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Antibacterial properties of plant extracts and isolated phytocompounds against *Listeria monocytogenes* and their effects on the activation of the general stress response

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EMILIJA IVANOVA STOJCHEVA

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EMILIJA IVANOVA STOJCHEVA

Degree in Biochemistry and Physiology

Thesis Director: Dr. José Carlos Quintela

Co-director: Prof. Mª Graciela Pucciarelli

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Fit for Doctor Title

Emilija Ivanova Stojcheva

Thesis director's permission

José Carlos Quintela

Thesis co-director's permission

Mª Graciela Pucciarelli Morrone

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ABSTRACT

Plants synthesize secondary metabolites in response to environmental stimuli. Among these metabolites are potent antimicrobial phytochemicals that serve as defense agents against bacterial pathogens. By sharing natural habitats, bacteria must necessarily be able of sensing the presence of plant antibacterial agents to survive and persevere. The ability of the ubiquitous pathogen *Listeria monocytogenes* to tolerate and survive harsh environmental conditions challenges food producers since it frequently contaminates food production facilities where it utilizes food vehicles to successfully transmit to human hosts and cause foodborne listeriosis. The alternative stress activated sigma factor σ^B (SigB) plays a key role in *L. monocytogenes* in sensing detrimental conditions and circumventing cell injury by reprogramming gene expression, expressing stress tolerance factors and ensuring cell survival. Developing a deeper understanding of how this pathogen resists the environmental stress encountered during its life cycle could facilitate the establishment of new and efficient food safety measures.

In this study, numerous plant samples constituting a mixture of plant extracts, fractions of active extracts and isolated phytocompounds were evaluated for their antibacterial effects against *L. monocytogenes* strain EGD-e wild-type and its isogenic Δ*sigB* mutant. Overall, the majority of the extracts did not affect the growth and survival of these strains, and the ones that did were categorized with either high, medium or low activities, based on their respective minimum inhibitory concentrations (MICs). The most active samples were: the purified hops bitter acids (HBAs) α-acids and β-acids from *Humulus lupulus* L.; oleanolic acid and hydroxytyrosol from *Olea europaea* L.; an *Eucalyptus globulus* L. fraction; two fractions from *Salvia officinalis* L.; and, an *Orthosiphon stamineus* Benth. fraction. The results indicated that there were no differences in the MIC values of the respective samples concerning *L. monocytogenes* wt or Δ*sigB*.

The monitoring in *L. monocytogenes* of the SigB-mediated general stress response following the exposure to a selection of plant antimicrobial agents revealed that not all agents trigger this response. Those agents that stimulated a strong SigB activity did not affect the cytosolic localization or phosphorylation pattern of the core stressosome protein RsbR1, although the results indicated that SigB location could be affected upon exposure to antibacterial stress from plant agents.

Finally, selected plant antimicrobial agents were tested in food matrices, and some agents, including the *H. lupulus* L. HBAs and *S. officinalis* L. products were able to maintain their growth inhibitory effects in the studied food systems. Moreover, selected samples were studied for activity against other food pathogens of interest to the food industry, including *Bacillus subtilis*, *Staphylococcus aureus*, *Bacillus cereus*, *Escherichia coli* and *Salmonella enterica* serovar Typhimurium. The results indicated that most samples active against *L. monocytogenes* were also active against the other Gram-positive food pathogens.

RESUMEN

Las plantas sintetizan varios metabolitos secundarios en respuesta a estímulos ambientales, incluyendo fuertes fitoquímicos antimicrobianos que sirven como agentes defensivos frente a patógenos bacterianos. Al compartir hábitats en la naturaleza, las bacterias deben tener la capacidad de detectar la presencia de agentes antibacterianos de las plantas para poder sobrevivir y persistir. La capacidad del patógeno alimentario, *Listeria monocytogenes*, de tolerar y sobrevivir en condiciones ambientales adversas es un reto al que se enfrentan los productores de alimentos ya que, con frecuencia, llega a contaminar las instalaciones de producción de alimentos, donde puede aprovechar los medios alimentarios para propagarse con éxito a los huéspedes humanos y causar listeriosis. El factor sigma alternativo σ^B (SigB) juega un papel clave en *L. monocyctogenes* en la detección de condiciones perjudiciales la reprogramación de la expresión génica, expresando factores de tolerancia al estrés y asegurando la supervivencia celular. Alcanzar un conocimiento más profundo de cómo esté patógeno resiste a las condiciones de estrés ambiental podría ser clave en el establecimiento de medidas de seguridad alimentaria más eficientes.

En este estudio, se evaluó el efecto antibacteriano de numerosas muestras de plantas, que constituyen una mezcla de extractos de plantas, fracciones de extractos activos y fitoquímicos aislados, frente a la cepa *L. monocytogenes* EGD-e wild-type (wt) y el mutante isogénico Δ*sigB.* La mayoría de los extractos evaluados no afectaron al crecimiento y la supervivencia del patógeno alimentario, y los que sí lo hicieron se clasificaron con baja, medida y alta actividad, en base a su concentración mínima inhibitoria (CMI). Las muestras que mostraron mayor actividad fueron: α-ácidos y β-ácidos purificados de *Humulus lupulus* L., ácido oleanólico e hidroxitirosol de *Olea europaea* L., una fracción de *Eucalyptus globulus*, dos fracciones de *Salvia officinalis* L., y una fracción de *Orthosiphon stamineus* Benth. Los resultados indican que no se observan diferencias entre la CMI de las diferentes muestras estudiadas con respecto a la cepa wt o Δ*sigB.* La activación de la respuesta general de estrés en *L. monocytogenes* por exposición a una serie de agentes antimicrobianos de plantas reveló que no todos los agentes son capaces de generar la respuesta regulada por SigB. Los que fueron capaces de producir una fuerte activación SigB no afectaron a la localización citosólica, ni a la fosforilación de la proteína central del estresosoma RsbR1, aunque los resultados indicaron que la localización de SigB puede verse afectada por la exposición al estrés antimicrobiano.

Finalmente, los agentes antimicrobianos de plantas se ensayaron en matrices alimentarias y, algunos agentes, incluidos los ácidos amargos del lúpulo y productos de *S. officinalis* L., mostraron su capacidad de mantener sus efectos inhibitorios en los sistemas alimentarios estudiados. Adicionalmente, las muestras seleccionadas fueron estudiadas por su actividad frente a otros patógenos de interés para la industria alimentaria, como *Bacillus subtilis*, *Staphylococcus aureus*, *Bacillus cereus*, *Escherichia coli* y

Salmonella enterica serovar Typhimurium. Los resultados indicaron que las muestras más activas frente a *L. monocytogenes* fueron también activas frente a los otros patógenos alimentarios Gram-positivos.

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ABBREVIATIONS

ANOVA	Analysis of Variance
BHI	Brain Heart Infusion
CFU	Colony forming units
CG	Catechin gallate
ECG	Epicatechin gallate
EGCG	Epigallocatechin gallate
EGFP	Enhanced Green Fluorescence Protein
EO	Essential oil
EtOH	Ethanol
EU FR2	Fraction isolated from *E. globulus* L.
FCM	Flow cytometry
GC	Gas Chromatography
GRS	Global Regulatory System
GSR	General Stress Response
HBA	Hops Bitter acids
HPLC	High Performance Liquid Chromatography
HT	Hydroxytyrosol
LAB	Lactic acid bacteria
LB	Luria-Bertani
LC	Liquid Chromatography
log	Common logarithm with base 10
MBC	Minimum Bactericidal Concentration
MeOH	Methanol
MIC	Minimum Inhibitory Concentration
MS	Mass Spectrometry
MSK	Reconstituted skimmed milk
NaCl	Sodium Chloride
o/n	overnight
OA	Oleanolic acid
OD	Optical density
OS FR2	Fraction isolated from *O. stamineus* Benth.

PBS	Phosphate Buffer Saline
pKa	Negative log of the acid dissociation constant
PI	Propidium Iodide
R^2	Pearson Coefficient
RMSE	Root Mean Square Error
RFU	Relative Fluorescence Units
RLU	Relative Luminescence Units
Rsb	Regulator of SigB
SAL FR2	Fraction isolated from *S. officinalis* L.
SAL FR4	Fraction isolated from *S. officinalis* L.
SIC	Sub-Inhibitory Concentration
SigB	Sigma B
SS	Sum of squares
TT	triterpenes
wt	Wild-type
Zn^+	Zink

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INTRODUCTION

1.1. Introduction

During recent years, the rise in popularity and usage of natural products in the global market has evidently increased. Plant preparations, including finished herbal products and starting materials for their production, are gaining stance into the international commerce and trade, which reflects their increased economic value and importance. For instance, the international market size of herbal extracts was assessed at US$48,5 billion in 2020, and is expected to grow at an annual rate of 9,3% to reach US$114 billion by 2030[1]. According to the World Health Organisation (WHO), even today, around 4 billion people worldwide widely utilize traditional medicine, and about 85% of traditional medicine involves the use of plants and plant extracts[2]. Apart from traditional uses, with advances in phytochemical practices, numerous active ingredients have been isolated from medicinal plants and have been introduced into contemporary health systems as effective drugs or drug templates for chemical transformations or *de novo* synthesis[3]. This makes plants a valuable source of bioactive compounds with favourable effects on the human health and well-being. Moreover, since thousands of plant preparations have a long history of use in traditional practices, they have generally been recognized as safe and broadly effective with little to no adverse effects.

Food products can be contaminated by a variety of pathogenic and spoilage microorganisms, the former causing foodborne diseases and the latter causing undesirable effects on the food properties. According to the global estimates from the WHO, each year worldwide consuming unsafe food causes 600 million cases of foodborne diseases and 420 000 deaths[4]. This number is probably even higher, since not all food poisoning cases are officially recorded or their causative agents identified. In this aspect, several foodborne bacterial pathogens have emerged as the main source of concern regarding the safety of foods, including, enteropathogenic *Escherichia coli*, *Campylobacter jejuni*, *Listeria monocytogenes*, *Bacillus cereus*, *Streptococcus aureus* and *Yersinia enterocolitica,* among others[5]. Apart from the devastating effects on human health, microbial food spoilage imposes a great economic burden on the food industry. The growth of microorganisms in foods has a negative impact on the shelf-life, textural characteristics and overall quality of the food products, affects the consumer choices, and results in significant commercial losses[6]. A report by the Food and Agriculture Organization of the United Nations has stated that as much as one-third of the food produced for human consumption is either spoiled or wasted[7], imposing an economic burden not only on farmers and food producers, but on consumers as well. Thus, prevention of microbial growth and elimination of foodborne pathogens constitutes a pertinent issue for the current globalized food production that requires the attention of various stakeholders.

