4.34	18.7	9.96	8.16	0	*Leuconostoc*	*Leuconostoc mesenteroides*
99	6.47	5.36	8.39	3.65	16.7	4.70	6.71	0	*Leuconostoc*	*Leuconostoc mesenteroides*
240	6.59	12.4	6.80	5.94	13.5	7.21	11.6	0	*Lacticaseibacillus*	*Lacticaseibacillus paracasei*

pH_6: pH value of milk broth after 6h at 30 °C; IDListeria37 and IDListeria10: diameter of inhibition (mm) of *L. monocytogenes* tested at 37 °C and 10 °C, respectively; IDStaphy37 and IDStaphy10: diameter of inhibition (mm) of *S. aureus* tested at 37 °C and 10 °C, respectively; IDSalmo37 and IDSalmo10: diameter of inhibition (mm) of *Salmonella* Typhimurium tested at 37 °C and 10 °C, respectively; Pact: diameter of proteolytic activity (mm).

Table 36. Antimicrobial, acidifying and proteolytic capacities of M17-isolated LAB along with the corresponding identified genus and species.

Isolate	pH_6	IDListeria37	IDSalmo37	IDStaphy37	IDListeria10	IDSalmo10	IDStaphy10	PAct	Genus	Species
105	6.12	6.91	0.00	0.38	13.7	5.65	3.21	0	*Enterococcus*	*Enterococcus faecalis*
115	5.43	0.00	4.50	0.54	2.97	2.87	2.30	0	*Lactococcus*	*Lactococcus cremoris*
118	6.19	8.28	3.67	0.59	12.7	5.51	2.57	0	*Enterococcus*	*Enterococcus faecalis*
120	6.12	7.86	4.91	0.40	12.7	5.49	4.08	0	*Enterococcus*	*Enterococcus faecalis*
121	5.28	0.00	4.14	0.35	1.34	5.38	1.90	0	*Lactococcus*	*Lactococcus lactis*
124	5.42	0.74	3.95	0.34	1.85	5.47	2.60	0	*Lactococcus*	*Lactococcus lactis*
125	5.28	0.00	4.22	0.25	2.23	3.24	2.20	0	*Lactococcus*	*Lactococcus cremoris*
127	5.49	0.75	3.84	0.39	2.36	3.17	2.03	0	*Lactococcus*	*Lactococcus lactis*
128	6.04	7.54	4.33	0.28	13.4	4.30	3.84	0	*Enterococcus*	*Enterococcus faecalis*
132	6.26	7.70	3.91	0.39	12.4	9.37	4.25	0	*Enterococcus*	*Enterococcus faecalis*
133	6.28	7.85	4.58	0.28	13.9	6.36	3.39	0	*Enterococcus*	*Enterococcus faecalis*
135	5.69	0.00	0.00	0.53	2.36	4.17	2.88	0	*Lactococcus*	*Lactococcus cremoris*
136	5.42	0.00	2.43	0.52	2.24	4.61	2.83	0	*Lactococcus*	*Lactococcus lactis*
138	5.48	0.00	3.56	0.36	2.01	6.68	2.73	0	*Lactococcus*	*Lactococcus cremoris*
140	5.52	0.00	4.06	0.54	2.22	5.62	2.42	0	*Lactococcus*	*Lactococcus lactis*
150	5.55	0.38	3.28	0.50	2.00	3.78	2.48	0	*Lactococcus*	*Lactococcus lactis*
151	5.53	0.34	4.12	0.51	1.81	4.42	2.87	0	*Lactococcus*	*Lactococcus lactis*
153	5.50	0.53	2.88	0.56	1.68	4.48	2.18	0	*Lactococcus*	*Lactococcus lactis*
155	5.58	0.38	3.80	0.41	1.73	4.47	1.87	0	*Lactococcus*	*Lactococcus lactis*
232	5.51	0.00	0.00	0.50	2.38	5.84	2.75	0	*Lactococcus*	*Lactococcus lactis*

pH_6: pH value of milk broth after 6h at 30 °C; IDListeria37 and IDListeria10: diameter of inhibition (mm) of *L. monocytogenes* tested at 37 °C and 10 °C, respectively; IDStaphy37 and IDStaphy10: diameter of inhibition (mm) of *S. aureus* tested at 37 °C and 10 °C, respectively; IDSalmo37 and IDSalmo10: diameter of inhibition (mm) of *Salmonella* Typhimurium tested at 37 °C and 10 °C, respectively; PAct: diameter of proteolytic activity (mm).

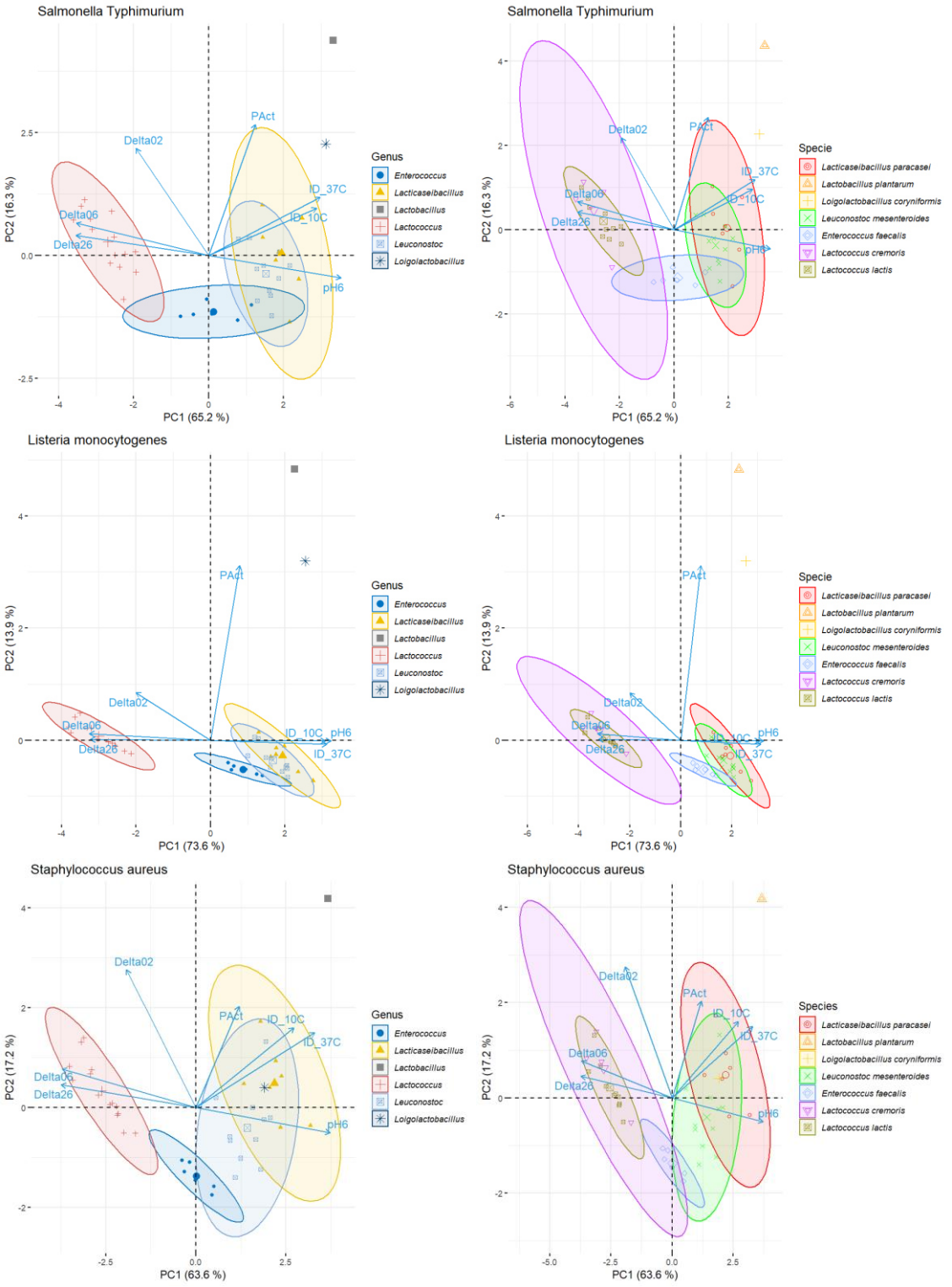

Figure 17. Maps of the first and second principal components of the tested properties of the subset of LAB. Legend: Delta02, Delta06, Delta26: pH decrease between t= 0h and t= 2h, t= 0h and t= 6h and t= 2h and t= 6h, respectively; pH6: pH value of milk broth after 6h at 30 °C; PAct: diameter of proteolytic activity (mm). For each pathogen: ID_10C and ID_37C: diameter of inhibition (mm) tested at 10 °C and 37 °C, respectively.

7.4 CONCLUSIONS

The detection of *Enterococcus faecalis* in artisanal goat's milk cheeses confirmed the importance of this study, whose main concern was related to the poor microbial quality of this product.

The genetic analysis of the isolates collected in this work showed a diverse lactic-acid-producing community, with various strains presenting antimicrobial activity against different pathogens, as well as acidifying and proteolytic capacities. The grouping of isolates by principal component analysis appeared to be useful for strain selection, based on advantageous characteristics for cheese production, and enabled to correlate the identified LAB genera with their main valuable property (antagonistic, acidifying and proteolytic). Overall, *Leuconostoc mesenteroides*, *Lactococcus lactis* and *Lacticaseibacillus paracasei* were the predominant organisms found in the subset of forty LAB with promising antimicrobial and acidifying properties selected from the initial 232 isolates collected.

The outcomes obtained indicate that application of indigenous LAB selected in this work, as a customised starter culture, may help prevent pathogen growth (biopreservation potential) and contribute to the proper acidification of milk during the cheese production process, thus promoting a stable microbiological environment and consequently improving the safety of this product.

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SECTION III

PREDICTIVE MODELLING

CHAPTER 8

THERMISATION TO IMPROVE THE SAFETY OF UNPASTEURISED MILK CHEESES

This chapter was based on the following submitted paper:

Silva, B.N.; Coelho-Fernandes; Teixeira, J.A.; Cadavez, V.; Gonzales-Barron, U. Modelling the kinetics of *Staphylococcus aureus* in goat's raw milk under different sub-pasteurisation temperatures. *Microb. Risk Anal.* (submitted)

8.1 INTRODUCTION

Artisanal cheeses are highly appreciated by consumers for their unique organoleptic properties, particularly their richness of taste, aroma and texture. They are frequently produced from goat and/or sheep raw milk due to tradition and the enhanced organoleptic properties attributed, at farm level, by small local dairies or by cheese industries working at regional level [1].

The use of raw milk in small-scale production plants, where the control of processing variables and of environmental parameters may be challenging, implies a potential risk of microbial contamination and growth [2]. Accordingly, *S. aureus* is among the main bacterial pathogens of interest concerning the safety of cheeses, particularly those made from raw milk [1,3,4]. The average incidence rates of *S. aureus* for goats' raw milk and goats' raw milk cheeses were estimated to be as high as 30-40% [1,5]. Moreover, several outbreaks attributed to milk and dairy products (including raw milk cheeses) caused by *S. aureus* have been reported in the past years, and raw milk cheeses have been involved in most of the outbreaks reported in relation with staphylococcal enterotoxins [6-8]. For these reasons, the presence of *S. aureus* in these products appears to remain a public health hazard [1].

To this, milk thermisation has been proposed as a strategy to improve the safety of cheeses made from unpasteurised milk [4, 9]. Thermisation is the generic description for a range of sub-pasteurisation (< 72 °C) heat treatments of milk prior to pasteurisation and/or cheese manufacture, generally from 57 to 68 °C, with a holding time of 5 seconds up to 30 minutes, which may promote a bacterial reduction of 3 to 4 log [9-14]. This milk treatment markedly reduces the number of spoilage bacteria, and, in the case of *S. aureus*, the log reduction is such that toxin formation in the cheese, which requires a microorganism count greater than 5 log CFU/g, is highly unlikely [13]. Simultaneously, thermisation causes minimum collateral heat damage to milk constituents and milk renneting properties, mild effect on the raw milk flora and the functionality of milk caseins and salts, and reduced impact on the sensory profile of the final cheeses [11-13,15,16]. For example, since the heat load is lower compared to that used in pasteurisation, enzymes involved in cheese flavour development, such as lipoprotein lipase, are less inactivated, thus avoiding changes in ripening and in aroma and flavour improvement of the cheese [13]. Pasteurisation of milk, on the other hand, modifies the biochemistry and the microbiology of ripening to a greater extent, as well as the flavour and texture of the cheese. This does not allow

for the characteristic and desirable special features of raw milk cheeses to emerge; thus, making this heat treatment inappropriate for such a product, unlike thermisation [17].

To our knowledge, there is no literature available describing the effects of thermisation against *S. aureus* in goats' raw milk. Only one study has reported on *S. aureus* populations after thermisation, but in a composite raw milk consisting of 90% ewes' and 10% goats' milk [16]. As a result, the temperature and time combinations needed to enhance the safety of goats' raw milk and reduce *S. aureus* counts are not characterised.

For this, a range of mathematical models can be used to estimate kinetic parameters from constant-temperature inactivation experiments, where microbial counts are modelled as a function of time (primary models). If properly formulated and validated, in conjunction with secondary models (which describe the effects of environmental factors, such as temperature, on the primary model parameters), these models facilitate prediction of the effects of a treatment regime and can be used for the design of thermal inactivation processes [18].

Frequently, primary and secondary models are fitted sequentially (two-step modelling) [19]. However, using a mixed-effects nonlinear regression approach (also known as omnibus or global modelling), a full dataset covering all experimental conditions can be modelled at once, fitting the primary and secondary models simultaneously [19-22]. The omnibus method has advantages compared to the two-step modelling as there is no loss of information associated with the uncertainty of the primary model kinetic parameters, and random effects can be used to account for the variability in parameters that environmental conditions may not explain [19].

In this context, the aim of our research was to characterise the heat resistance of *S. aureus* in goats' raw milk at sub-pasteurisation temperatures and to compare the standard two-step modelling approach with the omnibus modelling.

Through these models, it was possible to estimate the significant inactivation parameters and to determine the heat resistance of *S. aureus* at various temperatures, information that is valuable and can be employed to derive time/temperature tables to reach target *S. aureus* log reductions, which can be used by artisanal cheesemakers and improve the microbiological safety of cheeses made from unpasteurised milk.

8.2 MATERIALS AND METHODS

8.2.1 Inoculum preparation

Staphylococcus aureus ATCC 6538, obtained from the Polytechnic Institute of Bragança stock collection, was used. A loop of culture kept on Nutrient Agar slant was inoculated in 10 mL of Mueller Hinton broth (Ref. 4017412, Biolife, Italia). Broth tubes were incubated at 37 °C for 24 h, to achieve a concentration of approximately 8 log CFU/mL, verified by measurement of the absorbance at 600 nm using a spectrophotometer (Peak Instruments Inc., Version 1701).