Food preservation includes any food processing practices of treating and handling foods in order to stop or slow down food spoilage and maintain food quality. Chemical additives have revolutionized the food industry by significantly extending the shelf-life of food products, however, their excessive use has been criticized for their possible side effects and has eventually resulted in mistrust among consumers. The increased awareness on the safety of food additives and preservatives, have generated a significant number of studies and publications on the potential use of various natural substances, "generally recognized as safe" (GRAS), to be used as food preservatives[6]. In this regard, the use of plant-derived compounds with antimicrobial activities presents an intriguing case. On one side, people have used the healing powers of various plants for medicinal purposes since ancient times, including the treatment of infectious diseases. On the other, plants and their essential oils and extracts have also been recognized and used for centuries in food preservation. Essential oils were used by the early Egyptians and spices such as clove, cinnamon, mustard, garlic, ginger and mint have been used for centuries in Asian countries including China and India[8]. Moreover, antioxidant properties usually accompany the antimicrobial characteristic of plant products, making the product more effective and more valuable by providing dual beneficial properties. At present, the food industry mainly uses plant products as flavouring agents and functional additives, with a limited number of plant-based compounds, mostly essential oils, used for extending the shelf-lives of certain food products[119]. Further studies in this field can result in introducing novel natural compounds into the food industry and satisfy consumer needs.

1.2. Natural products of plant origin and their potential use as food antimicrobials

The plant kingdom is a deep well of chemically diverse compounds. It is estimated that there are between 250,000 and 500,000 species of plants on Earth and only a minor portion of them have been investigated for antimicrobial activity[9]. Plants have many attractive features for this purpose: they are readily available and cheap, extracts or compounds from plant sources often demonstrate high-level activity against microbial pathogens, and their long and extensive use in traditional practices has already confirmed they rarely have severe side effects. Moreover, rapid advancements in modern biotechnologies open up a way for obtaining natural products in environmentally friendly and low-toxic conditions.

The beneficial properties of plants are mainly due to a mixture of bioactive substances called secondary metabolites. The secondary metabolites of plants are a diverse group of molecules produced by the plant's cells that are non-nutritive and not vital for the plant's survival, but play important roles in interspecies competition and environmental defences. Some, such as terpenoids, give plants their odours,

others, like quinones and tannins are responsible for the plant pigment, many, such as, vanillin, capsaicin, limonene, cinnamaldehyde and others are responsible for the plant's flavour.

Up to date, nearly two-hundred thousand different secondary metabolites of plants have been isolated and identified[10]. These substances, known as phytochemicals, display an extensive range of desirable health effects on the human body and thus are broadly used in the pharmaceutical, cosmetic, agricultural and food industries. The secondary metabolites of plants can be classified based on their chemical structures and/or biosynthesis pathways. A simple classification includes three main groups: phenolic compounds (biosynthesized from shikimate pathways, containing one or more hydroxylated aromatic rings), terpenes and terpenoids (polymeric isoprene derivatives biosynthesized via the mevalonic acid pathway) and alkaloids (non-protein nitrogen-containing compounds, biosynthesized from amino acids, like tyrosine). Together, these groups make up about ninety percent of all plant secondary metabolites. Minor groups include saponins, lipids, carbohydrates, organic acids, aldehydes, ketones and others.

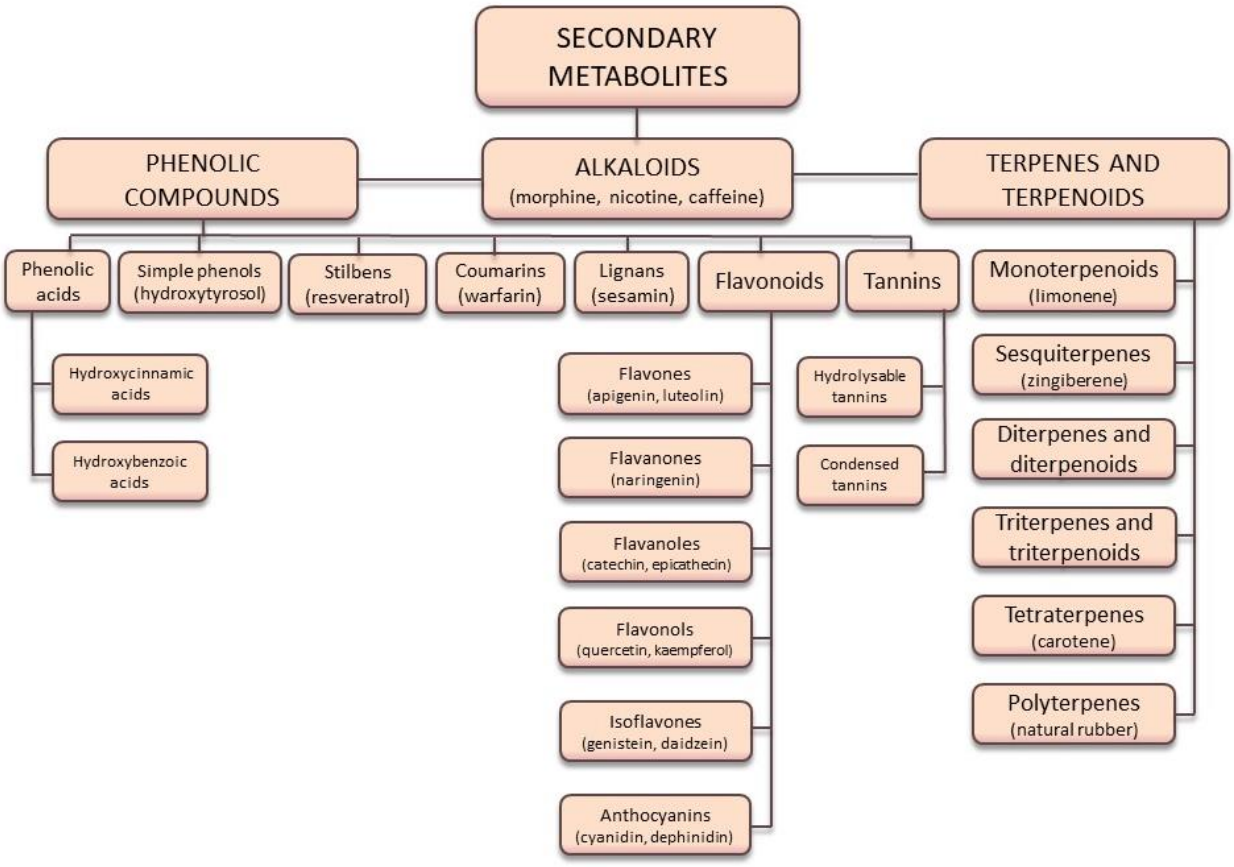


Figure 2. Main phytochemical classes and families

Phenolic compounds are the most abundant class of secondary metabolites in plants[11]. They are characterized by the presence of one or more phenolic functional groups and display a wide range of chemical complexity and diversity, ranging from simple compounds with one aromatic ring like gallic acid ($C_7H_6O_5$), to highly complex polymeric substances like punicalagins ($C_{48}H_{28}O_{30}$). A simple categorization of this broad class of compounds divides the phenolics into simple phenols and polyphenols. More fully, phenolics are classified into: simple phenols, phenolic acids, flavonoids, tannins, coumarins, stilbenes and lignans. The group of simple phenols and phenolic acids is widespread, and includes a variety of molecules that usually contain additional functional groups, including hydroxytyrosol (a phenylethanoid), vanillin (a phenolic aldehyde) rosmarinic, ferulic and caffeic acid (phenolic acids). **Flavonoids** are a family of phenolic compounds with high structural diversity, and several thousand substances belonging to this group have been identified. Flavonoids may be divided into various subclasses according to the oxidation level of the central ring in the flavonoid skeleton. The most common subdivision includes: flavonols, flavones, flavan-3-ols, flavanones, anthocyanidins and isoflavones. Some authors consider that flavonoids are synthesized by plants in response to microbial infection, since they have been found *in vitro* to be effective antimicrobial substances against a wide array of microorganisms [12,13]. **Tannins** are polymeric phenolic substances with high molecular weights. Unlike other phenolics, tannins are capable of precipitating protein, a property known as astringency. They are classified into two families according to their structure and biosynthetic origin, namely, hydrolysable and condensed tannins. The hydrolysable tannins represent esters of phenolic acids and polyols and are formed from the shikimate metabolic route. Depending on the type of phenolic acids, they are divided into gallotannins, which can be hydrolysed to gallic acid, and ellagitannins, which can be hydrolysed to ellagic acid. On the other hand, condensed tannins, also called proanthocyanidins, are highly hydroxylated polymers of flavan-3-ol units, synthesized from the phenylpropanoid metabolism. They do not contain sugar residues and yield anthocyanidins when depolymerized under oxidative conditions. Some tannins and resins produced by plants have antimicrobial and wound-healing properties[14]. **Coumarins** are a family of naturally occurring derivatives of benzo-α-pyrone, presumed to be produced by plants as a chemical defence against predation. Coumarins are known for their remarkable array of biochemical and pharmacological activities with coumarin itself representing a favoured molecular scaffold for medicinal chemists[15]. **Stilbenes** are a relatively small group of plant secondary metabolites found mostly as heartwood constituents with resveratrol as the most widely known representative, while lignans are low-molecular weight compounds found frequently in fibre-rich plants. Most phenolic compounds are water soluble and many are effective antioxidants and free radical scavengers, especially flavonoids. Certain phenolics are valued

pharmacologically for their bioactive properties, for example, quercetin is a known anti-inflammatory agent and silybin has antihepatotoxic properties. Genistein and daidzein are known soy phytoestrogens, while naringenin exerts cardioprotective properties[11].

Terpenes and terpenoids are a large and structurally and functionally diverse class of secondary metabolites produced predominantly by plants. Over 55,000 members of this class of metabolites have been isolated so far[16]. Terpenes have long provided humans with flavours, fragrances, hormones, medicines and even commercial products such as rubber[17]. While the terms terpenes and terpenoids are often used interchangeably, terpenes are a class of naturally occurring hydrocarbons characterized by the presence of isoprene subunits, while terpenoids are derived from terpenes and contain oxygen and cyclic groups. Most terpenes are hydrophobic in nature and are typically volatile, while terpenoids may be non-volatile or semi-volatile as they normally contain other polar moieties. Terpenes and terpenoids can be classified according to the number of carbon atoms they possess as follows: hemiterpenoids (C5), monoterpenoids (C10), homoterpenoids (C11,16), sesquiterpenoids (C15), diterpenoids (C20), sesterpenoids (C25), triterpenoids (C30), tetraterpenoids (C40), and polyterpenoids (C>40, higher-order terpenoids)[6]. Well known terpenoids are α-pinene, menthol and camphor from the monoterpenoids family, farnesol and artemisin (sesquiterpenoids), taxol and steviol from the diterpenoids, betulinic, oleanolic and ursolic acids (triterpenoids), carotenoids (tetraterpenoids) and many others.