8.2.2 Sample inoculation and heat treatment

The heat treatment trials were performed as described by Engstrom et al. [4] with some modifications. Fifty mL of raw goat's milk was pipetted into a sterile centrifuge tube and inoculated at 1% (v/v) with *S. aureus* to yield approximately 7 log CFU/mL. After vortexing, five mL aliquots of inoculated milk was pipetted into sterile sample bags, which were flattened to a uniform thickness.

Sample bags were then attached to a sampling rack to ensure their even distribution within a water bath and to allow for simultaneous and efficient immersion. The sampling rack was submerged in a stirred water bath (Clifton Range, United Kingdom) heated to 55 °C, 56.5 °C, 58 °C, 61 °C, 62.5 °C and 64 °C, and samples were removed at six appropriate pre-defined time intervals. At each sampling point, sample bags were removed and promptly immersed into an ice bath to reach approximately 15 °C. Chilled sample bags were removed from the ice bath, dried, and sanitised (on the outside) with ethanol 70% (v/v) before opening.

Determination of *S. aureus* counts was then performed. For every treatment, two runs were conducted.

8.2.3 Quantification of *S. aureus*

For every test unit, appropriate serial dilutions were prepared by homogenising the heat-treated milk in 45 mL of buffered peptone water (Ref. 414944.1210, PanReac AppliChem, Spain) for 30 seconds in a stomacher (BagMixer 400, Interscience, France). To determine *S. aureus* concentration, 0.1-mL aliquot of the dilutions was plated on Baird-Parker agar (Ref. 4011162, Biolife, Italy), supplemented with Egg Yolk Tellurite (Ref. FD046-100MLX5VL, HiMedia, India),

following ISO 6888-1:2001 [23]. Typical colonies were counted after 48 h after incubation at 37 °C. Microbiological determinations were done in duplicate.

8.2.4 Statistical analysis

The statistical analyses described below were performed in R software (version 4.1.0, R Foundation for Statistical Computing, Vienna, Austria) using the nlme and stats packages.

8.2.4.1 Two-step modelling approach

Primary model. For the survival curves obtained at 55 °C, 58 °C, 61 °C, 62.5 °C and 64 °C, *S. aureus* behaviour was modelled using the three-parameter Weibull equation as the primary model (i.e., a model describing microbial concentration as a function of time), defined as:

$$Y(t) = Y_0 - \left(\frac{t}{\chi}\right)^{\beta} \quad (1)$$

where Y_0 and $Y(t)$ represent the logarithms of microbial concentrations (log CFU/mL) at an initial time point (t = 0) and actual time t minutes, respectively; and χ and β are the scale and shape parameters of the underlying Weibull distribution, respectively. The scale parameter χ indicates the time for first decimal reduction (minutes), whereas the shape parameter accounts for upward concavity of a survival curve (β < 1), a linear survival curve (β = 1), or a downward concavity (β > 1) [24]. After separately fitting the Weibull primary model to each of the survival curves, the parameters Y_0, χ and β were extracted. To ensure that the estimated χ and β were positive, natural logarithmic transformations of those parameters were used for the fitting.

Secondary model. Since the survival experiments were conducted under different temperatures, secondary models (i.e., models describing one or more parameters of a primary model as a function of an intrinsic or extrinsic variable) were developed to assess the effects of temperature on χ and β.

Initially, the four estimates of the scale and shape parameters from the three-parameter Weibull models underwent a square root transformation ($\sqrt{\chi}$ and $\sqrt{\beta}$), to reduce heteroscedasticity. Then, the transformed estimates were plotted against the corresponding temperature, and two polynomial equations were adjusted to describe $\sqrt{\chi}$ and $\sqrt{\beta}$ as a function of temperature, as follows:

$$\sqrt{\chi} = a_1 + a_2 * Temperature + a_3 * Temperature^2 \quad (2)$$

$$\sqrt{\beta} = b_1 + b_2 * Temperature + b_3 * Temperature^2 \quad (3)$$

Model validation: The model was validated by parametric bootstrapping (1000 iterations) [25], comparing the set of experimental data collected at 56.5 °C with the predicted survival curve obtained by fitting the primary and secondary models at that temperature. For the bootstrapping, it was assumed that the residuals of the model follow a normal distribution with mean 0 and standard deviation 0.360 (calculated from the square root of the residual sum of squares). The confidence intervals were calculated at a significance level of α=0.05. For the evaluation of the performance of the model, two statistical internal validation indices were calculated from the observed and predicted values: the bias factor (Bf) and the accuracy factor (Af) [26].

8.2.4.2 Global modelling approach: omnibus model

An omnibus model is one that fits the primary and secondary models simultaneously, using all the data from the experimental curves and jointly estimating the parameters of both models [20-22].

The polynomial equations in Equation (2) and (3) were selected to be added to the omnibus model considering that the parameters of the Weibull model could be expressed as a function of the temperature, as shown by the previous stepwise-regressions tested, and that the goodness-of-fit measures (Akaike Information Criterion and Bayesian Information Criterion) and the behaviour of the residuals improved when using such equations for the global modelling approach.

The log CFU/mL concentration measured at time *i* when subjected to condition *j* was estimated as:

$$Y_{ij} = Y_{0_j} - \left(\frac{t}{\chi_j}\right)^{\beta_j} + \varepsilon_{ij} \quad (4)$$

$$Y_{0_j} = Y_{0\,mean} + u_j$$

$$\sqrt{\chi_j} = a_1 + a_2 * Temperature + a_3 * Temperature^2 + v_j$$

$$\sqrt{\beta} = b_1 + b_2 * Temperature + b_3 * Temperature^2$$

Since the initial microbial concentration Y_0 was different between conditions, this variability was accounted for by adding a random-effects term *u*. Another random-effects term *v* was added

to the mean of the intercept a_1 of the polynomial expression predicting $\sqrt{\chi}$. This was done because a fraction of the variability in the scale parameter could not be explained solely by its fixed-effect predictor. Hence, the random effects *u* and *v* were assumed to take in random shifts subject to a given condition *j* defined by the inactivation temperature. The two random effects were assumed to follow normal distributions with means zero and covariance matrix $[s_u^2, s_{uv}^2; s_{uv}^2, s_v^2]$. The residual error ε_{ij} followed a normal distribution with mean zero and variance s^2. Other mixed-effects models were assessed, but the model of Equation 4 fitted significantly better than the others and was the most parsimonious, therefore, it is the only one presented here.

Model validation. The model was validated by the "leave-one-out" method, also known as "internal validation", as it uses part of the data set [20,27]. Briefly, the procedure consisted of selecting and removing the inactivation data of one specific temperature (including the two replicates) from the full data set. Then, Equation (4) was re-fitted to the remaining data; and, using the new model parameters, the mean bacterial concentrations and confidence intervals along time were estimated for the specific temperature that was removed. Such predicted inactivation curve was then compared with the experimental values. This was repeated three times, each for one of the following temperatures: 58 °C, 61 °C and 62.5 °C. As for the two-step approach, the performance of the omnibus model was evaluated by calculating the bias and accuracy factors.

8.3 RESULTS AND DISCUSSION

8.3.1 Two-step modelling approach

Primary modelling. In the present study, *S. aureus* survival curves did not follow first-order kinetics (**Figure 18**). Instead, the inactivation curves presented either a downward concavity (55 °C and 64°C), an upward concavity (61 °C) or a sigmoid shape (58 °C and 62.5 °C), which may be due to the presence of subpopulations that differ in heat resistance, bacterial clumps [28,29], vital cellular component that are being destroyed before inactivation starts [30], among other explanations.

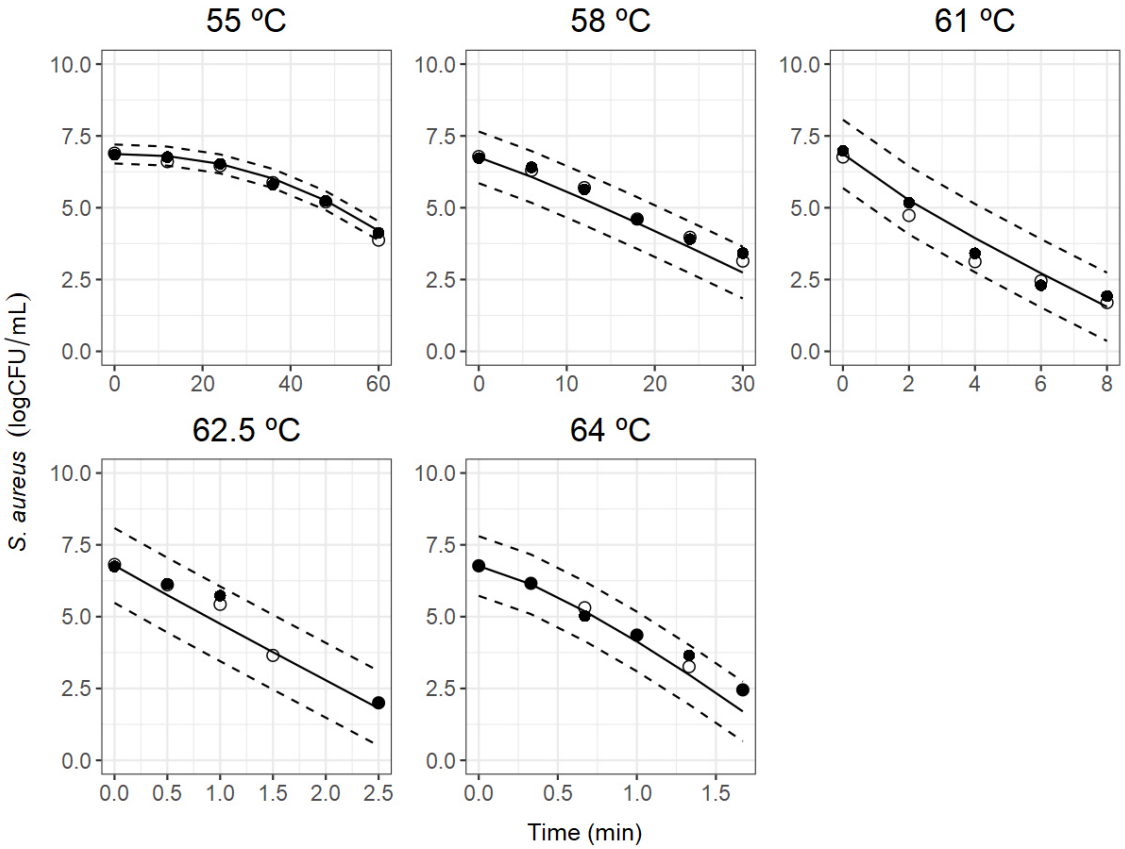

Figure 18. *S. aureus* experimental observations (markers), mean predicted values (full line) and 95% confidence intervals (dashed lines), as obtained by the two-step modelling approach, in goats' raw milk heated at 55, 58, 61, 62.5 and 64 °C over time. For each temperature, same markers represent observations from the same experiment.

Since the primary model selected had to be flexible to portray the various shapes observed in this study and considering that the Weibull model can be used to describe nonlinear survival curves and may be helpful to pinpoint relevant physiological effects caused by heating [24], the three-parameter Weibull equation was considered adequate and representative of all the survival curves.

Table 37 compiles the means and standard errors of the parameters of the Weibull equation fitted separately to each of the thermisation temperatures tested.

Table 37. Kinetic parameters of the Weibull decay model describing S. aureus behaviour in goats' raw milk heated at different thermisation temperatures (°C)

Thermisation temperature (°C)	Y_0 [1]	χ [2]	β [3]
55	6.819 ± 0.054 *	37.92 ± 1.036 *	2.241 ± 0.221 *
58	6.821 ± 0.006 *	9.703 ± 0.301 *	1.145 ± 0.058 ns
61	6.907 ± 0.169 *	0.573 ± 0.076 *	0.630 ± 0.041 ns
62.5	6.769 ± 0.161 *	0.791 ± 0.274 *	1.385 ± 0.371 ns
64	6.786 ± 0.019 *	0.455 ± 0.012 *	1.127 ± 0.048 ns

[1] Y_0: initial counts (log CFU/mL); [2] χ: scale parameter (minute); [3] β: shape parameter (dimensionless) (these parameters were expressed as means and standard error). Asterisks (*) represent the significance of the estimated parameter at $p < 0.05$; ns: non-significant ($p > 0.05$).

S. aureus initial concentrations (Y_0) were significant ($p < 0.05$), and a fast decline in their numbers with increasing thermisation temperature was observed, as suggested by the decreasing χ values (results for 61 °C are against this trend, as the shape of the curve is clearly different). These indicate smaller times for the first decimal reduction as temperature rises: for example, at 55 °C, the time needed for one log reduction is around 38 minutes, whereas at 64 °C approximately 27 seconds achieve the same decrease.

According to the shape parameter (β) of the Weibull model, temperatures of 55 °C, 58 °C and 61 °C progressively caused more damage and stress to *S. aureus*, as revealed by the decrease in β values (2.241, 1.145 and 0.630, respectively). However, at 62.5 °C and 64 °C, the shape parameters presented higher values (1.385 and 1.127) than at 61 °C, contrasting with the descending trend.

Even though the Weibull model is empirical, the value of β can be somewhat associated with the physiological effects of the heat treatment on the bacterial cells [24]. According to Van Boekel et al. [24], $\beta < 1$ suggests cell adaptation and $\beta > 1$ alludes to accumulated cell damage. In this sense, our results suggest that, at any point in the inactivation curve, the surviving bacteria become increasingly heat-susceptible in all of the temperature-specific experimental curves, although at 61 °C this behaviour was less evident.

Secondary modelling. The results from **Table 37** show that temperature has an impact on the inactivation kinetics of *S. aureus*. For this reason, the relationships between the transformed parameters $\sqrt{\chi}$ and $\sqrt{\beta}$ of the primary model and the thermisation temperatures were explored by scatter plots and, subsequently, by separate stepwise regression analyses (i.e., secondary models). The resulting polynomial secondary models predicting $\sqrt{\chi}$ and $\sqrt{\beta}$ as a function of temperature are displayed in **Figure 19** and the parameters are presented in **Table 38.**

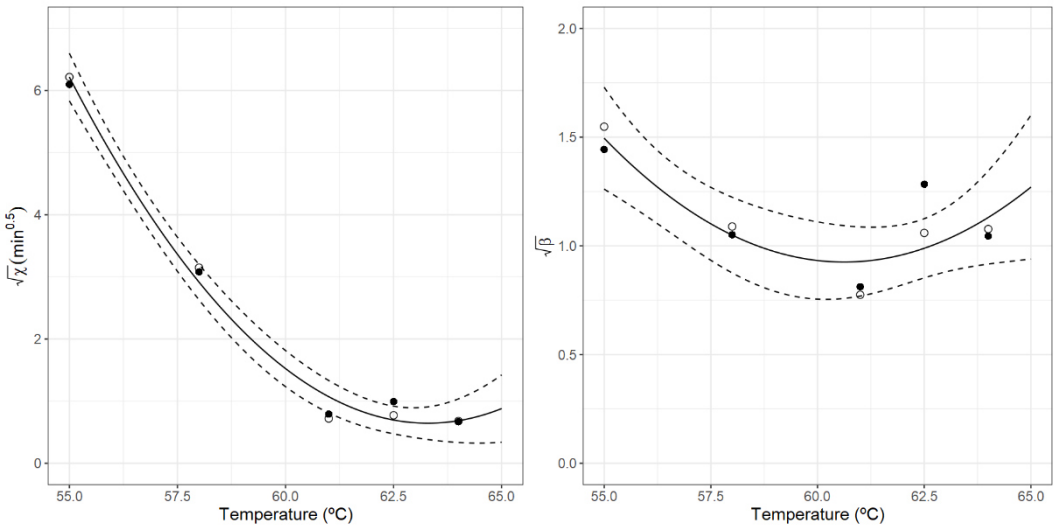

Figure 19. Mean (full line) and 95% confidence intervals (dashed lines) of the effect of temperature (°C) on the square root transformed scale parameter χ (left) and shape parameter β (right). For each temperature, different markers represent χ values obtained from different experiments.

Table 38. Parameter estimates of the polynomial secondary models predicting the square root transformed parameters χ and β in goats' raw milk as a function of temperature (°C).

Parameters	Mean	Standard error	Pr > |t|	AIC/BIC
Predictors of $\sqrt{\chi}$ ($min^{0.5}$)				
a_1 (Intercept)	325.0	32.58	<0.0001	
a_2 (Temperature)	-10.25	1.099	<0.0001	3.97/5.18
a_3 (Temperature^2)	0.081	0.009	<0.0001	
Variance				
s^2 (residual)	0.043		Adj. R^2	0.991
Predictors of $\sqrt{\beta}$				
b_1 (Intercept)	67.09	19.91	0.0119	
b_2 (Temperature)	-2.183	0.671	0.0140	-5.88/–4.67
b_3 (Temperature^2)	0.018	0.006	0.0153	
Variance				
s^2 (residual)	0.016		Adj. R^2	0.732

The negative linear effects of temperature (-10.25, $p < 0.0001$ for $\sqrt{\chi}$; -2.183, $p = 0.0140$ for $\sqrt{\beta}$) were anticipated since higher temperatures should lead to shorter inactivation times. In both models, the intercept estimates were positive, alluding to the concavity of the fitting and the quadratic effect of temperature on $\sqrt{\chi}$ ($p < 0.0001$; Table 2) and on $\sqrt{\beta}$ ($p = 0.0119$; Table 2) which means that the effect of temperature is not constant and that the change in $\sqrt{\chi}$ and $\sqrt{\beta}$ depends on its value.

The fitting capacity of both secondary models was reasonable (Adj. $R^2 > 0.73$), thus supporting their robustness. For both models, to further assess the quality of the fitting, the relationship between residuals and predicted values was assessed through scatter plots, which showed that the spread of the residuals over the fitted values was randomly distributed around the zero of the horizontal axis (plots not shown). Such results additionally corroborated the fitting quality of the models.

Model validation. The inactivation curve displayed in **Figure 20** was obtained by iteratively (N=10000) calculating the values of χ and β for the temperature of 56.5 °C using the polynomial

secondary models, considering that χ and β parameters follow a normal distribution with zero mean and constant variance, and placing such estimates of χ and β on the Weibull equation to obtain predicted *S. aureus* counts. From this iteration process, confidence intervals and predictions intervals could also be calculated and are presented in **Figure 20**.

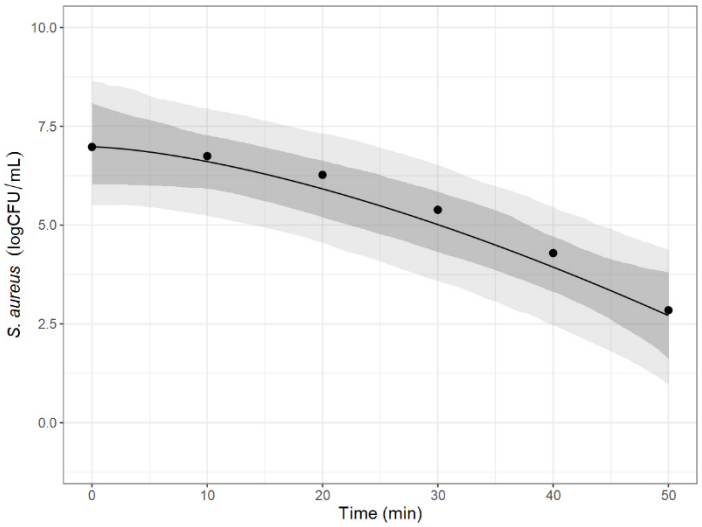

Figure 20. Mean (full line), 95% confidence intervals (dark grey) and 95% prediction intervals (light grey) of the concentration of *S. aureus* in goats' raw milk treated at 56.5 °C against time, as predicted by the two-step modelling approach. For each time point, a marker represents the mean of two replicates.

From this bootstrapping approach, using both primary and secondary models, it was possible to adequately describe the inactivation curve for the temperature of 56.5 °C, considering that it provided a good coverage of the experimental data points (all the observations are well within the 95% prediction bands), as shown in **Figure 20**.

The agreement between the predicted survival curve and the observed data was also verified by calculating the accuracy factor, Af = 1.12, and bias factor, Bf = 0.90. The Af is a measure of average deviation that indicates the spread of the results about the predictions [26] and, in this case, the Af value suggests that, on average, predictions are 1.12 factors of difference with respect to observations. The Bf, in turn, is a measure of the agreement between the predictions made by the model and the actual observations [26]. In this case, the Bf value suggests that the model may tend to underestimate the microbial concentrations by approximately 9%, and, for that reason, may be deemed as "fail-dangerous".

8.3.2 Global modelling approach: omnibus model

Omnibus model. The final omnibus model presented a total of ten parameters (Equation 4), from which seven were fixed effects or predictors of $\sqrt{\chi}$, $\sqrt{\beta}$ and $Y_{0\,mean}$, and two were variances of the random effects and the residual error. This global approach allowed to described well all the inactivation curves. **Table 39** compiles the parameter estimates for the omnibus model.

Table 39. Parameter estimates of the mixed-effects omnibus model predicting the non-log-linear decay of *S. aureus* in goats' raw milk as a function of temperature (°C).

Parameters	Mean	Standard error	Pr > |t|	AIC/BIC
Predictors of $\sqrt{\chi}$ ($min^{0.5}$)				
a_1 (Intercept)	343.6	14.87	0	
a_2 (Temperature)	-10.87	0.486	0	97.9/121
a_3 ($Temperature^2$)	0.086	0.004	0	
Predictors of $\sqrt{\beta}$				
b_1 (Intercept)	74.32	9.293	0	
b_2 (Temperature)	-2.427	0.310	0	
b_3 ($Temperature^2$)	0.020	0.003	0	
$Y_{0\,mean}$	6.888	0.088	0	
Variances				
s_u^2 ($Y_{0\,mean}$)	0.041			
s_v^2 (a_1)	1.023×10^{-20}			
s^2 (residual)	6.873×10^{-2}			

The positive intercepts a_1 and b_1, and the negative linear effects of temperature on $\sqrt{\chi}$ and $\sqrt{\beta}$ (reflected by a_2 and b_2) observed in the two-step modelling approach were also observed in the omnibus model. Analysing the standard errors of the predictors of $\sqrt{\chi}$ and $\sqrt{\beta}$, it can be stated that the omnibus model reduced the error associated with each parameter, when comparing with the standard errors obtained by the two-step modelling approach (**Table 38**). These results indicate that, by simultaneously fitting both primary and secondary models, this global approach minimises the error propagation that occurs when using the two-step methodology and, thus,

improves parameter estimation. With regards to the model's random effects, the two variances s_u^2 and s_v^2 were significant ($p < 0.05$). The correlation coefficient between the intercept of the linear predictor of $\sqrt{\chi}$, a_1, and the intercept of the linear predictor of $\sqrt{\beta}$, b_1, was 0.779, an observation that could not be made by fitting the primary and secondary models separately. In a Weibull model, a high correlation between its parameters may be anticipated as changes in $\sqrt{\chi}$ are normally compensated by changes in $\sqrt{\beta}$. This correlation has also been observed by other authors [21,24,31].

Model validation. The omnibus model was successfully validated using the leave-one-out method. Predictions for three temperatures (58, 61 and 62.5 °C) are shown in **Figure 21**, and, in all cases, the plots reveal a good agreement between the predicted survival curve for each temperature and its observed data (i.e., all the observations lay well within the 95% confidence bands).

Such agreement was further supported by the bias factors (0.916, 1.365 and 0.639) and accuracy factors (1.112, 1.365 and 1.595) of each model fitted (58, 61 and 62.5 °C, respectively). These bias factors suggest that the ability of the model to accurately estimate the microbial concentrations is dependent on the temperature for which the predictions are made. It is noticeable that as the model is adjusted for temperatures closer to the limits of the range of temperatures tested, it becomes less capable of accurately making predictions, and instead, overestimates (61 °C) or underestimates (62.5 °C) the microbial concentrations. The accuracy factors, in its turn, suggest that, on average, predictions are 1.1 to 1.6 times the value of observations, with increased discrepancy between observed and predicted values at higher temperatures.

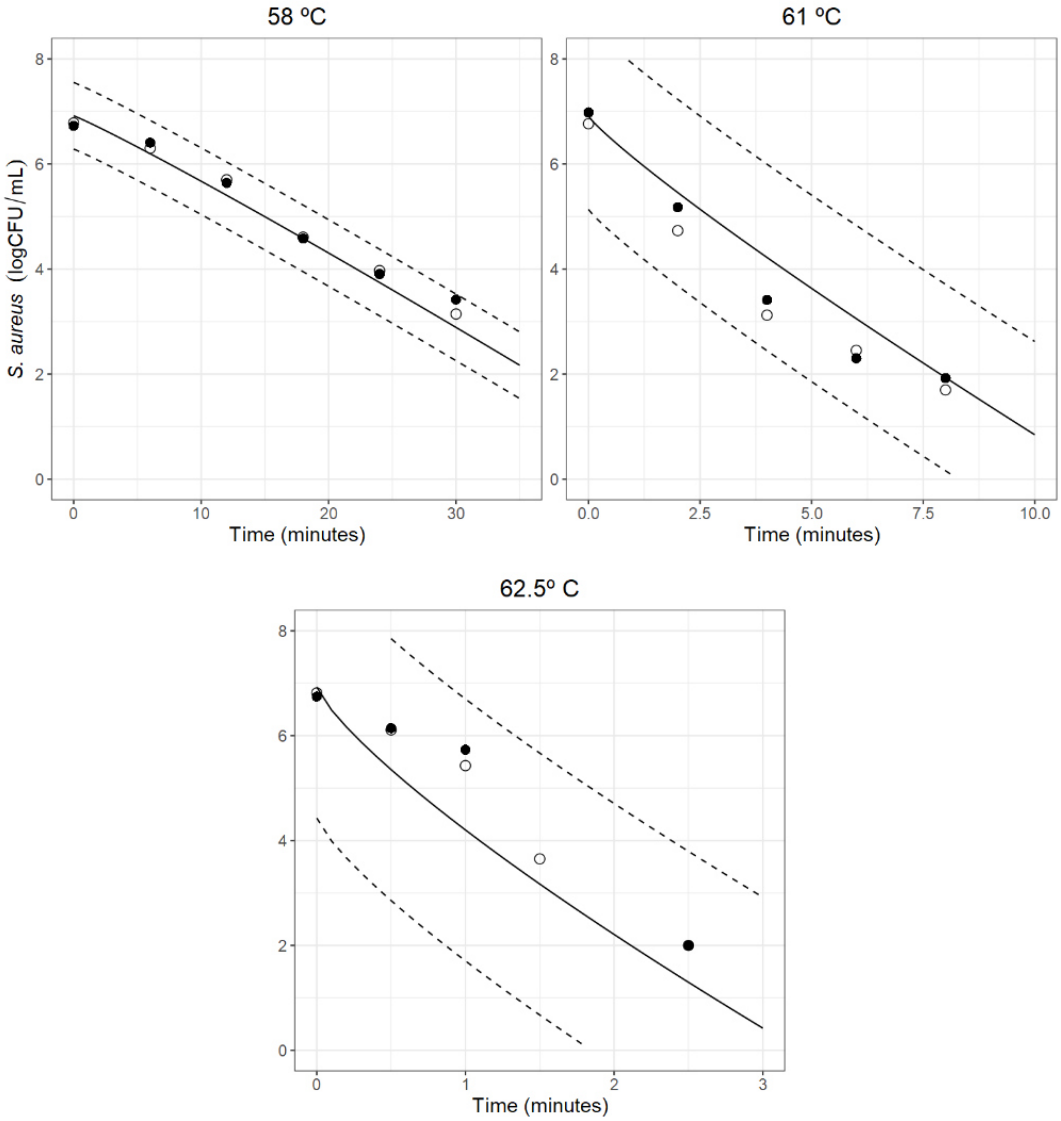

Figure 21. Mean (full line) and 95% confidence intervals (dashed lines) of the concentration of *S. aureus* in goats' raw milk against time, as predicted by the omnibus model refitted leaving out a randomly-selected temperature one at a time. Model validation for temperatures 58 °C (top left), 61 °C (top right) and 62.5 °C (bottom) is shown. For each temperature, same markers represent observations from the same experiment.

8.4 DISCUSSION

Comparing the estimates of **Table 38** and **Table 39**, there was general agreement between the outcomes of the secondary and the omnibus models. Nonetheless, our work shows that the omnibus approach is better at avoiding loss of information and error propagation, as occurs with the two-step method, which is reflected in the lower standard errors associated with the model

estimates. Moreover, the global approach allows to identify potential systematic errors in a dataset from one environmental condition and to explore them through an appropriate choice of fixed and random effects incorporated in the model [19].

To our knowledge, this is the first work using modelling to obtain *S. aureus* kinetics in goats' raw milk at different sub-pasteurisation temperatures, and it contributes to the body of work using predictive microbiology to describe pathogen heat-inactivation in milk, which is scarce, particularly if pasteurisation studies are disregarded. Lehotová et al. [32] studied the heat resistance of *S. aureus* in the 57–61 °C temperature range using the capillary method and broth containing glucose, tryptone and yeast extract. Then, the authors modelled the bacterial survival and estimated the fourth decimal reduction time t_{4D} and z-values through log-linear Bigelow and non-linear Weibull models. Although these models are useful, their accuracy to predict the real behaviour of bacteria in foods may be questioned, as using experimental data from homogeneously well-mixed broth media implies disregarding the food microstructure and composition [33]. For this reason, in our work, raw milk was chosen over broth media, aiming to produce meaningful estimates that may be used in real life applications.

Other authors have reported on the effects of heat treatments for pathogen inactivation using raw milk instead of broth media, but usually the results are conveyed as a comparison of microbial populations before and after the treatment, or as decimal reduction values (D-values). Samelis et al. [16], for example, observed an effective reduction of coagulase-positive staphylococci, from 3.3 log CFU/mL to < 2 log CFU/mL, when applying thermisation treatments of 60 °C and 67 °C for 30 seconds to a mixture of ewe's and goat's milk (90:10). Zottola et al. [34] applied sub-pasteurisation treatments of 147 to 150 °F (63.8 to 65.6 °C) for 16 to 21 seconds to raw milk, and reduced *S. aureus* concentration to such an extent that the pathogen was undetected. In turn, Engstrom et al. (2021) [4] determined and validated D-values for *L. monocytogenes* and STEC in raw milk at thermisation temperatures of 65.6, 62.8 and 60.0 °C (also at 57.2°C for *L. monocytogenes* only). The results from such studies are also valuable and validate the usefulness of thermal treatments for pathogen control and improved food safety. However, they do not enable predictions nor interpolations for other temperatures, which is an advantage of using predictive modelling.