Alkaloids are a class of basic, naturally occurring compounds that contain at least one nitrogen atom. Since they do not represent a homogeneous group of compounds from any standpoint, whether chemical, biochemical, or physiological, their definition and classification is somewhat problematic. Alkaloids have diverse and important physiological effects on humans. The activity of alkaloids against herbivores, toxicity in vertebrates, cytotoxic activity, mutagenic or carcinogenic activity, antibacterial, antifungal, antiviral and allelopathic properties have all been reported in literature considering this class of metabolites[11]. Well-known alkaloids include morphine, strychnine, quinine, ephedrine, and nicotine.

It is clear that the secondary metabolites of plants are a chemically diverse group of compounds with miscellaneous bioactivities. Going back to the antimicrobial properties of these compounds, some interesting examples include cranberry juice, *Vaccinium macrocarpon* L. (family: Ericaceae) which is used in the management of urinary tract infections and the prevention of recurrent cystitis[18]. Bearberry tea, *Arctostaphylos uva-ursi* L. (family: Ericaceae), is an antimicrobial agent used also for controlling urinary tract infections and is effective against several organisms including *Bacillus subtilis*, *E. coli*, *Mycobacterium smegmatis*, *S. aureus* and *Shigella* spp[19]. The antibacterial activity is attributed to its compound arbutin. Berberine has antibacterial activity against various strains of methicillin resistant *S. aureus* (MRSA). Lemon

balm, *Melissa officinalis* L. (family: Lamiaceae) and tea tree, *Melaleuca alternifolia* (Maiden & Betche) Cheel (family: Myrtaceae) are described as broad-spectrum antimicrobial agents and are taken as herbal teas, while the tea tree oil is applied as an ointment[21]. Garlic, *Allium sativum* L. (family: Alliaceae) has antimicrobial and antiseptic properties and is used for respiratory tract infections[22]. The essential oil constituents of plants, or their volatile compounds, also possess antimicrobial activity. It is said that plants produce phytoalexins in response to infections caused by fungi, viruses and bacteria that may infect them. Resveratrol is an example of a phytoalexin with antifungal activity, additional to its already recognized antioxidant and cardioprotective benefits. Other plant metabolites with antibacterial properties include anti-staphylococcal activities of the acylphloroglucinols and terthiophenes.[19]

Numerous studies have shown that the mechanisms of antimicrobial activity of plant extracts and their bioactive compounds are extensive and mainly include: promoting cell wall disruption and lysis or impairing cell wall synthesis, rupturing of the cell membrane, inducing the production of reactive oxygen species, inhibiting microbial replication, interfering with the proton motive force and depleting energy-rich substances such as ATP, inhibiting biofilm formation, attenuating virulence or reducing the production of bacterial toxins. This is due to the huge variety of plant compounds that introduce diverse chemical structures with copious mechanisms underlying antimicrobial activity. For example, the mechanism of action of many nonpolar terpenic compounds like thymol, eugenol, carvacrol and ursolic acid is bacterial membrane disruption, while the phenolics resveratrol, quercetin and luteolin are protein efflux pump inhibitors. Moreover, the catechin epigallocatechin gallate (EGCG) and the coumarin aegelinol inhibit DNA replication[23].

1.3. Bioassay-guided fractionation as an effective method of obtaining bioactive compounds

The discovery of novel natural products can follow different strategies. Plant extracts are complex mixtures of molecules, and purely analytical or synthetic approaches cannot be applied for obtaining new plant products with biological activities. Instead, an effect-directed analytical concept that combines the examination of biological effects with the identification and quantification of molecular entities is needed. A method that applies this approach is the process of bioassay-guided fractionation, a step-by-step sequence of operations with the final objective of obtaining a purified biologically active plant fraction or compound[24]. In the process of bioassay-guided or bioactivity-guided fractionation, a crude extract is tested for activity, then chemically separated, then the resulting fractions are tested for activity. The process continues by further separation of the most active fraction and testing its subfractions, repeating

until a single, or multiple active compounds are isolated. A schematical representation of the main steps involved in bioassay-guided fractionation is given in Figure 2.

Secondary steps using this approach can include determination of the active concentration of the compound, finding the amount of active compound present in the original extract and comparison of the activities of the active compound and the crude extract from which it was isolated. Depending on the potential of the biological compound, additional steps like synthesis, structural modification, testing of related compounds and investigation of the mode of activity can also be included.

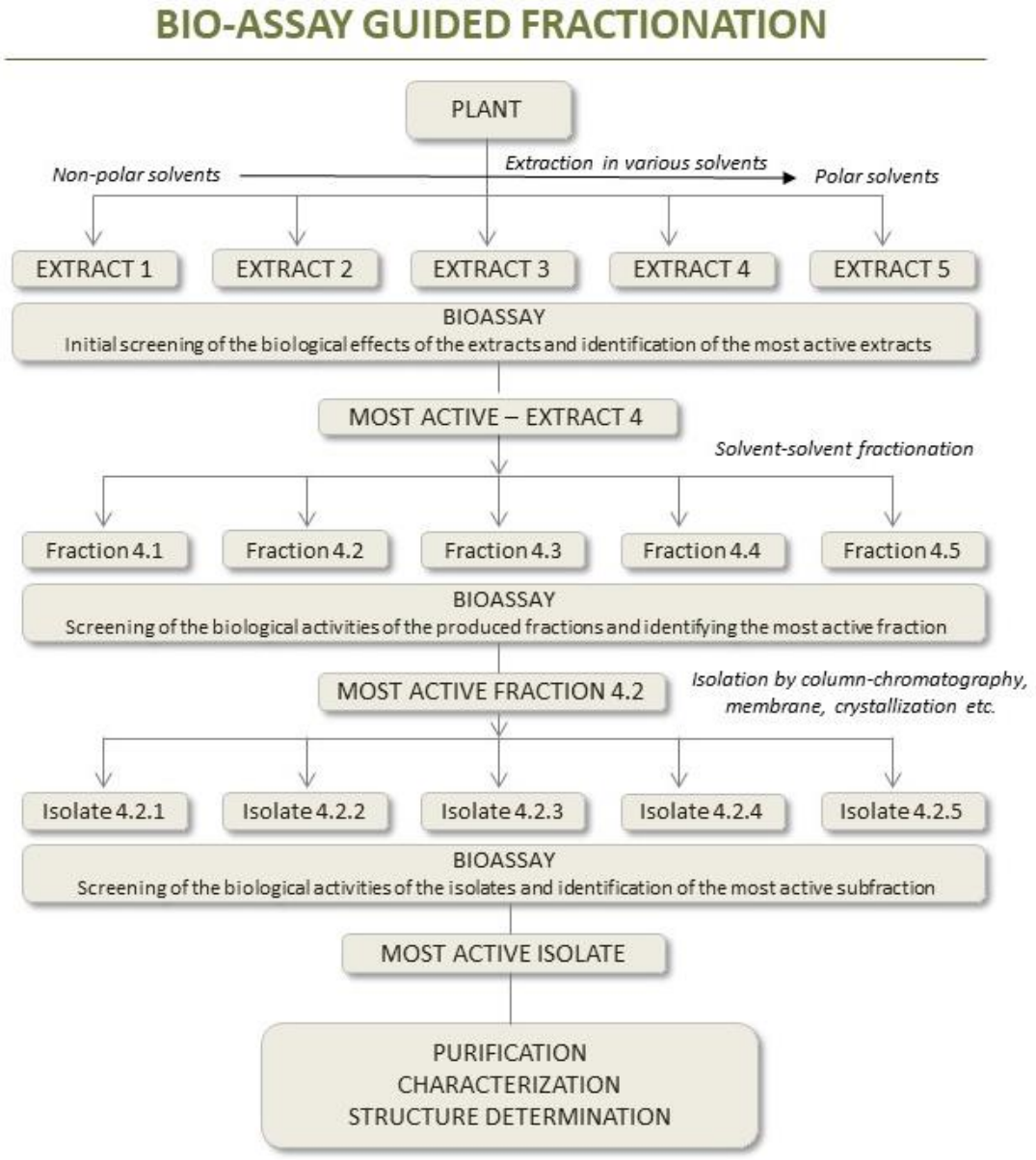


Figure 2. Schematic representation of bioassay-guided fractionation

1.4. Obtaining natural products: Extraction, fractionation and characterization

Extraction is the crucial first step in the process of discovering novel bioactive natural products, since it is first necessary to draw out the desired secondary metabolites from the plant before it undergoes further purification and characterization. By definition, extraction is the method of separation of soluble plant constituents from insoluble plant metabolites and the plant's cellular matrix using selective solvents. During extraction, the solvent penetrates into the plant material and dissolves chemical compounds with a related polarity. The basic parameters influencing the nature of the extract are the plant's part used as a starting material, the extraction solvent, the ratio between the plant and the solvent and the selected extraction technique. Methods such as maceration, infusion, percolation, decoction, Soxhlet extraction, heating under reflux, steam and hydro-distillation are considered classical extraction techniques, while modern techniques include microwave-assisted extraction, pressurized liquid extraction, supercritical fluid extraction, ultrasound extraction, counter-current extraction, surfactant-mediated extraction etc. In order to assess the extraction performance two main parameters are significant: global extraction yield and selectivity. Global yield is used to quantify the extraction recovery. It depends on the physicochemical nature of the extraction solvent and the presence of compounds of similar nature in the plant material. [25] The higher the yield of extraction, the better the extraction performance. However, there are cases when the goal is to extract one family or compound of interest over another. In this case, selectivity is considered to find the best extraction conditions that enrich the extract with the compounds of interest and avoid the unwanted ones. In this context, purity is the critical parameter to assess the extraction performance.

Once the primary extract is obtained and biologically evaluated, it can undergo further separation processes to remove the unwanted components and/or increase its potency by concentrating the active ones. This process is called fractionation. In fractionation, differences in solubility, size, shape, electrical charge, melting or boiling point between the compounds are exploited to achieve the desired separation. The most common methods used to achieve this goal include: precipitation, solvent-solvent extraction, distillation, membrane separations, chromatographic procedures, electrophoresis and solid-phase extraction. When choosing the fractionation method, the most important factor to be considered is the nature of the substances present in the extract. Unless preliminary chemical tests have been carried out, this knowledge may consist only of the approximate solubility of the components based on the type of solvent used to make the extract. For example, if water is used as the extracting solvent, the components present will be polar in nature and may include compounds carrying an electrical charge. On the other hand, if a non-polar solvent such as hexane has been used, mainly non-polar, non-charged substances will

form the major components of the mixture. Another important factor is the susceptibility of the components to degradation. Since stability of the substances present is often unknown, a good general principle is to carry out fractionations under the mildest possible conditions, including, minimization of temperature, protection from light and avoidance of reactive solvents and other chemical substances[26].