8.5 CONCLUSIONS

The present study estimated the inactivation parameters of *S. aureus* in goats' raw milk at several thermisation temperatures using the Weibull model in two distinct approaches: two-step modelling vs. omnibus modelling.

The results showed that the temperature influenced the time needed for the first decimal reduction, as expected, but also that it affects pathogen adaptability in goats' raw milk, as suggested by the different values of the shape parameter β. A quadratic relationship was found between each of the parameters of the Weibull model and the temperature, meaning that the effect of temperature is not constant over the range tested.

Validation of the models produced at temperatures within the models' domain was performed successfully, demonstrating their aptitude to predict inactivation kinetics of *S. aureus* in goats' raw milk. Nonetheless, the omnibus approach showed improved parameter estimation, considering the reduced standard errors associated, and revealed its value as a complementary approach to the traditional two-step modelling by enabling further exploration and insight of the experimental inactivation data.

The models described in this work can be used to design lethality treatments to achieve specific reductions of *S. aureus* in goats' raw milk, thus contributing to the enhancement of the microbiological quality and safety of raw milk cheeses.

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CHAPTER 9

DYNAMIC MODELLING TO DESCRIBE THE EFFECT OF PLANT EXTRACTS AND CUSTOMISED STARTER CULTURE ON THE SURVIVAL OF *S. AUREUS* IN GOAT'S RAW MILK SOFT CHEESES

This chapter was based on the following papers:

Silva, B.N.; Coelho-Fernandes, S.; Teixeira, J.A.; Cadavez, V.; Gonzales-Barron, U. Effect of Lemon Balm and Spearmint Extracts on the Survival of *S. aureus* in Goat's Raw Milk Cheese. *Biol. Life Sci. Forum* 2022, *18*, 15. https://doi.org/10.3390/Foods2022-12996

Silva, B.N.; Coelho-Fernandes, S.; Teixeira, J.A.; Cadavez, V.; Gonzales-Barron, U. Dynamic modelling to describe the effect of plant extracts and customised starter culture on the survival of *S. aureus* in goat's raw milk soft cheeses. *Foods* (submitted)

9.1 INTRODUCTION

The presence of *S. aureus* in milk and cheeses has been documented several times [1-6] and multiple dairy-related outbreaks have been linked to this pathogen [7-10]. Its occurrence is generally associated with subclinical or clinical mastitis in dairy cattle, which contaminates the milk [11], and with the lack of appropriate hygienic measures during cheese production, as *S. aureus* can be found on human skin or mucosa, nostrils, pharynx, in hair, and in gastrointestinal and urogenital tracts [12], even though water, milking equipment and the environment are other sources of contamination [1]. Contamination of milk with *S. aureus* is particularly relevant in the case of cheeses produced with raw milk, since there is no pathogen inactivation step such as pasteurisation, although *S. aureus* may also be found in pasteurised milk cheeses if unhygienic practices lead to the recontamination of thermally treated milk [12].

Various plant extracts have been added to cheeses and other dairy products as biopreservative agents, considering their antimicrobial capacities: Mohamed et al. successfully tested the use of *Moringa oleifera* leaves extract as an antimicrobial agent in cream cheese against several pathogens [13]; Carvalho et al. [14] used *Thymus mastichina* extracts to inhibit *Staphylococcus* spp. and *Enterobacteriaceae* in raw milk cheeses; and Shan et al. [15] investigated the antibacterial efficiency of cinnamon stick, oregano, clove, pomegranate peel, and grape seed extracts against *Listeria monocytogenes*, *Staphylococcus aureus*, and *Salmonella* enterica in cheese, reporting that all five extracts were active against the pathogens.

Intentionally added lactic acid bacteria (LAB) with known antimicrobial activity may be another strategy to improve the safety of milk and cheeses, and has also been previously tested by other researchers: for example, Gonzales-Barron et al. [16] observed a significant decline of *L. monocytogenes* during the ripening of artisanal Minas semi-hard cheese with addition of a cocktail of LAB strains with anti-listerial activity; Le Marc et al. [17] validated a commercial starter culture (Fresco 1010, Chr. Hansen, Hørsholm, Denmark) as effective to inhibit *S. aureus* growth during milk fermentation; and Alomar et al. [18] co-cultured, separately, *Lactococcus* and *Enterococcus*

strains with *S. aureus*, and the pathogen growth was inhibited after 6 h of incubation in microfiltered milk.

Nevertheless, only the use of LAB that belong to the genera *Lactococcus*, *Lactobacillus*, *Leuconostoc*, *Pediococcus*, and some *Streptococcus* is approved by the European Food Safety Authority (EFSA), which established its Qualified Presumption of Safety (QPS) status that indicates that there is reasonable evidence that such microorganisms do not raise safety concerns [19]. *Enterococcus* genus and some *Streptococcus* species, however, can be pathogenic and present virulence factors and resistance to a variety of antibiotics, and for that reason, they do not have QPS status [19].

Previous investigation from our research group [20,21] has demonstrated the in vitro antimicrobial capacity of lemon balm, spearmint and sage extracts against *S. aureus* by determination of their minimum inhibitory concentration (MIC) and suggested their potential to be incorporated in foods as preservatives against microbial spoilage. More specifically, hydroethanolic (70% (v/v)) lemon balm extract obtained by solid-liquid extraction presented a MIC of 2.5 mg/mL against *S. aureus*, whereas the equivalent extracts obtained from spearmint and sage showed a MIC of 1.25 mg/mL and 0.625 mg/mL against this pathogen, respectively [20]. A customised starter culture is also suggested in this work as a result of previous investigation from our research team [22]. After collecting LAB isolates (N = 232) from goat's raw milk cheeses, determining their antimicrobial, acidifying, and proteolytic activities, and conducting molecular characterisation, four strains were selected (*Leuconostoc mesenteroides*, *Lacticaseibacillus paracasei*, *Lactococcus cremoris* and *Lactococcus lactis*) to compose a cocktail of LAB that could be used as a starter culture in cheese production, considering its bactericidal and acidogenic capacities.

Mathematical models are useful tools for predicting microbial behaviour, as the determination of growth parameters of pathogens can be used to assess and manage the risk of foodborne illnesses [23]. In this sense, one of the objectives of this work was to mathematically determine the effect of a customised starter culture and of lemon balm, sage and spearmint extracts (when directly incorporated in curd, during cheese production) against *S. aureus* in goat's raw milk cheeses; and to characterise the survival kinetic parameters of this pathogen by means

of an extended Bigelow model. With this approach, values of decimal reduction time (*D*) can be described as a function of pH and incorporation of plant extract or starter culture, and the inactivation parameters of *S. aureus* may aid in the optimisation of the manufacturing process to ensure the microbial safety of cheeses. The second objective was to investigate the impact of the plant extracts and starter culture on the evolution of LAB, to ensure that this microbial community, crucial for the fermentation process, is not negatively affected; and to compare the behaviour of autochthonous LAB with that of LAB when a starter culture is added.

9.2 MATERIALS AND METHODS

9.2.1 Plant material and Extraction Procedure

Dried lemon balm, spearmint and sage aerial parts were provided by Pragmático Aroma Lda. ("Mais Ervas", Trás-os-Montes, Portugal) and mechanically ground. Extracts were obtained as described by Silva et al. 2021 [20]. Briefly, the extractions were performed using ethanol 70% (v/v) as solvent in a shaking water bath (150 rpm) at 60 °C for 90 minutes. The sample/solvent ratio was 1:20. The mixtures were filtrated (7–10 μm), and the ethanolic fraction was evaporated. The remaining aqueous fraction was frozen and lyophilised.

9.2.2 Bacterial strains

Staphylococcus aureus ATCC 6538, obtained from the Polytechnic Institute of Bragança stock collection, was used. A loop of culture kept on a fresh Nutrient Agar slant was cultivated twice at 37 °C, 200 rpm, for 16 h, first on tryptic soy broth (TSB) and then on tryptic soy broth with pH adjusted to 6.34, to mimic goat's milk pH. On the day of cheese production, the second subculture was centrifuged at 10640 g at 4 °C for 10 minutes, for removing debris and residual culture media. After centrifugation, the supernatant was discarded, and pellets were washed with sterile 0.9% physiological solution. Centrifugation and washing procedures were repeated twice and cells were re-suspended in sterile 0.9% physiological solution to reach approximately 7 log CFU/mL.

For the LAB cocktail, four strains of LAB (*Leuconostoc mesenteroides*, *Lacticaseibacillus paracasei*, *Lactococcus cremoris* and *Lactococcus lactis*) isolated from goats' raw milk artisanal cheeses and that presented antimicrobial and acidifying activity [22] were used in this study. For the preparation of individual LAB strains cell suspensions, the cryopreserved strains were thawed,

and a loop of culture of each strain was separately cultivated in MRS broth at 30 °C for 24 h. Two successive inoculations were then performed by placing 100 µL of the subcultures in 10 mL of MRS broth at 30 °C for 24 h. The following inoculation was carried out by placing 500 µL of the subculture in 200 mL of MRS broth at 30 °C for 18 h, to achieve a concentration of each strain of approximately 9 log CFU/mL, adjusted by measuring absorbance at 600 nm using a spectrophotometer (Peak Instruments Inc., Version 1701). Equal parts of each strain were then combined to obtain the selected LAB cocktail.

9.2.3 Inoculation of bacterial strains in milk and cheese production: incorporation of starter culture or plant extract

Laboratory-scale cheeses were prepared by adding the rennet (0.75 mL/L milk) and *S. aureus* inoculum (5 mL/L milk) to milk at approximately 34 °C, in the case of challenge tests with plant extracts; or rennet (0.75 mL/L milk), *S. aureus* inoculum (5 mL/L milk) and selected LAB cocktail inoculum (10 mL/L milk, 1% (v/v)), in the case of challenge tests with added starter culture. Through this procedure, each cheese reached a *S. aureus* target concentration of 4 to 5 log CFU/g, depending on the milk initial contamination.

After 30 minutes at 34 °C, curdled milk was cut and drained, and, for challenge tests with plant extracts, 1% (w/w) of lyophilised spearmint, lemon balm or sage extract was added to the curd and mixed. An inoculated control without extract or without starter culture was kept. Non-inoculated cheeses with starter culture were also produced.

Next, the curd was placed in 50 mL tubes and centrifuged at 6000 rpm at 20 °C for 3.5 minutes. The supernatant (whey) was removed, and cheeses of approximately 5 g were cut from the compacted curd and placed in a 15% (w/v) brine solution (ratio cheese: brine of approximately 90 g: 1.5L) for 10 minutes at 25 °C for salting. Finally, the weight in g of each cheese was annotated and cheeses were kept in a climate-controlled chamber (10 °C, 98% RH) for fermentation and maturation to take place for 15 days.

9.2.4 Microbiological and Physicochemical Analysis throughout cheese ripening

Analyses were conducted between day 0 (day of cheese production) and day 15. For the microbiological determinations, for every test unit, appropriate serial dilutions were prepared by homogenising the cheese in 50 mL of buffered peptone water for 60 seconds. To determine the concentration of *S. aureus*, 0.1 mL aliquot was plated on Baird-Parker agar, supplemented with

Egg Yolk Tellurite, following ISO norm [24]. Typical colonies were counted after 48 h following incubation at 37 °C.

LAB concentration was determined by incorporating 1 mL aliquot in MRS agar (for plant extracts challenge tests) or MRS and M17 agar (for the starter culture challenge test), and overlaying with 1.2% bacteriological agar, following ISO norm [25]. Plates were then incubated at 30 °C for 48 h before enumeration of typical colonies.

Physicochemical analyses during cheese ripening comprised the measurement of pH and water activity. The pH measurement was carried out using a pH meter (Hanna Instruments, model HI5522, USA) equipped with a HI1131 glass penetration probe. To measure a_w, samples were transferred into the cuvette of an Aqualab meter (4TE Decagon, WA, USA), and the value was recorded after measurement stabilization.

9.2.5 Modelling of *S. aureus* and LAB behaviour during cheese ripening

9.2.5.1 *S. aureus* behaviour during cheese ripening

Since the pH of cheese varies during ripening at 10 °C, and changes in *S. aureus* counts were primarily driven by the drop in pH, dynamic kinetic analysis was employed to assess *S. aureus* kinetic parameters in cheese.

For every treatment, a three-parameter empirical decay function was used to describe the pH change over the maturation time, as follows:

$$pH_t = (pH_0 - pH_{res}) \times e^{-kt} + pH_{res} \quad (1)$$

Where k is the pH decay rate (day^{-1}), pH_t the pH at time t, pH_0 the pH at time 0, and pH_{res} the asymptotic pH.

Then, a log-decay function with shoulder and tail in differential form as primary model (as described by Geeraerd et al. 2000, 2005 [26,27]), with varying D-value, coupled to a secondary model Bigelow equation of D-value as a function of pH (with parameters $\log D_{ref}$ at pH 7.0 and z_{pH}) was adjusted, as follows:

$$\frac{dN}{dt} = -kN\left(\frac{1}{1+C_c}\right)\left(1-\frac{N_{res}}{N}\right)$$
$$\frac{dC_c}{dt} = -kC_c \quad (2)$$

$$D = \frac{\ln(10)}{k}$$

$$\log D = \log D_{ref} - \left(\frac{pH - pH_{ref}}{z_{pH}}\right)^2 \quad (3)$$

In Equation (2), N is the population density (CFU/g), k is the inactivation rate (day^{-1}), C_c is related to the existence or absence of a shoulder region in the inactivation curve and N_{res} is the residual population density and related to the tailing phenomenon in the inactivation curve (CFU/g). In Equation (3), D (days) is the decimal reduction time (days) at the constant temperature (10 °C) and at the pH of the cheese, pH_{ref} is the reference pH (set to 7.0), z_{pH} is the distance of pH from pH_{ref} which leads to a ten-fold change in decimal reduction time, and D_{ref} is the decimal reduction time at pH_{ref} (days). First, C_c and N_{res} were approximated by fitting the Equation (2) to each data set in integrated form; and subsequently z_{pH} and $\log D_{ref}$ were estimated by fitting the dynamic model defined by Equations (2) and (3).