Once separation of the phytochemicals with similar properties is achieved, the next important step is the isolation and characterization of the active compounds. The benefits of using methods to purify or isolate individual phytochemicals are mostly related with improving their efficacy or reducing cytotoxicity[27]. Purifying compounds from a mixture of other, possibly structurally related phytocompounds is a complex process that largely depends on the chemical nature of the target compound and the impurities present. Recovering a target phytochemical from a mixture of compounds with similar physico-chemical properties requires a series of individually designed steps, each one removing some of the unwanted compounds and concentrating the desired one. This renders purification a unique process specifically tailored for the desired phytochemical.

The successfulness of purification of a natural compound is assessed by determining the purity of the isolated product with different analytical methods. Techniques that are commonly used to analyse the biologically active phytochemicals exploit the differences in partition coefficients of the compounds and include chromatography, crystallization, sublimation, distillation and differential extraction. High performance liquid chromatography (HPLC) is a versatile, robust, and widely used technique for both the isolation and separation of natural products and their subsequent identification. The remarkable resolving power of HPLC is ideally suited for analysing complex multicomponent samples of biological origin where the active entity can be present as a major or minor component[28]. A similar analytical technique is gas chromatography (GC), a chromatographic technique that is based on the same separation principles but is mainly used for separating gases and volatile compounds that can be vaporized without decomposition. HPLC and GC usually require the use of reference standards and previously established standard curves to identify components. While they are perfect for confirmation of the presence or absence of the wanted phytochemical, they can impose difficulties when the compounds are unknown. For this reason, HPLC and GC are often coupled to mass spectrometry, since the individual capabilities of each technique for separation and structural identity combined with high molecular specificity and detection sensitivity are enhanced synergistically providing an ideal identification tool in this combination.

Plant extracts are a mixture of secondary metabolites out of which some have biological activities. Sometimes, only one component of this mixture is the bioactive entity, but in many cases the extract contains several bioactive compounds that all contribute to the observed grade of biological activity.

Hence, even though identification of the bioactive compound is useful and should be carried out when possible, in many cases once the major and minor compounds are separated, the bioactivity declines.

1.5. Choice of biological assay

Once the desired plant product is obtained, the biological activities of the candidate plant product, including its effects on prokaryotic and eucaryotic cell lines, studies in food models, animal models or human volunteers need to be screened for assessing its bioactive potential. In this matter, the choice of good and satisfactory *in vitro* and/or *in vivo* assays are essential for identifying potential industry use and developing end product formulations. Considering plant-derived products aimed for use as antimicrobials, evaluating the potential of these preparations or substances to kill pathogenic microorganisms or inhibit their growth is fundamental. Unlike the standards and guidelines for antimicrobial susceptibility testing of antibiotics established by the Clinical and Laboratory Standards Institute[29], a universal reference method for assessing the antimicrobial properties of natural products has not been settled. Nonetheless, a variety of *in vitro* assays have been developed and are used. These assays rely on the inherent properties of the antimicrobial agents and microorganisms, and include, among others, the common methods like microdilution methods and broth assays, agar well diffusion and disc diffusion methods, time-kill and time to detection assays and biofilm assays; and less common methods like gradient assays, bioautography, bioluminescence assays, flow cytometric assays and others.

Dilution methods are the reference methods for antimicrobial susceptibility testing against which other methods, such as disk or well diffusion, are calibrated[30]. They allow quantitative assessment of the antimicrobial susceptibility by determining the lowest concentration of the agent capable of inhibiting the growth of the tested organism known as the *Minimum Inhibitory Concentration* (MIC)[31]. Furthermore, the *Minimum Bactericidal Concentration* (MBC), the lowest concentration of the antimicrobial agent needed to kill the tested microorganism can also be determined. MIC methods are widely used in the comparative testing of new agents. Dilution methods can be subdivided into broth and agar dilution methods, depending on whether the tests are taking place in liquid media or on solid agar plates[32]. In the case of broth dilutions, both macro- and microdilution methods are similar and well established. Redox indicators and changes in turbidity are often utilized for results interpretation in broth dilution methods. Changes in turbidity can be evaluated either visually or by measuring the optical density of the solution at 600 nm, while colorimetric changes in the indicator color can represent absence or presence of bacterial growth.

Even though using broth dilution methods for assessing the antimicrobial activity of plant extracts is a very common occurrence, they can entail difficulties that can result in misinterpretation of the results.

The main reason for the difficulties is the dispersion problem of water insoluble compounds in the liquid growth medium. Despite the use of dispersing and emulsifying agents, hydrophobic or oily substances are often poorly soluble in liquid medium and separation of the oil–water phases can occur. In such circumstances, even contact between the test organism and the agent is not unequivocally ensured. Furthermore, the determination of the MIC value becomes problematic when the opacity of oil–water emulsions conceals the turbidity of bacterial growth. Similarly, strongly coloured compounds or extracts, also make the MIC value impossible to determine by broth dilution because they preclude distinction between bacterial growth and the medium. To help resolve some of these difficulties, the agar dilution method can be used for assessing and/or confirming the antibacterial activities of problematic water-insoluble extracts[33].

1.6. Use of microbial biosensors in bioactivity assays

Microorganisms, due to their low cost, long lifetime and a wide range of pH and temperature tolerance, have been widely employed as the biosensing elements in designing biosensors[34]. In the construction of effective microbial biosensors, the physical signal from the recognition element can be linked to a given transducer either by methods that immobilize whole cells onto the transducer or by microorganisms that are genetically modified with different types of measurable signals. Various methodologies based on genetic/protein engineering and synthetic biology to construct microorganisms with the required signal outputs, sensitivity and selectivity have been developed. One of them is reporter gene expression under the control of a specific regulatory network, a powerful technique that offers increased sensitivity and provides a simple and easy sensor platform[35]. In recent years, many different genetically modified microbial biosensors have been constructed to measure gene expression and study cell-trafficking mechanisms. Commonly used reporter genes are enzymes with activities for colorimetric, fluorescent or luminescent readouts. Between them, the most frequently used are: β-galactosidase (β-gal) for colorimetric detection, luciferase for blue-green light emission detection, fluorescent proteins like enhanced green fluorescent protein (eGFP) for the detection of fluorescence signals and others.

1.7. Listeria monocytogenes is an important foodborne pathogen

Listeria spp. are Gram-positive, facultative anaerobic bacteria that do not form spores, do not have a capsule and are motile at temperatures from 10 to 25°C. The bacterial species included in this genus are of low G+C content and are closely related to the genera *Bacillus*, *Clostridium*, *Enterococcus*,

Streptococcus, and *Staphylococcus*[36]. *Listeria* spp. are widely distributed in the environment and can be isolated from a broad range of environmental sources including, but not limited to, soil, water, silage and effluents, and a large variety of foods and food processing environments. Until recently, the genus *Listeria* had consisted of eight species and two subspecies, however, new species have been identified and this number has now increased to 17 species[37]. Only two species of this genus, *L. monocytogenes* and *L. ivanovii*, are considered pathogenic to humans and ruminants, respectively.

The natural habitat of *L. monocytogenes* is soil and decomposing plant matter, in which it lives as a saprophyte. Given its ubiquitous distribution in the environment along with its ability to form biofilms, it frequently contaminates the raw materials used in the preparation of industrially processed foods as well as food processing and production plants. Moreover, this bacterium is well equipped to survive harsh environments and food processing technologies[38]. It can tolerate high salt concentrations and relatively low pH, and most concerning of all, it is capable of multiplying at refrigeration temperatures (0-4°C). All these factors render *L. monocytogenes* a serious threat to food safety and ranks these bacteria among the microorganisms of highest concern to the food industry[39,40,41,42].

The infectious diseases caused by *L. monocytogenes* are known as listeriosis. For many years, the epidemiology of listeriosis was unresolved, however, at the end of the 1970s and the start of the 1980s, the number of reports on *Listeria* isolations begun to increase, and from 1983 onwards, a series of epidemic outbreaks in humans clearly established listeriosis as an important foodborne infection[36]. Even though listeriosis is a relatively rare disease with 0.1 to 11.3 cases per 1 million people per year depending on the country and region of the world, the high mortality (20%–30%) and hospitalization (91%) rate following infection make listeriosis a significant public health concern[43]. Listeriosis can manifest as a non-invasive disease with mild symptoms in healthy adults and a severe invasive disease in certain risk groups including pregnant women, neonates, elderly or immunocompromised people. The invasive form of listeriosis is a serious infection characterized by fever, myalgia, septicaemia, meningitis and meningo-encephalitis, and can cause miscarriage and stillbirth in pregnant women. In Europe, human listeriosis peaked in incidence during the 1980s, showed a general decline during the 1990s and stabilized in the 2000s[44]. However, more recent data show a statistically significant increasing trend of human invasive listeriosis cases reported in the EU/European Economic Area (EEA) in the last ten years, mostly as a result of large foodborne outbreaks. From 2012–2016, between 1,754 and 2,555 *L. monocytogenes* cases were reported annually by 30 EU/EEA countries, and according to the latest European Union One Health Zoonoses Report from EFSA and the European Centre for Disease Prevention and Control (ECDC), the number of people affected by listeriosis in the EU in 2018 was 2,549 cases, similar to the cases reported

in 2017 which was 2,480[45]. A constantly increasing trend of listeriosis cases has also been observed in Spain, where 5,696 listeriosis hospitalizations occurred between the period of 1997-2015. It was concluded that the age group ≥65 years old was the most represented (50%) in the hospitalizations, while pregnant women and neonates accounted for 7% and 4% of hospitalizations, respectively. In this period, death occurred in 17% of patients, more frequently among those ≥65 years old (67.5%)[44]. The results of this study are in line with the listeriosis rate reported to EFSA in 2012 which concluded that Spain had the second highest listeriosis rate of any country in the European Union (EU) (0.93, vs an EU-wide incidence rate of 0.41/100,000 population)[46], indicating that listeriosis is an emergent public health problem in Spain and more attention needs to be given to preventing and controlling of this disease.

1.8. Foodborne transmission and the intracellular life cycle of *L. monocytogenes*

Listeriosis is usually caused after eating foods contaminated with *L. monocytogenes* and outbreaks of this disease have generally involved the ingestion of foods containing high doses of bacteria. The most frequently implicated foods are soft cheeses and dairy products, pâtés, sausages and other delicatessen, smoked fish, salads, raw fruits and vegetables and minimally processed, ready-to-eat (RTE) food products[43,45]. Upon ingestion of contaminated food by the host, the gastrointestinal tract is the primary site of entry for *L. monocytogenes.* The bacterium is capable of colonizing the intestinal epithelium, and at this stage the listeriosis is non-invasive, but if the immune system is not able to control the infection it can progress to become invasive as the pathogen can traverse the intestinal epithelial barrier into the lamina propria and disseminate through the mesenteric lymph nodes to the bloodstream and towards its primary target organs, the liver and spleen[39,40]. From here, *L. monocytogenes* can then cross the blood–brain barrier in immuno-compromised persons or the fetoplacental barrier in pregnant women. Release of the bacteria into the bloodstream can also result in septicaemia[40,47,48].