9.2.5.2 LAB behaviour during cheese ripening

Cheeses with plant extracts. To evaluate the impact of plant extracts on the growth of LAB in cheese, the integrated Huang model [28] as described in Equation (4) was used:

$$Y(t) = Y_0 + Y_{max} - \ln\left(e^{Y_0} + (e^{Y_{max}} - e^{Y_0})e^{-\mu_{max}\beta(t)}\right)$$

$$\beta(t) = t + \frac{1}{\alpha}\ln\left(\frac{1+e^{-\alpha(t-\lambda)}}{1+e^{\alpha\lambda}}\right) \quad (4)$$

In Equation (4), Y_0, Y_{max} and Y are the natural logarithms of bacterial counts at time 0, at maximum level and at the "real time" t, respectively; μ_{max} is the maximum growth rate (ln CFU/g day^{-1}); $\beta(t)$ is the transition function; λ is the lag time (day) of the growth curve; α is the lag phase transition coefficient (dimensionless); and t is the time (day) under a constant temperature (10 °C). The parameter α was given a value of 4.0, as recommended by Huang (2013) [29]. The estimated parameters from Equation (4) were Y_0, Y_{max} and μ_{max}. It is acknowledged that the growth rate of LAB in cheese is affected by the changing pH of the matrix; nonetheless, a dynamic model was not fitted to the LAB data since the objective was to compare the effect of the extracts on LAB growth, and not to characterise the kinetic parameters of the pool of indigenous lactic acid bacteria, which were largely unknown at that time.

Cheeses with selected LAB cocktail. To estimate the kinetic parameters of LAB taking into consideration the pH decay during storage at constant temperature (10 °C), dynamic kinetic analysis was used. This was done by simultaneously fitting a primary growth model in differential form with an explicit secondary model of the specific growth rate as a function of cheese pH. The Huang model [28] was chosen as the primary model describing the growth of LAB in cheese during ripening, and the cardinal parameter model for pH was chosen for secondary modelling [30]. Accordingly, the following model, labelled as Huang-Cardinal, was fitted to the data:

$$\frac{dY}{dt} = \frac{\mu_{max}}{1 + e^{-\alpha(t-\lambda)}}(1 - e^{Y-Y_{max}})$$

$$\mu_{max} = \mu_{opt}\left\{\frac{(pH-pH_{min})(pH-pH_{max})}{(pH-pH_{min})(pH-pH_{max})-(pH-pH_{opt})^2}\right\} \quad (5)$$

As in Equation (4), in Equation (5), Y_{max} and Y are the natural logarithms of bacterial counts at maximum level and at the "real time" t, respectively; μ_{max} is the maximum growth rate (ln CFU/g day^{-1}); λ is the lag phase duration (day) of the growth curve (set to zero); α is the lag phase transition coefficient (dimensionless, set to 4.0 [29]); and t is the time (day) under a constant temperature (10 °C). The terms pH_{min} and pH_{max} refer to the pH below or above which no microbial growth occurs, whereas pH_{opt} is the pH at which the μ_{max} is optimal.

The estimated parameters from Equation (5) were Y_0, Y_{max} and μ_{opt}. The latter parameter can be interpreted as the optimum growth rate at 10 °C of LAB in goats' raw milk cheese at the optimum pH (pH_{opt}), assuming that the variation in water activity during ripening is negligible. The cardinal parameters of LAB (pH_{min}, pH_{opt} and pH_{max}) are not estimable from our data since the monitored pH (5.04-6.61) of the cheese correspond to narrow-ranged values. For that reason, literature data [31-45] was used to set average cardinal values of the LAB strains used in the cocktail (*L. mesenteroides*, *L. paracasei*, *L. cremoris* and *L. lactis*): pH_{min}= 4.00, pH_{opt}=6.50 and pH_{max}=9.00.

9.2.5.3 Estimation of parameters

Numerical methods can be used to solve ordinary differential equations (ODE) such as Equation (2) and (5), which do not have an analytical solution. Numerical optimisation consists of searching for the model parameters resulting in least residual sum of squares (RSS) of the errors. Herein, the 4th order Runge-Kutta method was used to resolve ODE [28], while the unknown kinetic

parameters were determined by least-square optimisation, employing the 'deSolve' and 'FME' libraries from the R software (version 4.1.0, R Foundation for Statistical Computing, Vienna, Austria).

The mean absolute error (MAE, Equation (6)) and root mean square error (RMSE, Equation (7)) were also computed to assess the fitting capacities of the models,

$$MAE = \frac{\sum|Y_{obs\,i} - Y_{fit\,i}|}{n} \quad (6)$$

$$RMSE = \sqrt{\frac{\sum(Y_{obs\,i} - Y_{fit\,i})^2}{df}} \quad (7)$$

where $Y_{fit\,i}$ and $Y_{obs\,i}$ designate for each of the *i*-th *S. aureus* or LAB concentrations adjusted by the model and its corresponding observation, respectively. The degrees of freedom (df) are determined as 'n-np', where n is the amount of data points of a trial growth curve and np is the number of parameters of the adjusted model.

9.3 RESULTS AND DISCUSSION

9.3.1 pH decay during cheese ripening

The estimated parameters of the empirical decay function used to describe the pH change over the maturation time are presented in **Table 40**. Change in pH throughout cheese ripening is shown in **Figure 22** (plant extracts) and **Figure 23** (selected LAB cocktail).

The natural course of the fermentation process appeared to be impacted by the presence of spearmint and lemon balm extracts, as suggested by the lower pH drop rates, k, of the treatments (spearmint: 0.194 $days^{-1}$; lemon balm: 0.223 $days^{-1}$) in comparison to the corresponding controls (spearmint: 0.262 $days^{-1}$; lemon balm: 0.240 $days^{-1}$), whereas sage extract had no effect on the pH drop rate (0.521 $days^{-1}$ and 0.522 $days^{-1}$ for cheeses with and without sage extract, respectively). In this sense, among the extracts tested, spearmint affected the pH drop rate the most.

However, sage extract also affected the fermentation process, but in this case it was the extract with the biggest impact on the final pH achieved: cheeses with this extract present a greater difference between their pH_{res} (5.377) and the pH_{res} of the control treatment (5.172), in comparison to cheeses with and without spearmint (5.584 and 5.418, respectively) and with and without lemon balm extracts (5.286 and 5.115, respectively). Nevertheless, in all cases, the pH_{res} was higher in cheeses with plant extracts, compared to the controls.

Table 40. Effect of the addition of spearmint, lemon balm or sage extract in curd or of a cocktail of selected LAB on the parameters of the empirical decay function used to describe the pH change over the maturation time in goat's raw milk cheese, along with goodness-of-fit measures (variance, S^2, root mean square error, RMSE, and mean absolute error, MAE) and estimated pH decay ($\boldsymbol{pH_0 - pH_{res}}$) throughout maturation.

Treatment	Parameters	Mean ± SE	Pr (>|t|)	Goodness-of-fit measures	$\boldsymbol{pH_0 - pH_{res}}$
S. aureus + Spearmint 0%	pH_0	6.581 ± 0.058	<.0001	S^2=0.006	1.163
	pH_{res}	5.418 ± 0.050	<.0001	RMSE=0.075	
	k	0.262 ± 0.041	<.0001	MAE=0.061	
S. aureus + Spearmint 1%	pH_0	6.530 ± 0.062	<.0001	S^2=0.007	0.946
	pH_{res}	5.584 ± 0.107	<.0001	RMSE=0.079	
	k	0.194 ± 0.058	0.008	MAE=0.067	
S. aureus + Lemon balm 0%	pH_0	6.567 ± 0.046	<.0001	S^2=0.004	1.452
	pH_{res}	5.115 ± 0.043	<.0001	RMSE=0.059	
	k	0.240 ± 0.025	<.0001	MAE=0.047	
S. aureus + Lemon balm 1%	pH_0	6.502 ± 0.053	<.0001	S^2=0.005	1.216
	pH_{res}	5.286 ± 0.055	<.0001	RMSE=0.069	
	k	0.223 ± 0.034	<.0001	MAE=0.050	
S. aureus + Sage 0%	pH_0	6.142 ± 0.061	<.0001	S^2=0.007	0.970
	pH_{res}	5.172 ± 0.031	<.0001	RMSE=0.079	
	k	0.522 ± 0.092	<.0001	MAE=0.063	
S. aureus + Sage 1%	pH_0	6.265 ± 0.036	<.0001	S^2=0.002	0.888
	pH_{res}	5.377 ± 0.018	<.0001	RMSE=0.046	
	k	0.521 ± 0.058	<.0001	MAE=0.037	
S. aureus without LAB cocktail	pH_0	6.461 ± 0.044	<.0001	S^2=0.003	0.885
	pH_{res}	5.576 ± 0.031	<.0001	RMSE=0.057	
	k	0.330 ± 0.047	<.0001	MAE=0.045	
S. aureus with LAB cocktail	pH_0	6.509 ± 0.042	<.0001	S^2=0.003	0.853
	pH_{res}	5.656 ± 0.029	<.0001	RMSE=0.054	
	k	0.337 ± 0.047	<.0001	MAE=0.043	
LAB cocktail only	pH_0	6.440 ± 0.044	<.0001	S^2=0.004	1.190
	pH_{res}	5.250 ± 0.051	<.0001	RMSE=0.058	
	k	0.263 ± 0.035	<.0001	MAE=0.044	

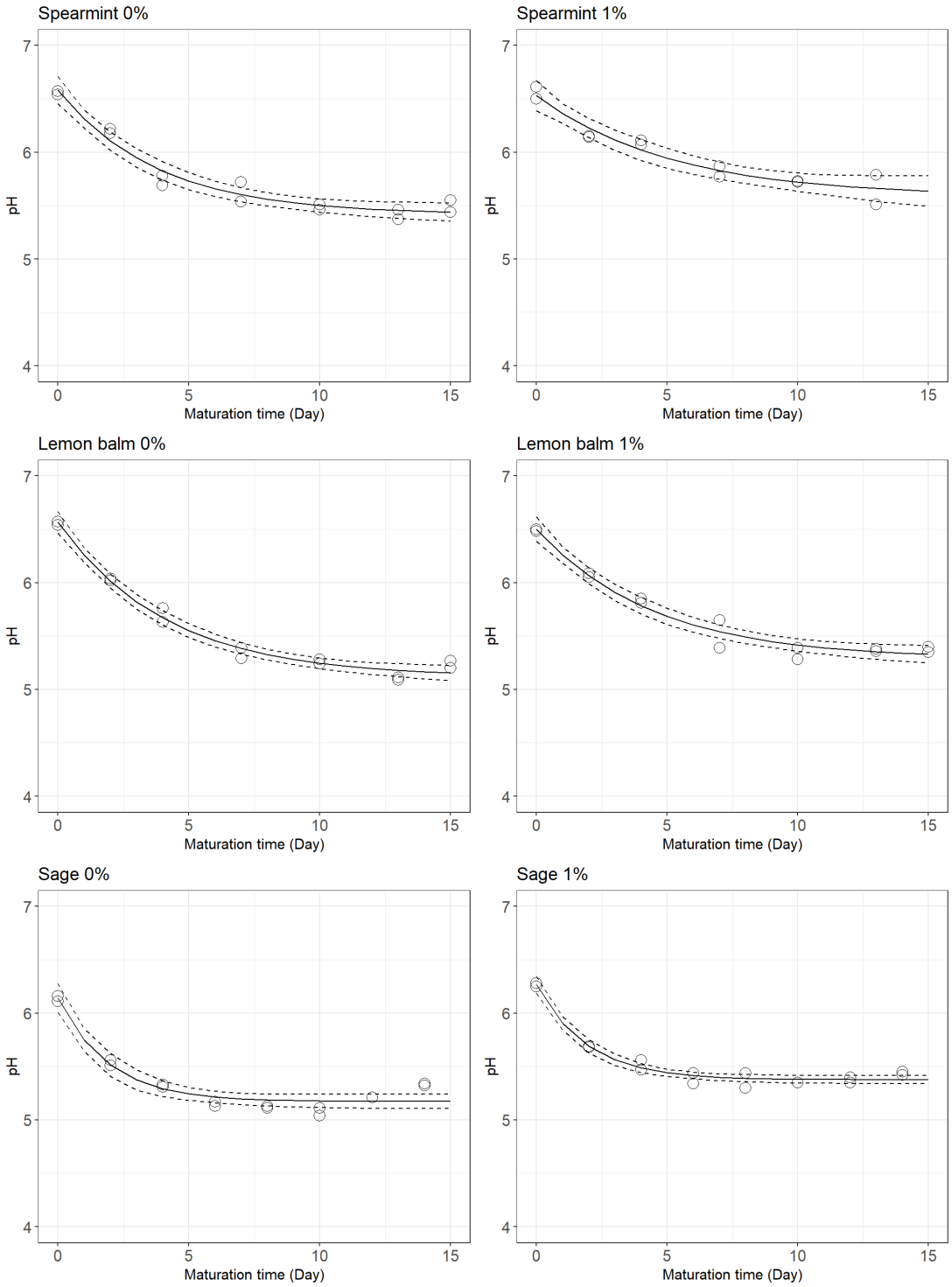

Figure 22. Change in pH of lab-scale cheeses made from goats' raw milk without (left) and with (right) addition of 1% (w/w) spearmint, lemon balm and sage extracts, described by a three-parameter empirical decay function (full line) with 95% confidence intervals, CI (dashed lines).

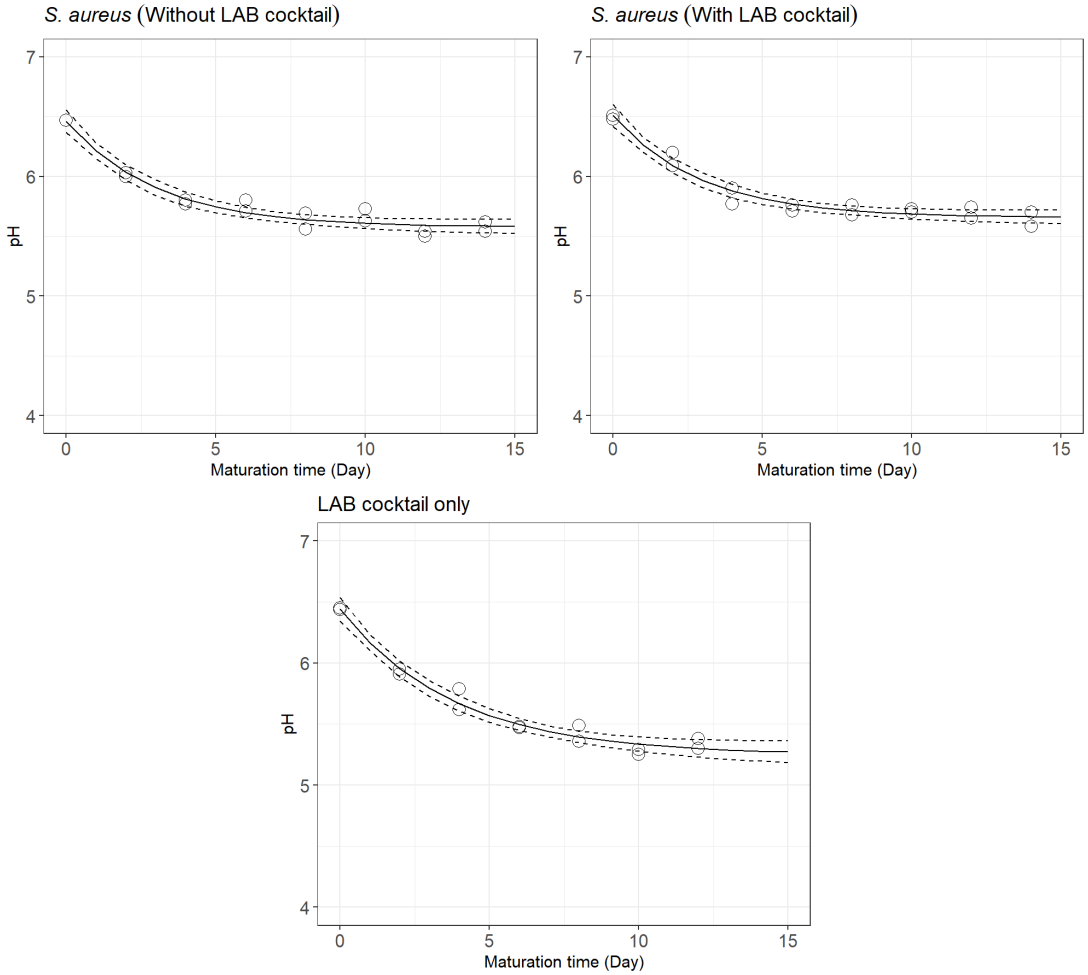

Figure 23. Change in pH of lab-scale cheeses made from goats' raw milk inoculated with *S. aureus* without (top left) and with (top right) addition of a cocktail of selected LAB, and cheeses non-inoculated with *S. aureus* with selected LAB cocktail (bottom), described by a three-parameter empirical decay function (full line) with 95% CI (dashed lines).