L. monocytogenes is an intracellular pathogen, which indicates that it can survive and reproduce inside host cells. This pathogen triggers the entry into non-phagocytic cells like epithelial cells or phagocytic cells like macrophages and dendritic cells by interaction of its surface adhesion proteins called internalins, InlA and InlB, with their respective host cell surface receptors E-cadherin and Met[40,]. This then leads to internalization of the bacterium inside the host cell into an endocytic vacuole which is ruptured through the activity of secreted virulence effectors, the pore-forming toxin listeriolysin O (LLO) and the phosphatidylinositide phospholipase C (PI-PLC)[58]. Once into the cytosol, the bacteria use cytosolic nutrients to replicate and to stimulate the polymerization of cellular actin comet trails by the surface protein ActA, which promotes actin-based motility and cell-to-cell spread[39,40,49]. The mechanism of

transmission, spread to target organs and intracellular cycle of *L. monocytogenes* into host cells are depicted in Figure 3.

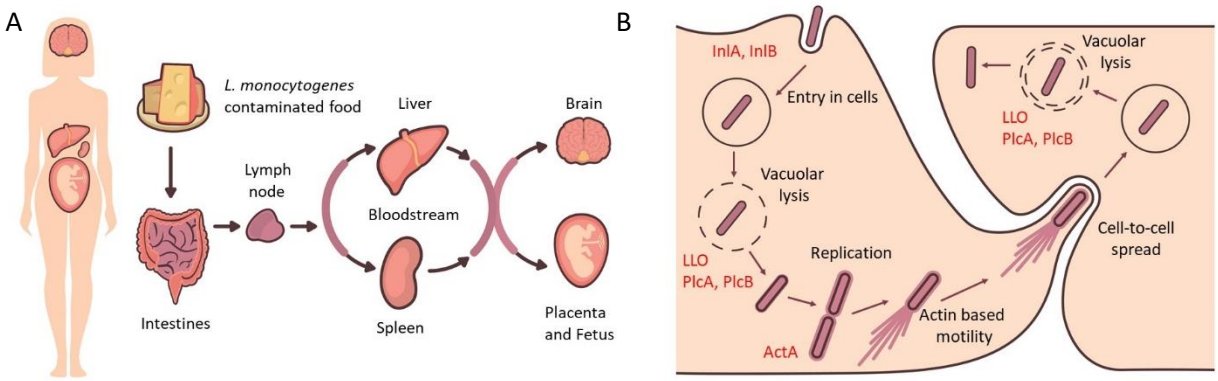


Figure 3. Dissemination of *L. monocytogenes* to target organs and scheme of its intracellular cell cycle. A) *L. monocytogenes* infection of a human host. After ingestion of contaminated food, *L. monocytogenes* traverses the intestinal barrier and spreads into the bloodstream through the lymph nodes to disseminate to target organs, the liver and spleen. In at risk individuals, *L. monocytogenes* the blood–brain barrier or fetoplacental barrier and provokes potentially fatal meningitis, sepsis, premature birth or abortion. B) *L. monocytogenes* invades host cells through receptor-mediated endocytosis (Inl, internalin) and mediates vacuolar escape using potent virulence factors such as the hemolysin Listeriolysin O (LLO), and the phospholipases PlcA and PlcB. Upon vacuolar escape, *L. monocytogenes* polymerizes actin (ActA, actin assembly-inducing protein) and spreads from cell to cell through actin-based motility.

Considering the ubiquitous distribution of *L. monocytogenes* in the environment and the high contamination rate of raw and industrially processed foods[50], ingestion of these bacteria is likely to be a common event. Nonetheless, as it was previously mentioned, the incidence of human listeriosis is low, up to 10 sporadic cases annually per million population in Europe and the United States[45,51]. This is consistent with the relatively high 50% lethal dose (LD_{50}) values reported for experimentally infected mice by the oral (10^9) or parenteral (10^5 to 10^6) routes[52]. The minimum dose of *L. monocytogenes* required to cause clinical infection in humans has not been clearly established, however, the number of *L. monocytogenes* bacteria detected in foods responsible for epidemic and sporadic cases of listeriosis was typically higher than 10^5 CFU per serving[36]. On the other hand, it cannot be excluded that lower doses may cause infection in the identified risk groups, like immunocompromised individuals, the elderly and pregnant women, where levels of contamination as low as 10^2 to 10^4 *L. monocytogenes* cells per g of food have been associated with cases of listeriosis[53,54]. Additionally, the infectious dose may also vary according to the virulence potential and pathogenicity of the *L. monocytogenes* strain involved in the infection.

The increase in listeriosis incidence rates can mostly be attributed to two distinct elements. On one hand, the ageing of the population, the appearance of the 'fourth age' group and the increase in life expectancy of immunocompromised patients due to advances in medicine and, on the other, to changes in the ways food is produced, stored, distributed and consumed around the world[43]. Over the past several

years, consumer interest in consumption of minimally processed and RTE foods has increased because of their freshness, convenience, and healthy attributes. According to the Scientific Opinion on *L. monocytogenes* contamination of RTE foods and the risk for human health in the EU published by the Panel on Biological Hazards of the European Food Safety Authority (EFSA) in 2015, the RTE food categories typically associated with human listeriosis, i.e. meat and meat products, fish and fish products, and milk and milk products continue to have a significant public health impact. Moreover, plant-derived foods or even frozen foods have also been implicated in several outbreaks lately (for example cantaloupe, caramel apples, ice cream), illustrating that almost all RTE foods may support growth and/or have the potential to contribute to the burden of listerioses in Europe[43]. In Spain, a recent study has found that the most frequently contaminated RTE food category with *L. monocytogenes* was smoked fish, although the most recent outbreak of August 2019 was associated with the consumption of a chilled roasted pork meat product manufactured in Spain and sold under the brand name "La Mechá"[44,55]. The U.S. Food and Drug Administration and U.S. Department of Agriculture Food Safety and Inspection Service have established a zero tolerance for *L. monocytogenes*, meaning no detectable level in RTE food products including minimally processed fresh fruits and vegetables[56]. In Europe, including Spain, food safety criteria (FSC) for *L. monocytogenes* follow the European Commission (EC) regulations to keep the concentration of *L. monocytogenes* in RTE foods below 100 CFU/g[57].

1.9. L. monocytogenes is one the most stress resilient food pathogens

Apart from its widespread distribution, one of the most significant factors contributing to the persistence of *L. monocytogenes* in the environment is its ability to withstand adverse environmental conditions[50,59,60]. Along the food chain, bacteria are continuously exposed to a wide range of stress factors affecting their physiological activity and viability, with a final goal to limit their growth and/or reduce their number. These stresses can either be intrinsic to the food matrix or extrinsic factors intentionally applied to preserve the food product. The ability of *L. monocytogenes* to withstand these stresses allows for successful colonization in various niches along the food production facilities and formation of reservoirs for contamination[50,61]. The stresses encountered by *L. monocytogenes* in food products include various methods of preservation, as the traditional ones like using organic acids or lactic acid bacteria to acidify the food product and lower its pH, osmotic stress introduced by increasing the concentrations of osmolytes like salt or sugar, and, more contemporary measures like adding growth inhibition substances[38]. At the same time, there are more technical measures of food preservation that are designed either to kill food pathogens and spoilage microorganisms at the processing stage - applying high

temperatures or high pressure, pulsed electric fields, ionization and radiation, or to protect the food product during storage, including low temperatures, low oxygen concentrations, presence of protective gases in the package atmosphere and others.

A number of transcriptional regulators important for stress response and virulence gene expression are central in the adaptation of pathogenic bacteria to harmful environments. The bacterial responses to stress factors can either be exerted in the form of immediate (shock) response or long-term adaptation. In many cases, immediate and long-term adaptation responses are generated through similar "shock proteins" and the synthesized proteins that are a product of the resistance mechanisms may be effective against a single or multiple stress factors. Various stress responses have been extensively described in literature for *L. monocytogenes*, including among others: i) a *cold stress response,* mediated by highly conserved chaperones designated as cold shock proteins (Csps) that control uptake of cryoprotective osmolytes and increased membrane permeability; ii) a *heat shock response,* promoted by the increased production of highly conserved chaperones and ATP-bounded proteases like GroES, GroEL and DnaK known as heat shock proteins (Hsps); iii) *osmotic stress adaptation*, mediated by protein transport systems like BetL, Gbu and OpuC that accumulate substances that help maintain the intracellular osmotic pressure; iv) an *acid tolerance response*, mainly controlled by the glutamate carboxylase (GAD) system and a H^+ATPase multi-subunit enzyme system; and, v) an *oxidative stress response* controlled by the bacterial ROS (reactive oxygen species) detoxification systems and others[38,62,63]. In many of these, the involvement of small regulatory RNA molecules, hypothesized to initialize the response by modulating protein translation has been recently shown [188,189].

Ultimately, the stress responses of *L. monocytogenes* are not only important for survival in challenging external and food-associated conditions, but also for survival in hostile host environments. The previous exposure to environmental stress challenges that lead to the activation of the stress response systems prior to interaction with susceptible hosts can significantly contribute to the pathogenicity of *L. monocytogenes* and its ability to infect humans and cause foodborne listeriosis[38, 64]. A deeper understanding of the mechanisms used by *L. monocytogenes* to survive and proliferate in food products may help food specialists to design efficient preservation methods that will extend shelf lives and at the same time provide a better protection of consumers against this pathogen.

1.10. Sigma B (σ^B) controls the general stress response in *L. monocytogenes*

The physiological changes resulting from the response to particular environmental stress stimuli in *L. monocytogenes* are a consequence of changes at gene transcription and/or protein expression levels.

Over the past years, it has become clear that, apart from regulating the expression of specific single genes, bacteria have evolved global regulatory networks that control the simultaneous expression of a large number of genes. These systems are called Global Regulatory Systems (GSR), and they respond to a variety of environmental stresses, such as changes in temperature, pH, nutrients, and oxidation[65]. The existence of GSRs in bacteria that control the expression of large regulons was implied by the observation of induction of large groups of proteins in response to specific environmental stimuli[190]. In controlling transcriptional regulation, the most crucial role is played by sigma factors, dissociable multi-domain subunits of the prokaryotic RNA-polymerase that largely determine which genes are transcribed at any time by directing the transcriptional machinery to the appropriate promoter sequences[66].

Most *L. monocytogenes* strains have five sigma factors, including the principal housekeeping sigma factor σ^A (also named SigA for the gene that encodes it) and four alternative sigma factors, σ^B, σ^C, σ^H, and σ^L (SigB, SigC, SigH and SigL)[70,71,72]. σ^B is a stress-activated sigma factor that controls the general stress response (GSR) in *L. monocytogenes* with more than 300 genes (approximately 10% of the genome) under its positive control[67]. The *sigB* locus has so far been described in three genera of Gram-positive bacteria, *Bacillus, Listeria* and *Staphylococcus*, where it has been recognized as a general stress-responsive sigma factor that contributes to survival under environmental and energy stress conditions[68]. While this sigma factor has been positively identified in low-GC-content, Gram-positive bacteria, it does not appear to be present in other Gram-positive bacteria, although, a σ^B-like alternative sigma factor, often designated as σ^F, has been identified in some high-GC-content, Gram-positive bacteria, such as *Mycobacterium tuberculosis*[69]. For *L. monocytogenes*, σ^B has demonstrated an important role in controlling the expression of genes associated with acid and bile tolerance, in osmotic stress responses, cold and freezing stresses, oxidative stress, in response to certain antimicrobials, in visible light recognition and high hydrostatic pressure stress regulation[70,73,74,75]. A mutant strain lacking σ^B activity shows increased sensitivity and reduced survival rate under a wide range of stresses compared to its wild-type parent, not only environmentally associated, but also during intracellular survival, which was shown in a murine infection model[76]. Today, the role that σ^B plays in virulence and host cell invasion of *L. monocytogenes* has been clearly established both by directly regulating the transcription of PrfA[77,78], the master regulator of virulence in *L. monocytogenes*, and indirectly regulating PrfA activity by moderating the expression of PrfA dependent genes during infection[79,80,81,112]. Overall, the current understanding is that σ^B plays a pivotal role in the early gastrointestinal stages of infection, whereas the virulence regulator PrfA dominates during systemic spread and the intracellular stages of the infectious cycle[70,72,82,83].

1.11. Model of Sigma B (σ^B) regulation and the σ^B signaling cascade

Activation of the correct stress response systems required for *L. monocytogenes* survival is only possible by previous assimilation of information about the surrounding environment and the possibility to process this information quickly in order to adapt to the challenging conditions. While the importance of σ^B is paramount in adequately controlling the GSR, ensuring adaptation and survival[38,63,68,84], σ^B itself is carefully regulated by a complex signal transduction pathway encoded by a set of *rsb* (regulator of sigma B) genes. This signal cascade culminates in the release of σ^B from an inhibitory complex with its primary regulator, the anti-sigma factor RsbW, which is followed by its activation and binding to the promoters it regulates. From extensive research in *B. subtilis*, a model has been developed describing the activation of σ^B by environmental or energy stress signals. In this model, the stress signals are sensed by a large 1.8 mDa multi-protein complex called the 'stressosome' that initiates a cascade of partner-switching protein interactions culminating in increased levels of free σ^B [86,87]. While evidence for the existence of the stressosome complex within *L. monocytogenes* has only recently been obtained[92,93], BLAST comparisons between the genomes of *L. monocytogenes* and *B. subtilis* have shown high levels of sequence homology among the stressosome components and the σ^B signalling cascade, suggesting that the model established for *B. subtilis* represents a solid guide for outlining the structural components and signalling cascade that lead to the activation of σ^B in *L. monocytogenes*[69,70,].

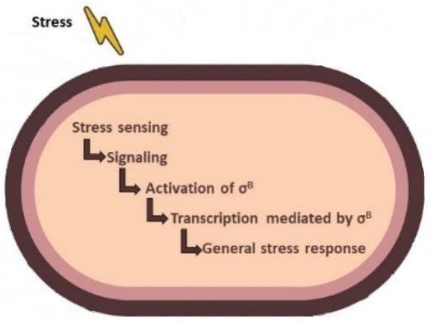


Figure 4. The pathway from stress sensing to activation of σ^B and protection. Once a stress is sensed, a signal is relayed that leads to the activation of σ^B. Then σ^B associates with core RNA polymerase and transcription of the σ^B regulon is initiated. The protein products encoded by the genes under σ^B control confer stress protection to the cell.

In *B. subtilis*, the *sigB* operon which encodes σ^B, includes eight genes whose products are involved in σ^B regulation (*rsbR*, *rsbS*, *rsbT*, *rsbU*, *rsbV*, *rsbW*, *sigB*, and *rsbX*). RsbV and RsbW form a set of proteins that directly regulate σ^B activation by protein–protein interactions, known as the partner-switching mechanism[89]. In unstressed cells, σ^B is held inactive by RsbW, preventing it from interacting with RNA-

polymerase, and RsbV is mainly found in its phosphorylated form, RsbV-P. During stress, the serine phosphatase RsbU is activated and dephosphorylates RsbV-P, and the unphosphorylated form of this anti-σ^B antagonist competes for binding to RsbW. Since RsbW has a higher affinity for the dephosphorylated RsbV, as the concentration of the newly formed alternative complex RsbV-RsbW increases, the concentration of free σ^B increases, allowing σ^B to bind to the RNA-polymerase. RsbW has also an important role in deactivating σ^B since it has a functional role of a serine kinase, the substrate for which is RsbV. Phosphorylation of RsbV induces the dissociation of the RsbV-RsbW complex and RsbW can then interact again with σ^B thus enabling its inactivation[86,87,88,89,90]. The signal integration hub known as the stressosome that acts upstream of RsbU is composed of three "core" proteins, RsbR, RsbS, and RsbT. The stressosome is thought to sense stress through the N-terminal domain of RsbR, leading to the phosphorylation of RsbR and RsbS by RsbT, and subsequently to the dissociation of RsbT from the multi-protein complex[91]. Upon dissociation, RsbT interacts with RsbU and activates its phosphatase activity. In the absence of stress, another phosphatase protein called RsbX has the role of resetting the stressosome to its dephosphorylated form by dephosphorylating RsbS, thus enabling RsbT to reassociate with the RsbR:RsbS complex instead of with RsbU[88,90] (Figure 5).

The atomic structure of the *L. monocytogenes* stressosome has recently been determined by cryo-electron microscopy at 3.38 Å resolution[92]. The structure has revealed great similarities in arrangement of the *L. monocytogenes* stressosome and the one of *B. subtitlis*. In *L. monocytogenes*, the truncated icosahedral complex contains 20 dimers of RsbR and 10 dimers of RsbS arranged in repeating units of 2 dimers of RsbR and 1 dimer of RsbS. The structure contains a core region made up of RsbS:RsbT complexes, into which the conserved STAS (Sulfate Transport and Anti-anti-Sigma factor) C-terminal domain of RsbR is embedded, leaving the N-terminal region of the RsbR protein which is more consistent with a sensory perception role to protrude out in the form of a turret[70,92]. In addition to RsbR, four RsbR proteins paralogues with high levels of sequence similarity in the C-terminal domain are believed to co-exist with RsbR in the stressosome complex[92,93]. In *B. subtilis*, RsbR and its paralogs are designated RsbRA, RsbRB, RsbRC, RsbRD, and YtvA[86,90,91,94], while their homologs in *L. monocytogenes* are named RsbR1 (*lmo0889*), RsbR2 (Lmo0161), RsbL (Lmo0799), RsbR3 (Lmo1642), and RsbR4 (Lmo1842)[93,128]. As previously mentioned, the role of the kinase RsbT in the stressosome is to phosphorylate RsbR and RsbS leading to its subsequent dissociation and activation of RsbU[90]. Similar to *B. subtilis*, the substrates for this phosphorylation in *L. monocytogenes* are conserved serine (Ser59) and threonine (Thr175 and Thr 209) residues in RsbS and RsbR respectively[69,79]. Structural analysis has revealed that these conserved residues are located close to the surface of the stressosome and would be easily accessible for phosphorylation by

RsbT[92]. Nevertheless, the mechanism by which RsbT's kinase activity is activated upon the perception of a stress signal is so far not understood.

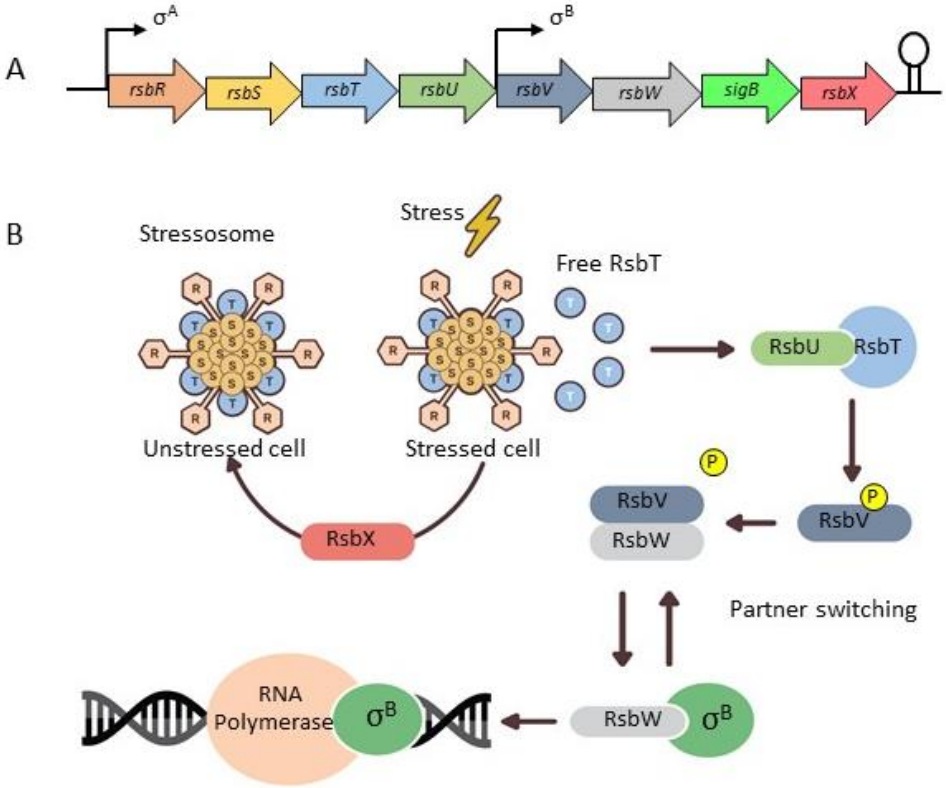


Figure 5. The *sigB* operon and model of the σ^B signalling cascade in *L. monocytogenes*. (A) The *sigB* operon of *L. monocytogenes* includes eight genes whose products are involved in σ^B regulation and activation. The position of the σ^A- and σ^B-dependent promoters is indicated. (B) The regulatory mechanism that leads to the activation of σ^B: The stressosome is composed of two dimers of RsbR, one dimer of RsbS, and two monomers of RsbT. When a stress signal is detected, conformation changes in the protein complex initiate the kinase activity of RsbT, resulting in the phosphorylation of RsbR and RsbS, and releasing of RsbT from the stressosome. Free RsbT interacts with and activates the RsbU phosphatase, which acts on phosphorylated RsbV. Simultaneously, the anti-sigma factor RsbW that binds and prevents σ^B from interacting with the R- polymerase, releases σB and preferentially interacts with non-phosphorylated RsbV. σ^B can then bind to the R- polymerase forming a holoenzyme. Once the stress signal is removed, RsbX dephosphorylates the stressosome, resulting in the sequestration of RsbT back into the stressosome and inactivating the signal transduction pathway.