The pH drop rate in *S. aureus*-free cheeses with addition of the selected LAB cocktail was lower (k=0.263 days^{-1}; **Figure 23**, bottom plot) than that of cheeses inoculated with *S. aureus* (without LAB cocktail: k=0.330 days^{-1}; with LAB cocktail: k=0.337 days^{-1}; **Figure 23**, top plots). However, the pH of *S. aureus*-free cheeses with the selected LAB cocktail by the end of the challenge test was much lower ($pH_{res} = 5.250$) when compared to that of cheeses inoculated with *S. aureus*, either with ($pH_{res} = 5.656$) or without ($pH_{res} = 5.576$) the selected LAB cocktail. While the strains composing the customised starter culture presented acidifying capacity in vitro [22], it seems that, in this challenge test, they were not able to accelerate the pH decay during fermentation, as would have been expected [46]. In any case, the selected LAB cocktail promoted a decay more prolonged in time, which enabled reaching a lower pH_{res} by the end of maturation.

Comparing cheeses inoculated with *S. aureus* but not the selected LAB cocktail (**Figure 23**, top left plot) with those inoculated with both (**Figure 23**, top right plot), it can be seen that the starter culture modified only slightly the pH drop rate (without LAB cocktail: k=0.330 $days^{-1}$; with LAB cocktail: k=0.337 $days^{-1}$) and that cheeses inoculated with *S. aureus* and the selected LAB cocktail were not able to reach a pH value as low as those inoculated with *S. aureus* only ($pH_{res} = 5.656$ vs. $pH_{res} = 5.576$).

Changes in water activity were observed during cheese ripening, with values oscillating between 0.932 and 0.984 without a specific trend (data not shown), so no function could be fitted to describe the water activity evolution over time.

9.3.2 *S. aureus* behaviour during cheese ripening

Bigelow-type secondary models were used to describe the inactivation of *S. aureus* in goat's raw milk cheeses during maturation as affected by spearmint, lemon balm and sage extracts and a cocktail of selected LAB. The survival curves of *S. aureus* in cheese with plant extracts and with a selected LAB cocktail, as depicted by dynamic models, are presented in **Figure 24**. The dynamic inactivation model was not fitted for the treatment without the selected LAB cocktail as *S. aureus* decay did not occur. The results of this particular control (slight growth of *S. aureus*) were therefore not quite aligned with those of the control treatments for the extracts (survival of *S. aureus*). Since the experimental work conducted was the same for the controls of all runs, the normal lot-to-lot variations in composition, microbiota and microbiological quality of the goat's raw milk are likely to explain the slight deviation observed in such a control treatment.

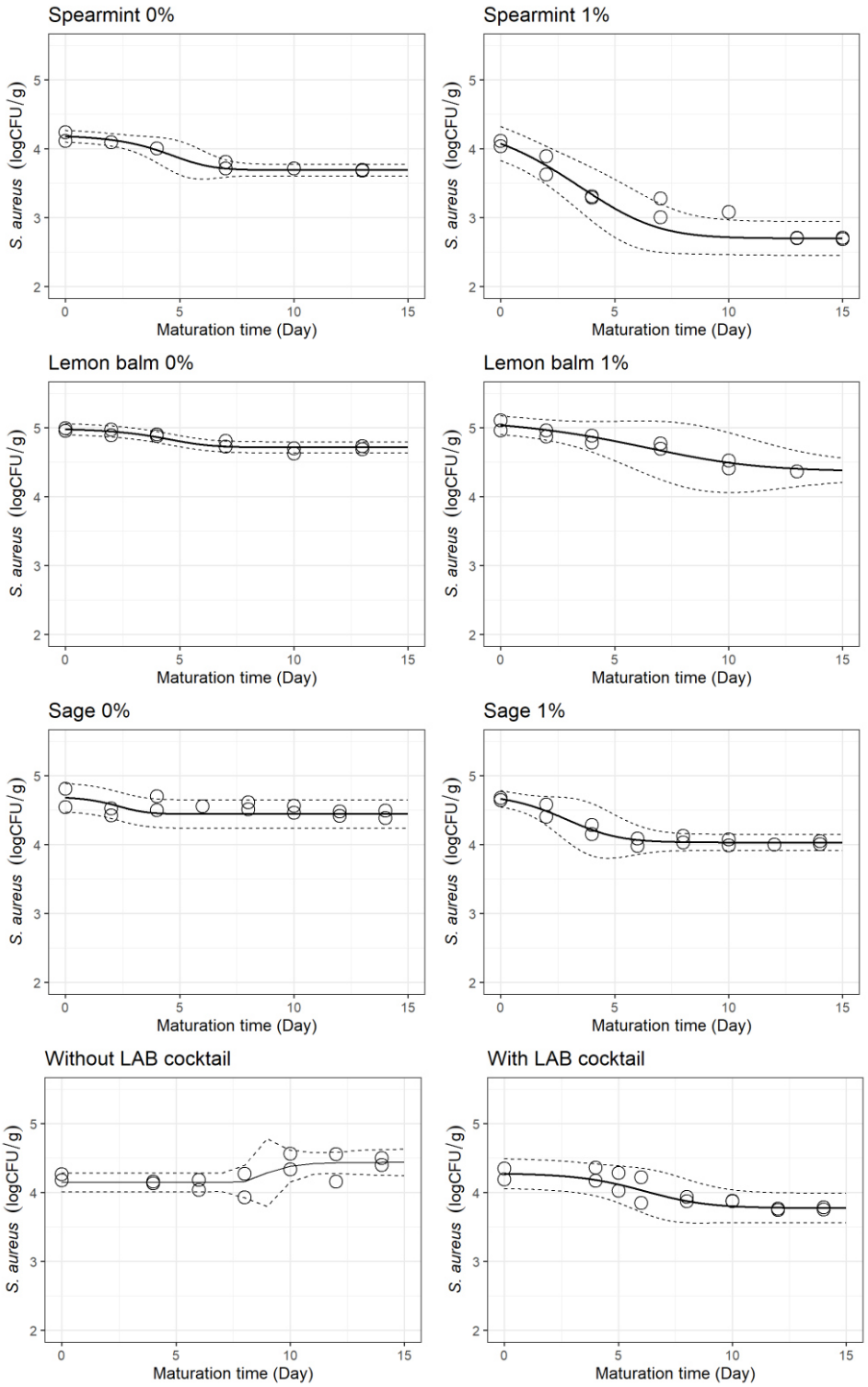

Figure 24. *S. aureus* behaviour in goat's raw milk cheese without (left) and with (right) 1% (w/w) of extract of spearmint, lemon balm or sage, and without (left) and with (right) a cocktail of selected LAB as starter culture, as depicted by dynamic inactivation (full lines) with 95% CI (dashed lines). As an exception, the integrated Huang model was fitted to the curve produced without LAB cocktail.

The results of the Bigelow parameters for each treatment are shown in **Table 41**. The dynamic models adequately fitted the survival curves, with root mean square errors (RMSE) of 0.116, 0.063, 0.057 and 0.103 for spearmint, lemon balm, sage and selected LAB cocktail, respectively (**Table 41**), producing significant parameter estimates ($p < 0.05$) in all cases.

Table 41. Effect of the addition of spearmint, lemon balm or sage extract in curd or of a cocktail of selected LAB on the Bigelow's inactivation parameters of *S. aureus* in goat's raw milk cheese during maturation, along with goodness-of-fit measures (variance, S^2, root mean square error, RMSE, and mean absolute error, MAE) and *S. aureus* mean total inactivation (log CFU/g) after 12 days.

Treatment	Parameters	Mean ± SE	Pr (>|t|)	Goodness-of-fit measures	$\triangle Y_{0\text{-}12}$ (log CFU/g)
Spearmint 0% ($C_c(0)$=1.5)	$\log D_{ref}$	0.993 ± 0.190	0.001	S^2=0.002 RMSE=0.040 MAE=0.035	0.491
	z_{pH}	1.599 ± 0.358	<.0001		
Spearmint 1% ($C_c(0)$=0.01)	$\log D_{ref}$	0.621 ± 0.061	<.0001	S^2=0.015 RMSE=0.116 MAE=0.098	1.373
	z_{pH}	3.172 ± 0.655	<.0001		
Lemon balm 0% ($C_c(0)$=1.5)	$\log D_{ref}$	0.996 ± 0.056	<.0001	S^2=0.002 RMSE=0.037 MAE=0.033	0.262
	z_{pH}	1.851 ± 0.066	<.0001		
Lemon balm 1% ($C_c(0)$=0.01)	$\log D_{ref}$	1.190 ± 0.200	<.0001	S^2=0.004 RMSE=0.063 MAE=0.056	0.611
	z_{pH}	2.340 ± 0.835	0.019		
Sage 0% ($C_c(0)$=1.5)	$\log D_{ref}$	0.796 ± 0.068	<.0001	S^2=0.010 RMSE=0.098 MAE=0.077	0.238
	z_{pH}	2.054 ± 0.131	<.0001		
Sage 1% ($C_c(0)$=0.01)	$\log D_{ref}$	0.996 ± 0.278	0.003	S^2=0.003 RMSE=0.057 MAE=0.047	0.634
	z_{pH}	2.006 ± 0.677	0.010		
With LAB cocktail ($C_c(0)$=3)	$\log D_{ref}$	0.756 ± 0.067	<.0001	S^2=0.011 RMSE=0.103 MAE=0.078	0.493
	z_{pH}	2.490 ± 0.487	<.0001		

From **Table 41**, the parameter $\log D_{ref}$ was affected by the addition of extracts (0.621 ± 0.061 days for spearmint; 1.190 ± 0.200 for lemon balm; 0.996 ± 0.278 for sage) in comparison

to the controls (0.993 ± 0.190 days for spearmint; 0.996 ± 0.056 for lemon balm; 0.796 ± 0.068 for sage).

In the case of cheeses with spearmint extract, $\log D_{ref}$ was lower than that of the control (0.621 ± 0.061 vs. 0.993 ± 0.190 days), implying a greater inactivation rate of the pathogen. Moreover, the survival curves presented in **Figure 24** show that the incorporation of spearmint extract reduced the initial shoulder and promoted *S. aureus* inactivation earlier in maturation. In turn, comparing the survival curves of the treatments without and with the selected LAB cocktail, it seems that the customised starter culture completely inverted the behaviour of *S. aureus*, as it inhibited the pathogens' growth observed in the control and started promoting *S. aureus* decay after around five days of ripening.

Oppositely, when adding lemon balm or sage extract to the cheese, the estimated $\log D_{ref}$ were higher (1.190 ± 0.200 for lemon balm; 0.996 ± 0.278 for sage) than that of the controls (0.996 ± 0.056 for lemon balm; 0.796 ± 0.068 for sage), thus suggesting a lower inactivation rate. However, when these extracts were incorporated in cheese, *S. aureus* inactivation was steadier and more prolonged throughout maturation, compared to control cheeses, in which *S. aureus* inactivation phase was rather short and the tail region appears sooner (**Figure 24**).

The addition of plant extracts decreased the time to achieve one log reduction, which in practical terms corresponded to a reduction of 0.634 log CFU/g (sage), 0.611 log CFU/g (lemon balm), and 1.373 log CFU/g (spearmint) after 12 days of maturation (**Table 41**). Without the incorporation of plant extracts, *S. aureus* decay was still observed but less pronounced, with a decline in pathogen concentration between 0.238 and 0.491 log CFU/g in the same period. Considering these results, the usefulness of incorporating spearmint, lemon balm and sage extracts to reduce *S. aureus* burden in this dairy product is confirmed. The addition of the selected LAB cocktail also reduced the time necessary for a log decrease, and in practice corresponded to a reduction of 0.493 log CFU/g after 12 days of maturation. These results are consistent with previous works reporting on the antimicrobial capacities of selected LAB strains [16,47-51] and plant extracts [13-15,52,53] against various microorganisms in cheeses.

The higher z_{pH} values of cheeses with extract of spearmint (3.172 ± 0.655) and extract of lemon balm (2.340 ± 0.835) in **Table 41** indicate that a greater difference between pH and pH_{ref} is necessary to lead to a tenfold change in D when incorporating these plant extracts in cheese, than the one needed for the same variation in D in the controls (spearmint: 1.599 ± 0.358; lemon balm: 1.851 ± 0.066). This would imply that, for the same pH variation, *S. aureus* in cheeses

with incorporated spearmint or lemon balm extract would suffer a smaller reduction than *S. aureus* in control cheeses; however, a phenomenon of interaction should be also considered in the interpretation, since the addition of extracts to the curd retarded the pH drop (**Table 40**). Other inhibitory mechanisms apart from pH decay may promote inactivation, and in the mathematical equations, these could manifest themselves in the shortening of the shoulders. On the other hand, the z_{pH} value of cheeses with sage extract (2.006 ± 0.677) was close to that of the control (2.054 ± 0.131), suggesting that the difference between pH and pH_{ref} that leads to a tenfold change in D is virtually the same in both cases.

Overall, the results of the Bigelow-type secondary models adjusted indicate that the plant extracts and selected starter culture tested may be used for the control of *S. aureus* in cheeses, but that each biopreservative influences different factors. The results showed that the main effect of adding 1% lemon balm extract or 1% sage extract in curd was on the delay of the tailing phenomenon and on the z_{pH} parameter, whereas 1% spearmint extract affected *S. aureus* shoulder, z_{pH} and $\log D_{ref}$. In turn, the main effect of the selected LAB cocktail was on z_{pH} and $\log D_{ref}$, as it inverted the behaviour of *S. aureus* from growth to survival.

Considering the multiple impacts on the pathogen and the reduction promoted, among all options, spearmint extract appears to be more efficient in controlling *S. aureus* in goat's raw milk cheeses. This was despite the previously determined MIC of spearmint against *S. aureus*, which was not the lowest among the three extracts tested (lemon balm extract: 2.5 mg/mL; spearmint: 1.25 mg/mL; sage: 0.625 mg/mL) [20], thus demonstrating the effect of the matrix on the antimicrobial potential of biopreservatives [54].

From our previous work [20], spearmint extract did not present the highest total phenolic content but revealed the highest concentration of rosmarinic acid (333 mg/L extract) when compared to sage (170 mg/L extract) and lemon balm extracts (185 mg/L extract). Rosmarinic acid is recognised for its high antimicrobial capacity [55,56], and although the mechanisms of action are not clearly known, Honório et al. [57] reported cell shrinkage and appearance of blebbing-like structures on *S. aureus* cell surfaces, and Bais et al. [58] also described damaged cell surface when treating *A. niger* with rosmarinic acid. Ferulic acid, ellagic acid, naringin, hesperidin, resveratrol and quercetin were also detected in our spearmint hydroethanolic extract, but in lower concentration compared to rosmarinic acid [20]. The antimicrobial potential of these compounds against *S. aureus* has also been reported in literature [59-64].

9.3.3 LAB behaviour during cheese ripening

Cheeses with plant extracts. The Huang model parameters describing the behaviour of LAB in goat's raw milk cheese with and without plant extracts during maturation are shown in **Table 42**. The corresponding fitted models are depicted in **Figure 25**.

Table 42. Kinetic parameters (initial and maximum microbial concentration, Y_0, Y_{max} in ln CFU/g, maximum growth rate, μ_{max} in ln CFU/g day^{-1} and lag duration, λ in days) of LAB in goat's raw milk cheese during maturation with and without plant extracts and inoculated or not with *S. aureus*, as estimated by the Huang model, along with goodness-of-fit measures (variance, S^2, root mean square error, RMSE, and mean absolute error, MAE).

Treatment	Parameters	Mean ± SE	Pr (>|t|)	Goodness-of-fit measures
Spearmint 1%	Y_0	13.94 ± 0.366	<.0001	S^2=0.277
	Y_{max}	19.70 ± 0.216	<.0001	RMSE=0.508
	μ_{max}	1.088 ± 0.160	<.0001	MAE=0.445
S. aureus + Spearmint 0%	Y_0	14.11 ± 0.360	<.0001	S^2=0.254
	Y_{max}	20.01 ± 0.195	<.0001	RMSE=0.486
	μ_{max}	1.421 ± 0.189	<.0001	MAE=0.438
S. aureus + Spearmint 1%	Y_0	15.00 ± 0.299	<.0001	S^2=0.249
	Y_{max}	19.99 ± 0.358	<.0001	RMSE=0.481
	μ_{max}	0.503 ± 0.076	<.0001	MAE=0.421
Lemon balm 1%	Y_0	16.40 ± 0.307	<.0001	S^2=0.252
	Y_{max}	27.79 ± 0.349	<.0001	RMSE=0.275
	μ_{max}	1.219 ± 0.074	<.0001	MAE=0.208
S. aureus + Lemon balm 0%	Y_0	17.31 ± 0.168	<.0001	S^2=0.081
	Y_{max}	26.75 ± 0.157	<.0001	RMSE=0.275
	μ_{max}	0.960 ± 0.038	<.0001	MAE=0.208
S. aureus + Lemon balm 1%	Y_0	16.71 ± 0.346	<.0001	S^2=0.361
	Y_{max}	26.87 ± 0.350	<.0001	RMSE=0.579
	μ_{max}	0.967 ± 0.073	<.0001	MAE=0.490
Sage 1%	Y_0	17.80 ± 0.435	<.0001	S^2=0.461
	Y_{max}	22.52 ± 0.457	<.0001	RMSE=0.654
	μ_{max}	0.643 ± 0.138	<.0001	MAE=0.539
S. aureus + Sage 0%	Y_0	18.19 ± 0.325	<.0001	S^2=0.262
	Y_{max}	23.73 ± 0.232	<.0001	RMSE=0.496
	μ_{max}	0.806 ± 0.102	<.0001	MAE=0.369
S. aureus + Sage 1%	Y_0	18.27 ± 0.338	<.0001	S^2=0.179 RMSE=0.408 MAE=0.336
	Y_{max}	24.05 ± 0.229	<.0001	
	μ_{max}	1.133 ± 0.174	<.0001	
	λ	1.749 ± 0.565	0.011	

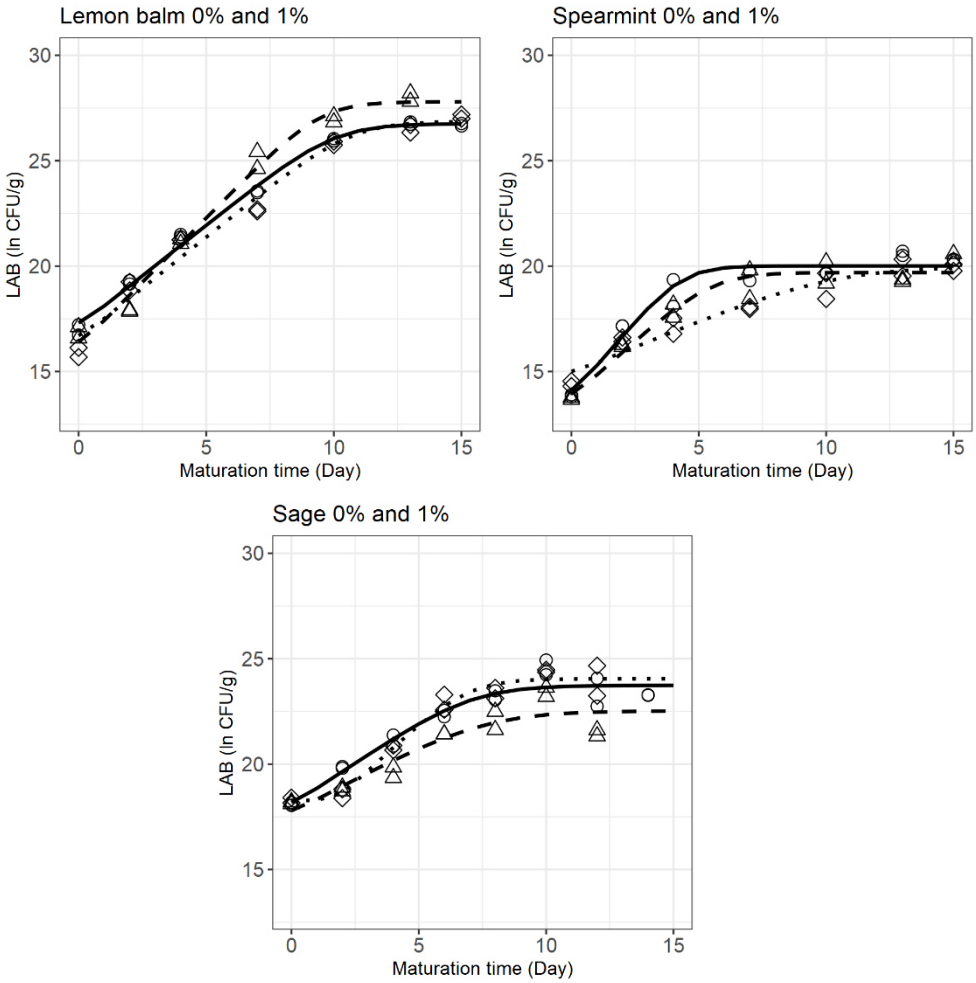

Figure 25. Growth of LAB in goat's raw milk cheese inoculated with *S. aureus*, with (···◇···) and without (–○–) plant extracts; and non-inoculated with plant extracts (- -Δ- -), as depicted by the Huang model. Same markers represent observations from the same experiment.

The Huang model adequately fitted each of the growth curves, with root mean square errors (RMSE) between 0.275 and 0.654 and produced significant parameter estimates ($p < 0.05$).

In the case of inoculated cheeses produced with sage extract, the growth curve of LAB presented a lag phase ($\lambda = 1.749 \pm 0.565$ days; **Figure 25**), which did not happen in non-inoculated cheeses with sage. This suggests that the combined presence of *S. aureus* and sage extract acts as a hurdle against LAB, inducing a period of adaptation before cell growth is possible. The other extracts tested did not produce this response.

The estimated initial LAB concentration, Y_0, varied between different experiments (from 13.94 ± 0.366 to 18.27 ± 0.338 ln CFU/g), a consequence of the high microbial variability of the raw milk used for cheese production.

In inoculated cheeses (with *S. aureus*), significant differences ($p < 0.05$) were found between the initial concentration of LAB in cheeses without and with lemon balm (17.31 ± 0.168 and 16.71 ± 0.346, respectively), and also without and with spearmint extracts (14.11 ± 0.360 and 15.00 ± 0.299 ln CFU/g day^{-1}, correspondingly), although no differences were found in Y_0 between cheeses produced with and without sage extracts (18.27 ± 0.338 and 18.19 ± 0.325 ln CFU/g day^{-1}, respectively).

Regarding the μ_{max} parameter, in inoculated cheeses, the growth rate of LAB was not affected by the incorporation of lemon balm extract, as reflected by the estimated values of 0.960 ± 0.038 and 0.967 ± 0.073 ln CFU/g day^{-1} ($p > 0.05$) and the identical shape of the growth curves in **Figure 25**. On the other hand, spearmint and sage extracts modified considerably ($p < 0.05$) the exponential phase of LAB in cheeses with *S. aureus* (observe the distinct growth curve shapes in **Figure 25**). Spearmint incorporation triggered a lower growth rate of LAB (0.503 ± 0.076 compared to 1.421 ± 0.189 ln CFU/g day^{-1} for cheeses without extract), whereas sage reduced the cell doubling time, i.e., increased the growth rate (1.749 ± 0.565 vs. 0.806 ± 0.102 ln CFU/g day^{-1} for cheeses without extract).

In the case of cheeses with lemon balm and spearmint extracts, the negative impact of the presence of *S. aureus* on LAB growth rate was observable, as significant differences ($p < 0.05$) were found between μ_{max} of inoculated and non-inoculated cheeses (the latter being higher). However, in cheeses produced with sage extract, the opposite was observed, as inoculated cheeses revealed higher μ_{max} (1.133 ± 0.174 ln CFU/g day^{-1}) than those non-inoculated (0.643 ± 0.138 ln CFU/g day^{-1}). Regardless of the direction of change, differences in μ_{max} between the two treatments may be partly explained by microbial competition mechanisms between LAB and *S. aureus*.

The extracts did not have an impact on the maximum LAB concentration, as no significant differences were detected between the Y_{max} values of cheeses with and without either of the plant extracts (in inoculated samples). However, the presence of *S. aureus* in cheeses with lemon balm and sage extracts appears to influence Y_{max}, as visible in the plots of **Figure 25**: non-inoculated cheeses with lemon balm reached higher LAB final concentration (27.79 ± 0.349 ln CFU/g), whereas in the case of cheeses with sage extract, inoculated samples were the ones achieving greater Y_{max} values (24.05 ± 0.229 ln CFU/g).

Even though cheeses with lower Y_0 presented lower Y_{max} values (spearmint 0% and 1%), it could not be inferred that the maximum concentration achieved is influenced by the initial LAB numbers, since treatments with higher Y_0 (sage 0% and 1%) did not present the highest Y_{max}.

Considering these results, lemon balm extract appears to be the one affecting LAB behaviour the least. In turn, spearmint extract greatly reduces (by more than half) the growth rate of LAB, even though, by the end of maturation, the same concentration is achieved. Taking into account the results in **Table 41**, where the high antagonist effect of this extract against *S. aureus* is observed, it seems that spearmint extract exhibits high antimicrobial capacity against both microbial communities. Sage extract and *S. aureus* contamination caused a period of little to no cell division (lag phase) but the higher growth rate allowed the cells to reach the stationary phase earlier, when comparing inoculated cheeses with and without sage, with no impact on the final LAB concentration reached.

Cheeses with selected LAB cocktail. The Huang-Cardinal model parameters describing the behaviour of LAB in goat's raw milk cheese with and without a cocktail of selected LAB during maturation are shown in **Table 43** and **Table 44**, for LAB isolated in MRS agar and M17 agar, respectively. All models adequately fitted the growth curves, with root mean square errors (RMSE) between 0.120 and 0.248; and produced significant parameter estimates (p< 0.05). Nonetheless, it is noteworthy to point out that the estimates of the kinetic parameters of M17-grown LAB (**Table 44**) are associated with higher standard errors than those isolated in MRS agar (**Table 43**). This is a consequence of the lower selectivity of M17 agar compared to MRS agar, which causes higher variability in the results of the microbiological analysis (plate counting) and therefore affects the precision of the estimation of parameters.

From **Table 43**, the Huang-Cardinal models showed that the addition of selected LAB with antimicrobial activity reduced the μ_{opt} (1.198 ± 0.260 and 1.144 ± 0.091 ln CFU/g day^{-1}) and increased Y_{max} (20.22 ± 0.199 and 20.40 ± 0.071 ln CFU/g) of MRS-grown LAB in comparison to cheeses without addition of the selected LAB cocktail (μ_{opt}: 1.560 ± 0.260 and 1.343 ± 0.145 ln CFU/g day^{-1} and Y_{max}: 18.54 ± 0.137 and 18.83 ± 0.085 ln CFU/g). The estimates in **Table 44** suggest the same tendencies in cheeses with intentionally-added LAB: a reduction of the optimum growth rate, μ_{opt} (0.979 ± 0.236 and 1.372 ± 0.246 ln CFU/g day^{-1}), and an increase of Y_{max} (21.20 ± 0.265 and 20.88 ± 0.236 ln CFU/g). In addition to the anticipated increase in Y_{max}, the initial concentration Y_0, was also higher in cheeses with incorporation of the selected LAB (MRS: 16.37 ± 0.144 and 16.65 ± 0.340 ln CFU/g; M17: 16.72 ± 0.464 and 15.99 ± 0.392 ln CFU/g).

Table 43. Kinetic parameters (initial and maximum microbial concentration, Y_0, Y_{max} in ln CFU/g, and optimum growth rate, μ_{opt} in ln CFU/g day^{-1}) of LAB isolated in MRS agar in goat's raw milk cheese during maturation with and without a cocktail of selected LAB and inoculated or not with *S. aureus*, as estimated by the Huang-Cardinal model, along with goodness-of-fit measures (variance, S^2, root mean square error, RMSE, and mean absolute error, MAE).