Even though great advances have been made in the elucidation of the structure of the stressosome complex and the downstream σ^B signalling cascade, how the stressosome perceives stress signals from the environment has remained elusive even today[70,85,93]. Understanding the details of how pathogens integrate stress signals from their surrounding environment is critical, since their immediate fate is determined by the responses it catalyses in order to subvert or adapt to the existing stress. A deeper knowledge of how the mechanisms of sensory perception that ultimately lead to the activation of σ^B work could be a vital step in developing novel strategies to undermine σ^B-mediated protective functions and, ultimately, to prevent this pathogen from surviving in the food chain and the human host.

OBJECTIVES

2. OBJECTIVES

Plants synthesize a diverse range of secondary metabolites with bioactive properties, some of which are known to offer protection against pathogenic bacteria. A significant environmental impact on bacteria is stress, which, in effecting a myriad of adaptive and protective responses, alters gene expression patterns and cell physiology in ways that influence antimicrobial susceptibility. *L. monocytogenes* is a ubiquitous bacterium that shares its natural habitat with plants, so we hypothesize that *L. monocytogenes* will be able to detect environmental stresses associated with plant antimicrobial compounds.
The objectives of this thesis were the following:

1. To identify newly prepared and existing plant extracts from the extract library of Natac Biotech affecting the growth and survival of *Listeria monocytogenes*
2. To fractionate the active extracts, and purify and characterise the bioactive compounds mediating such antibacterial effects
3. To dissect whether the antibacterial effect occurs by interference with general stress responses
4. To evaluate the possibility of developing an antibacterial method for the identification of novel antimicrobials using stress-sensing biosensors
5. To escalate the most promising compounds to real food trials
6. To test the most effective compounds on other food pathogens, including *Bacillus subtilis*

MATERIAL & METHODS

3. MATERIAL & METHODS

3.1. Bacterial strains and culture conditions

All strains used in this study are listed in Table 1. below.

Table 1. Bacterial strains used in the study

Bacterial strains	*Used in Chapter*	*Reference / Source*
Listeria monocytogenes		
EGD-e wild-type	1, 2, 3	Glaser *et al*.[71]
EGD-e Δ*sigB*	1, 2, 3	Guerreiro *et al*.[191]
EGD-e WT/pKSV7-$P_{lmo2230}$::eGFP	2	Utratna *et al*.[110]
EGD-e Δ*sigB*/pKSV7-$P_{lmo2230}$::eGFP	2	Utratna *et al*.[110]
EGD-e pPL2*lux*P_{help} (EGDe*lux*)	3	Riedel *et al*.[125]
EGD-e Δ*rsbR1*	2	O'Donoghue *et al*.[111]
EGD-e *rsbR1* T175A	2	Dessaux *et al*.[128]
EGD-e *rsbT* N49A	2	Dessaux *et al*.[128]
ScottA	3	Briers *et al*.[192]
2F	3	NIZO2608
6E	3	NIZO2603
Bacillus subtilis		
BG214	3	Alonso L.C.
168 wt	3	Kunst *et al*.[193]
Escherichia coli		
ATCC 29532	3	ATCC® 29532™
MG1655	3	Blattner *et al*.[194]
Raw cheese isolate	3	NIZO350
Milk isolate	3	NIZO352
Salmonella enterica subsp. enterica		
Serovar Typhimurium 14028s	3	Jarvik *et al*.[195]
Serovar Typhimurium ATCC 13311	3	ATCC® 13311™
Serovar Infantis	3	NIZO4678
Serovar Putten	3	NIZO4679
Staphylococcus aureus		
Newman	3	Baba *et al*.[196]
ATCC 19095	3	ATCC® 19095™
Raw milk isolate	3	NIZO846
Farm cheese isolate	3	NIZO 3808
Bacillus cereus		
ATCC 14579	3	ATCC® 14579™
Fermented dairy products isolate	3	NIZO4153
Bacillus weihenstephanensis WSBC 10204	3	DSM 11821

All *L. monocytogenes* strains were grown in Brain Heart Infusion broth (BHI; Bacto™ BD) at 37°C, 30°C or room temperature (RT) with continuous shaking (120 rpm). *B. subtilis*, *E. coli*, *S. enterica*, *S. aureus* and *B. cereus* were grown in Luria-Bertani (LB) broth at 37°C with continuous shaking (120 rpm). All strains were periodically recovered on agar plates (BHI agar or LB agar) from culture stocks stored at -80°C in 20% sterile glycerol. The bacteria were kept on plates short term at 4°C for two weeks, expect *L. monocytogenes* EGD-e WT/pKSV7-$P_{lmo2230}$::eGFP which was kept at 4°C for one week maximum as a higher level of basal fluorescence was detected for older cultures of this strain. Chloramphenicol was used to maintain the plasmids in strains EGD-e WT/pKSV7-$P_{lmo2230}$::eGFP, EGD-e ΔsigB/pKSV7-$P_{lmo2230}$::eGFP and EGD-e pPL2luxP$_{help}$ (EGDe*lux*) at a concentration of 10 µg/mL.

3.2. Plant material, reagents and analytical standards

Dried plant material was acquired from the warehouse of Natac Biotech. The plant material used in this study met the requirements of the European Pharmacopoeia for the industrial production of plant extracts. The originating plant material was further processed as described in Section 3.5. All samples were processed and analyzed within 12 months of collection.

All solvents and reagents from various suppliers (VWR International, LLC and Sigma-Aldrich, Darmstadt, Germany) were of the highest purity needed for each application. Analytical standards included aescin, apigenin-7-O-glucoside, betulin, betulinic acid, caffeic acid, carnosic acid, carnosol, catechin, p-coumaric acid, ellagic acid, epicatechin, epicatechin gallate (ECG), epigallocatechin gallate (EGCG), gallic acid, hesperidin, hydroxytyrosol, hyperoside, hops bitter acids international standard, isoflavones, maslinic acid, oleanolic acid, oleuropein, phloridzin, punicalagins, proanthocyanidins, quercetin, resveratrol, rosmarinic acid, rutin, silymarin, tannic acid, ursolic acid, verbascoside, vitexin and xanthohumol. The analytical standards were purchased from Extrasynthese, Genay Cedex, France and Sigma-Aldrich, Darmstadt, Germany.

3.3. Preparation of the initial plant extracts

The plants, extraction conditions and analytical markers of the initial 120 plant extracts used in the general screening are provided in Table 2. All other *de novo* extractions, fractionations and purifications of the active extracts not discarded after the general screening are provided in Section 3.5.

Table 2. Code, plant origin, plant part, manner of obtaining and analytical markers of the extracts used in the initial study.

CODE	PLANT	PLANT PART	EXTRACTION	ANALYTICAL MARKER	CONCENTRATION
PS-001	*Cynara scolymus* L.	leaves	Solid-liquid extraction, 100% water	Cynarine and derivatives	5.8%
PS-002	*Vaccinium myrtillus* L.	fruit	Solid-liquid extraction and chromatographic purification (elution 50% ethanol)	Anthocyanins	36%
PS-003	*Vaccinium macrocarpon* L.	fruit	Solid-liquid extraction and chromatographic purification (elution 60% ethanol)	Proanthocyanidins	80%
PS-004	*Coffea arabica* L.	fruit	Solid-liquid extraction, 50% ethanol	Chlorogenic acid and derivatives	50%
PS-005	*Camelina sativa* L.	seed	Solid-liquid extraction, 80% ethanol	Polyphenols	10.2%
PS-006	*Aesculum hippocastanum* L.	seed	Solid-liquid extraction, 90% ethanol	Aescin	21,8%
PS-007	*Citrus sinensis* L.	pericarp	Solid-liquid extraction, 100% water (pH 12), and crystallisation at pH 4	Hesperidin	60%
PS-008	*Curcuma longa* L.	root	Solid-liquid extraction, 96 % ethanol	Curcuminoids	95%
PS-009	*Echinacea purpurea* L. (Moench)	root	Solid-liquid extraction, 70% ethanol	Chicoric acid	4%
PS-010	*Punica granatum* L.	pericarp	Solid-liquid extraction, 70% ethanol and acid hydrolysis	Ellagic acid	40%
PS-011	*Punica granatum L.*	pericarp	Solid-liquid extraction, 70% ethanol	Punicalagins	40%
PS-012	*Lippia citriodora* L.	leaves	Solid-liquid extraction, 96% ethanol	Verbascoside	20%
PS-013	*Zingiber officinale* Rosc.	root	Supercritical fluid extraction with 5% ethanol as a co-solvent	Gingerols	20%
PS-014	*Humulus lupulus* L.	hop cones	Supercritical fluid extraction with 5% ethanol as a co-solvent	Hop bitter acids	35%
PS-015	*Pirus malus* L.	pericarp	Solid-liquid extraction and chromatographic purification (elution 60% ethanol)	Phlorizine	5%
PS-016	*Chamaemelum nobile* L.	flowers	Solid-liquid extraction, 70% ethanol	Apigenins	5%
PS-017	*Olea europaea* L.	fruit	Chromatographic purification of the aqueous fraction of the fruit	Hydroxytyrosol	20%
PS-018	*Olea europaea* L.	leaves	Solid-liquid extraction of the leaf, 60% ethanol	Oleuropein	20%
PS-019	*Olea europaea* L.	fruit	Solid-liquid extraction of the fruit, 96% ethanol	Maslinic acid	25%
PS-020	*Olea europaea* L.	leaves	Solid-liquid extraction of the leaf, 96% ethanol	Pentacyclic triterpenes	20%
PS-021	*Rosmarinus officinalis* L.	leaves	Solid-liquid, 96% ethanol, concentration, addition of water, precipitation and recuperation of the insoluble fraction	Carnosic acid	20%