Treatment	Parameters	Mean ± SE	Pr (>|t|)	Goodness-of-fit measures
Without LAB cocktail	Y_0	15.01 ± 0.258	<.0001	S^2=0.040 RMSE=0.183 MAE=0.135
	Y_{max}	18.54 ± 0.137	<.0001	
	μ_{opt}	1.560 ± 0.260	0.009	
With LAB cocktail	Y_0	16.65 ± 0.340	<.0001	S^2=0.074 RMSE=0.248 MAE=0.198
	Y_{max}	20.22 ± 0.199	<.0001	
	μ_{opt}	1.198 ± 0.260	0.019	
S. aureus without LAB cocktail	Y_0	15.16 ± 0.174	<.0001	S^2=0.021 RMSE=0.134 MAE=0.120
	Y_{max}	18.83 ± 0.085	<.0001	
	μ_{opt}	1.343 ± 0.145	0.0007	
S. aureus with LAB cocktail	Y_0	16.37 ± 0.144	<.0001	S^2=0.016 RMSE=0.120 MAE=0.096
	Y_{max}	20.40 ± 0.071	<.0001	
	μ_{opt}	1.144 ± 0.091	<.0001	

Table 44. Kinetic parameters (initial and maximum microbial concentration, Y_0, Y_{max} in ln CFU/g, and optimum growth rate, μ_{opt} in ln CFU/g day^{-1}) of LAB isolated in M17 agar in goat's raw milk cheese during maturation with and without a cocktail of selected LAB and inoculated or not with *S. aureus*, as estimated by the Huang-Cardinal model, along with goodness-of-fit measures (variance, S^2, root mean square error, RMSE, and mean absolute error, MAE).

Treatment	Parameters	Mean ± SE	Pr (>|t|)	Goodness-of-fit measures
Without LAB cocktail	Y_0	15.27 ± 0.531	<.0001	S^2=0.190 RMSE=0.404 MAE=0.349
	Y_{max}	19.57 ± 0.249	<.0001	
	μ_{opt}	1.705 ± 0.475	0.023	
With LAB cocktail	Y_0	16.72 ± 0.464	<.0001	S^2=0.168 RMSE=0.3791 MAE=0.342
	Y_{max}	21.20 ± 0.265	<.0001	
	μ_{opt}	0.979 ± 0.236	0.014	
S. aureus without LAB cocktail	Y_0	15.35 ± 0.321	<.0001	S^2=0.074 RMSE=0.253 MAE=0.232
	Y_{max}	19.96 ± 0.164	<.0001	
	μ_{opt}	1.407 ± 0.221	0.003	
S. aureus with LAB cocktail	Y_0	15.99 ± 0.392	<.0001	S^2=0.102 RMSE=0.292 MAE=0.261
	Y_{max}	20.88 ± 0.236	<.0001	
	μ_{opt}	1.372 ± 0.246	0.011	

Overall, the results of the Huang-Cardinal models built indicate that, regardless of the presence or absence of *S. aureus*, at 10 °C and pH = 6.50 (assumed as optimum), autochthonous LAB grow at a higher rate than those present in cheeses with the addition of the selected LAB cocktail, although they do not reach such high final concentrations. Previous work by Cadavez et al. [23] observed the same trend in terms of reduction of the LAB growth rate, since treatments without addition of a selected anti-listerial LAB cocktail presented higher values of growth rates for LAB than those with addition of the customised starter, as estimated by Jameson-effect models. Gonzales-Barron et al. [16] and Campagnollo et al. [47] also observed higher growth rates of native LAB in comparison to the growth rates of LAB in treatments with a selected and deliberately added starter culture. In these studies, the authors pointed out that the lower growth rate of LAB in cheeses with addition of a selected LAB cocktail could be a consequence of the initial LAB concentration, Y_0, being higher, and therefore, closer to the maximum carrying capacity, and/or a

result of intra-species competition between native LAB and intentionally-added LAB [16,23]. These explanations could also apply to our study.

The influence of the pH on the specific growth rate of LAB can be appreciated in **Figure 26**, which shows values predicted by the underlying cardinal model. The plots illustrate that cheese acidification during ripening causes a reduction of the specific growth rate of LAB for all cheese treatments, i.e., the pH evolution of cheese is towards the lower limit for bacterial growth. To this, as maturation elapses, the lower pH values may directly affect the cells or cause an increase of the degree of dissociation of organic acids [65], thus reducing the growth potential of the LAB.

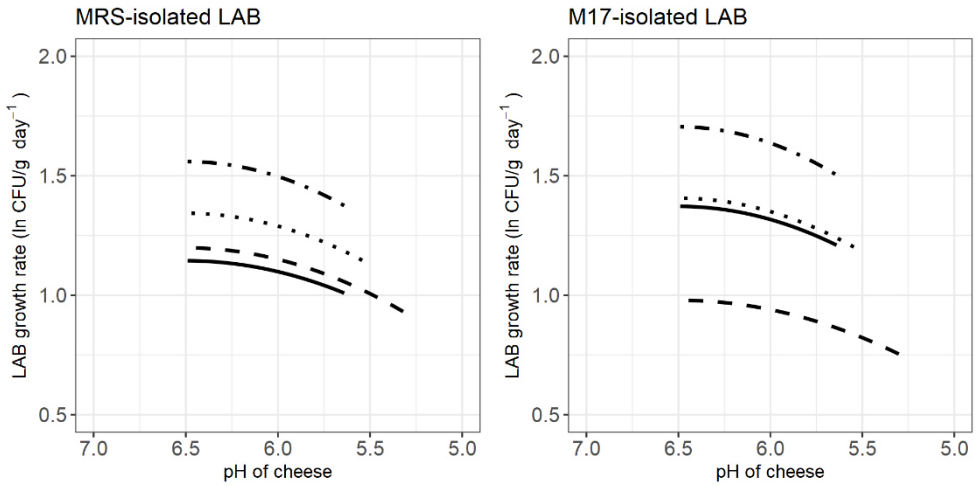

Figure 26. Effect of pH on the specific growth rate (ln CFU/g day^{-1}) of MRS-isolated (left) and M17-isolated LAB (right) in goat's raw milk cheese inoculated with *S. aureus*, with (—) and without (··) the selected LAB cocktail; and non-inoculated, with (- - -) and without the selected LAB cocktail (-•-), as depicted by the Huang-Cardinal parameter model.

9.4 CONCLUSIONS

The Bigelow-type secondary models were able to characterise *S. aureus* survival parameters in goat's raw milk cheese produced with plants extracts (lemon balm, sage, spearmint) or with a customised LAB starter culture during cold maturation; and were able to confirm and quantitatively describe the inhibitory effect of the selected plant extracts and selected LAB cocktail on *S. aureus*.

The results of the Bigelow-type secondary models indicate that both parameters, $\log D_{ref}$ and z_{pH}, were affected by the addition of extracts and the use of the starter culture. z_{pH} values increased with the addition of extracts as a compensatory effect of the slower pH drop caused by the extracts. The dynamic models also demonstrated that the addition of any of the biopreservation

strategies tested reduced the time needed to reduce *S. aureus* by one log, thus showing their ability to act as antimicrobial agents during cheese maturation.

The Huang models pointed lemon balm extract as the one affecting LAB behaviour the least, whereas spearmint extract greatly reduced the growth rate of LAB, although the same final concentration was achieved than that of the control. In turn, the results of the Huang-Cardinal models revealed that autochthonous LAB grow at a higher rate than those of cheeses with a cocktail of selected LAB, and this was independent of the inoculation of *S. aureus*.

The models developed in this work validate the biopreservatives tested as adequate strategies to reduce *S. aureus* contamination and improve the safety of raw milk cheeses. Furthermore, the results also point to the effects of such preservatives on the fermentation parameters. The importance of monitoring the pH decay of cheeses during maturation when incorporating plant extracts was evidenced. In case the appropriate pH drop during fermentation is compromised by the addition of herbal extracts and this affects the quality of the final product, it may be necessary to investigate and implement a solution to overcome this hindrance. Further challenge tests may be directed towards investigating the combined effects of using a starter culture with high acidification capacity and adding herbal extracts of proven inhibitory effects against *S. aureus*. Finally, it is important to note that the models in this work do not account for the temperature effect, so they cannot be used to estimate kinetic parameters at temperatures other than 10 °C.

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SECTION IV

CONCLUSIONS AND SUGGESTIONS FOR FUTURE WORK

CHAPTER 10

GENERAL CONCLUSIONS AND FUTURE PERSPECTIVES

This chapter includes the final remarks, general conclusions and some suggestions for future activities on the bases of the obtained results.

10.1 CONCLUDING REMARKS

Artisanal raw milk cheeses are part of the cultural heritage and tradition of a country, and they are highly appreciated by consumers for their unique organoleptic characteristics. Raw milk cheeses are mainly produced by small-scale local artisans, but given the issues related to their microbial quality and safety, and with increasingly strong food safety legislation, many choose to produce cheese with pasteurised milk, or even stop producing cheeses. Both options imply the loss of ancient, traditional cheese manufacturing practices, with major economic consequences particularly for rural communities that depend on these niche products for their livelihoods.

In this sense, the present thesis aimed to provide specific biopreservation options that could be used to improve the microbiological quality and safety of artisanal goats' raw milk cheeses regarding *S. aureus* contamination. Furthermore, novel modelling approaches were developed for a better assessment of the biopreservation options.

The work developed tested milk thermisation as a mild heat treatment to be used prior to cheese manufacture and evaluated the application of plant extracts and a customised starter culture in cheese production, then assessing their antimicrobial effects against *S. aureus* through predictive microbiology models.

Several methodologies (infusion, decoction, hydroethanolic maceration, solid-liquid, Soxhlet) and non-toxic solvents (water and ethanol) were tested to obtain extracts rich in bioactive compounds from basil, French lavender, lemon balm, sage, spearmint and tarragon. The chemical and bioactive characterisation of the extracts was carried out, and those with the most promising biological capacities (sage, spearmint and lemon balm hydroethanolic solid-liquid extracts) were selected for incorporation in cheese to test their antimicrobial properties in situ during the ripening stage. Additionally, this work compiled a library of indigenous LAB isolated from artisanal goats' raw milk cheeses and evaluated their antimicrobial, acidifying and proteolytic characteristics, also conducting genetic analysis for a subset of the library. From the results, a customised starter culture with bactericidal and acidogenic capacities was built to evaluate the effects of the selected LAB in cheese during maturation.

Mild thermisation was applied by subjecting the raw milk to several treatments at different temperatures, being the inactivation parameters of *S. aureus* estimated through the Weibull model using two modelling approaches (two-step vs. omnibus modelling). Lastly, challenge tests were conducted and Bigelow-type secondary models, Huang models and Huang-Cardinal models were

adjusted to the data sets to describe the effect of the select plant extracts and of the customised starter culture on *S. aureus* survival parameters and LAB behaviour.

The main contributions of this thesis can be summarised as follows:

- Meta-regressions models described the effects of added LAB and essential oils on the inactivation of *L. monocytogenes*, *S. aureus* and *Salmonella* spp. in cheese and showed that the effectiveness of these biopreservatives is conditioned by storage temperature, exposure time, pathogen's inoculum size, antimicrobial concentration and method of application of the biopreservative. The models also revealed two important issues, one related to experimental design and the other related to the need for further investigation on how the pathogen's inoculum size affects microbial kinetics measurements in challenge studies (Chapter 3).
- Basil, French lavender, lemon balm, sage, spearmint and tarragon extracts revealed a wide range of phenolic compounds, including phenolic acids and flavonoids. The extracts showed different phenolic profiles, depending on the plant, extraction methodology and solvent used, with a few exceptions (Chapters 4, 5 and 6).
- Hydroethanolic (70% of ethanol) solid-liquid extracts of lemon balm, sage and spearmint showed great potential as biopreservatives due to their high phenolic contents, antioxidant activities and antimicrobial capabilities (Chapter 4).
- Lemon balm, sage and spearmint infusions revealed antimicrobial, antioxidant, anti-inflammatory and antiproliferative capacities and absence of toxicity against non-tumour cells, although other extraction methods offered higher concentrations of phenolic compounds (Chapter 5).
- French lavender infusion and decoction presented high total phenolic content, antioxidant and antimicrobial activity and selectivity for tumour cells. Basil infusion, decoction and hydroethanolic extract revealed anti-inflammatory power, as did tarragon decoction and hydroethanolic extract (Chapter 6).
- A diverse community of LAB, composed predominantly of *Leuconostoc mesenteroides*, *Lactococcus lactis* and *Lacticaseibacillus paracasei*, was found in artisanal goats' raw milk cheeses from Mirandela, Portugal, with numerous strains presenting antimicrobial and acidifying capacities, which suggests their usefulness when intentionally added to milk to guarantee an adequate pH decrease and to prevent pathogen growth during cheese fermentation and ripening (Chapter 7).

- *S. aureus* inactivation parameters in goats' raw milk at several thermisation temperatures were estimated using the Weibull model in two distinct approaches (two-step vs. omnibus). The omnibus approach improved the estimation of parameters and enabled greater insight of the experimental inactivation data. The temperature influenced the pathogen adaptability in goats' raw milk (Chapter 8).
- The incorporation of lyophilised lemon balm, sage and spearmint hydroethanolic (70% of ethanol) solid-liquid extracts in cheese curd, and the addition of a customised starter culture composed of the strains *Leuconostoc mesenteroides*, *Lacticaseibacillus paracasei*, *Lactococcus cremoris* and *Lactococcus lactis* in milk reduced the time needed to achieve a reduction of one log of *S. aureus*, as revealed by dynamic Bigelow-type secondary models, even though the fermentation of cheeses with these biopreservatives was affected (lower pH decay rates). The integrated Huang models revealed that LAB behaviour was least affected by lemon balm extract, whereas that of spearmint greatly diminished the growth rate of this microbial group. Moreover, the Huang-Cardinal models indicated that indigenous LAB showed a higher growth rate than those of cheeses with the customised starter culture, regardless of the inoculation or not of *S. aureus* (Chapter 9).

In conclusion, this work emphasised the value of various plant extracts as natural food ingredients to prevent spoilage and potentially replace artificial additives, which agrees with current trends in the food industry. Furthermore, this work suggested the application of a customised starter culture to be used in cheese production as well as a mild thermal treatment of milk, using modelling approaches that can be used to design lethality treatments to reduce *S. aureus* contamination in artisanal goats' raw milk cheeses, therefore enhancing their microbiological quality and safety.

Overall, the work developed is also expected to have a significant impact on the development of artisanal and traditional production of cheeses, thus contributing not only to the maintenance of local production systems while maintaining the quality and safety of the products, but also to the sustainable development of local communities and production systems.

10.2 FUTURE PERSPECTIVES

Using predictive microbiology models to unveil the effects of thermisation and incorporation of plant extracts and selected LAB on *S. aureus* survival in goats' raw milk and cheese, this thesis demonstrated different alternatives to control the development of this pathogen in such products.

However, further studies are needed to guarantee their applicability and functionality in goats' raw milk cheeses and to establish the best strategy for their application. Moreover, the production of enterotoxins by *S. aureus* may also be investigated, as they impose a serious health threat to consumers.

In this sense, the following topics are suggested as future research to complement this thesis:

- The proposed extraction procedures must be up-scaled.
- The cytotoxicity, stability and dose-response effects of sage, lemon balm and spearmint hydroethanolic (70% of ethanol) solid-liquid extracts incorporated in cheese must be assessed, as well as their impact on the sensory profile of cheeses. This additional information will help to define the appropriate doses of plant extracts in cheese.
- The impact of the customised starter culture on the sensory characteristics of the final product must be evaluated.
- The substances produced by the customised starter culture that cause *S. aureus* inhibition must be identified.
- The synergetic effect of milk thermisation and use of plant extracts and/or customised starter culture in cheese production may be tested.
- The formation of staphylococcal enterotoxins in goats' raw milk cheeses and the effect of the strategies tested on their concentration.