CODE	PLANT	PLANT PART	EXTRACTION	ANALYTICAL MARKER	CONCENTRATION
PS-022	*Rosmarinus officinalis* L.	leaves	Solid-liquid extraction, 96% ethanol, concentration, addition of water and recuperation of the soluble fraction	Rosmarinic acid	6%
PS-023	*Ruscus asculeatus* L.	rhizome	Solid-liquid extraction, 96 % ethanol	Ruscogenins	5%
PS-024	*Salvia officinalis* L.	leaves	Solid-liquid extraction of the leaf, 96% ethanol, concentration, addition of water, precipitation and recuperation of the insoluble fraction	Ursolic acid	15%
PS-025	*Salvia officinalis* L.	leaves	Solid-liquid extraction of the leaf, 96% ethanol, concentration, addition of water and recuperation of the soluble fraction	Polyphenols	10%
PS-026	*Camellia sinensis* L.	leaves	Solid-liquid extraction y chromatographic purification (elution 60% ethanol)	Flavan-3-oles	60%
PS-027	*Vitis vinifera* L.	seed	Solid-liquid extraction y chromatographic purification (elution 60% ethanol)	Proanthocyanidins	95%
PS-028	*Vitis vinifera* L.	fruit	Aqueous extraction solid-liquid of grape skins and chromatographic purification (elution 60% ethanol)	Anthocyanins	5%
PS-029	*Polygonum cuspidatum* Houtt.	root	Solid-liquid extraction of the root, 96% ethanol, hydrolysis and chromatographic purification of the aglycone	Resveratrol	98%
PS-030	*Vitis vinifera* L.	vine shoots	Solid-liquid extraction of vine shoots	Viniferine	5%
PS-031	*Olea europaea* L.	leaves	Solid-liquid extraction, 96% ethanol, crystallization	Oleanolic acid	50%
PS-032	*Olea europaea* L.	leaves	Solid-liquid extraction, 96% ethanol, concentration, addition of water and recuperation of the precipitate	Pentacyclic triterpenes	40%
PS-033	*Olea europaea* L.	fruit and leaves	Successive extractions (alcohol, hydroalcoholic mixtures and water) of the fruit and leaf followed by purification of the principal groups of active compounds from olive	Hydroxytyrosol	3.2%
				Oleanolic acid	11.7%
				Maslinic acid	3.0%
				Tocopherols	3.1%
PS-034	*Olea europaea* L.	fruit	Solid-liquid extraction, 96% ethanol	Maslinic acid	10%
				Hydroxytyrosol	2%
PS-035	*Valeriana officinalis* L.	rhizome	Solid-liquid extraction, 70% ethanol	Valerenic acids	0.80%
PS-036	*Fucus vesiculosus* L.	talus	Solid-liquid extraction, water	Iodine	0.2%
PS-037	*Cynara scolymus* L.	leaves	Solid-liquid extraction, water	Caffeoylquinic acids	10%

CODE	PLANT	PLANT PART	EXTRACTION	ANALYTICAL MARKER	CONCENTRATION
PS-038	*Trifolium pratense* L.	leaves	Solid-liquid extraction and chromatographic purification (elution 60% ethanol)	Isoflavones	20%
PS-039	*Verbascum thapsus* L.	flowers	Solid-liquid extraction, 60% ethanol	ratio plant:extract	4:1
PS-040	*Raphanus sativus* L.	radix	Solid-liquid extraction, 60% ethanol	ratio plant:extract	5:1
PS-041	*Cupressus sempervirens* L.	cone	Solid-liquid extraction, 60% ethanol	Polyphenols	2%
PS-042	*Citrus aurantium* var. *amara* L.	pericarp	Solid-liquid extraction, 90% ethanol	Synephrine	10%
PS-043	*Eleutherococcus senticosus* (Rupr. & Maxim.)Maxim.	root	Solid-liquid extraction, 70% ethanol	Eleutherosids	0.8%
PS-044	*Rosa canina* L.	fruit	Solid-liquid extraction, water	ratio plant:extract	4:1
PS-045	*Garcinia gummi-gutta (L.) N. Robson*	pericarp	Solid-liquid extraction, water + precipitation $Ca(OH)_2$	Hydroxycitric acid	60%
PS-046	*Triticum aestivum* L.		Solid-liquid extraction, 90% ethanol	ratio plant:extract	4:1
PS-047	*Ginkgo biloba* L.	leaves	Solid-liquid extraction and chromatographic purification (elution 60% ethanol)	Flavone glycosides	24%
PS-048	*Punica granatum* L.	fruit	Pulverized juice	ratio plant:extract	10:1
PS-049	*Glycine max* L.	seed	Solid-liquid extraction and chromatographic purification (elution 60% ethanol)	Isoflavones	40%
PS-050	*Glycine max* L.	seed	Solid-liquid extraction and chromatographic purification (elution 60% ethanol)	Aglycone Isoflavones	32%
PS-051	*Lepidium meyenii* Walp.	root	Solid-liquid extraction, 96% ethanol	Isoflavones	40%
PS-052	*Melissa officinalis* L.	leaves	Solid-liquid extraction, 70% ethanol	Rosmarinic acid	5%
PS-053	*Mentha piperita* L.	leaves	Solid-liquid extraction, 70% ethanol	Essential oil	2%
PS-054	*Urtica dioica* L.	leaves	Solid-liquid extraction, 70% ethanol	Chlorogenic acid	2%
PS-055	*Urtica dioica* L.	root	Solid-liquid extraction, 70% ethanol	Betasitoesterol	0.8%
PS-056	*Orthosiphon stamineus* Benth.	leaves	Solid-liquid extraction, 70% methanol	Flavonoids	3%
PS-057	*Orthosiphon stamineus* Benth.	leaves	Solid-liquid extraction, 90% ethanol	Sinensetin	0.2%
PS-058	*Passiflora incarnata* L.	flowers	Solid-liquid extraction, 70% ethanol	Flavonoids	4%
PS-059	*Citrus paradisi* Macfad.	pericarp	Solid-liquid extraction, water (pH=12), and crystallization at pH=4	Flavonoids	45%
PS-060	*Glycyrrhiza glabra* L.	root	Solid-liquid extraction, 96% ethanol	Glycyrrhizic acid	20%
PS-061	*Rhodiola rosea* L.	root	Solid-liquid extraction, 70% ethanol	Rosavin	5%
				Salidroside	2%

CODE	PLANT	PLANT PART	EXTRACTION	ANALYTICAL MARKER	CONCENTRATION
PS-062	*Cassia angustifolia* Mill.	leaves	Solid-liquid extraction, 60% ethanol	Sennosides	20%
PS-063	*Tilia cordata* Mill.	leaves	Solid-liquid extraction, 70% ethanol	ratio plant:extract	6:1
PS-064	*Vaccinium myrtillus* L.	fruit	Solid-liquid extraction y chromatographic purification (elution 50% ethanol)	Anthocyanins	36%
PS-065	*Aloe vera* L.	leaves	Solid-liquid extraction, 60% ethanol	Hydroxyanthracene derivatives	13.6%
PS-066	*Vaccinium macrocarpon* L.	fruit	Solid-liquid extraction and chromatographic purification (elution 70% ethanol)	Proanthocyanidins	80%
PS-067	*Cinnamomum zeylanicum* J.Presl	bark	Solid-liquid extraction, 70 % ethanol	ratio plant:extract	10:1
PS-068	*Allium sativum* L.	bulb	Solid-liquid extraction, 70% ethanol	Allicin	0.5%
PS-069	*Panax ginseng* Baill.	root	Solid-liquid extraction, 96% ethanol	Ginsenosides	12%
PS-070	*Althaea officinalis* L.	leaves	Solid-liquid extraction, 70% ethanol	ratio plant:extract	4:1
PS-071	*Paullinia cupana* Kunth	seed	Solid-liquid extraction, water	Caffeine	10%
PS-072	*Pimpinella anisum* L.	fruit	Solid-liquid extraction, water	ratio plant:extract	4:1
PS-073	*Humulus lupulus* L.	cones	Solid-liquid extraction y chromatographic purification	Xanthohumol	25%
PS-074	*Silybum marianum* L.	seed	Solid-liquid extraction, acetone:water (80:20)	Silymarin	50%
PS-075	*Origanum vulgare* L.	leaves	Solid-liquid extraction, 70% ethanol	ratio plant:extract	4:1
PS-076	*Ribes nigrum* L.	leaves	Solid-liquid extraction, 60% ethanol	Flavonoids	5%
PS-077	*Cichorium intybus* L.	root	Solid-liquid extraction, water	ratio plant:extract	6:1
PS-078	*Plantago lanceolata* L.	seed	Solid-liquid extraction, water	powder	1:1
PS-079	*Humulus lupulus* L.	cones	Supercritical fluid extraction followed by solid-liquid extraction and chromatographic purification	Xanthohumol	2.5%
				Hops bitter acids	10%
PS-080	*Crocus sativa* L.	stigmas	Solid-liquid extraction, 80% ethanol	Safranal	2%
PS-081	*Euterpe oleracea* Mart.	fruit	Solid-liquid extraction, 50% ethanol	Anthocyanidins	2%
PS-082	*Sambucus nigra* L.	flowers	Solid-liquid extraction, 70% ethanol	ratio plant:extract	4:1
PS-083	*Verbena officinalis* L.	aerial part	Solid-liquid extraction, 70% ethanol	ratio plant:extract	4:1
PS-084	*Mangifera indica* L.	fruit	Solid-liquid extraction, 70% ethanol	ratio plant:extract	10:1
PS-085	*Hypericum perforatum* L.	flowers	Solid-liquid extraction, 70% ethanol	Hyperforin	10%
PS-086	*Hypericum perforatum* L.	flowers	Supercritical fluid extraction (CO_2)	Hypericin	0.3%

CODE	PLANT	PLANT PART	EXTRACTION	ANALYTICAL MARKER	CONCENTRATION
PS-087	*Schisandra chinensis* (Turcz.) Baill.	fruit	Solid-liquid extraction, 70% ethanol	Schisandrins	2%
PS-088	*Monascus purpureus* Went.	yeast	Solid-liquid extraction, 96% ethanol	Lovastatin	3%
PS-089	*Psidium guajava* L.	leaves	Solid-liquid extraction, 70% ethanol	Quercetin	1%
PS-090	*Camelina sativa* L.	seed	Solid-liquid extraction, 70% methanol	ratio plant:extract	5:1
PS-091	*Thymus vulgaris* L.	leaves	Steam distillation	Essential oil	10%
PS-092	*Dioscorea villosa* L.	root	Solid-liquid extraction, 70% ethanol	ratio plant:extract	4:1
PS-093	*Eruca vesicaria* (L.) Cav.	leaves	Solid-liquid extraction, 70% ethanol	ratio plant:extract	4:1
PS-094	*Berberis vulgaris* L.	root	Solid-liquid extraction, 70% ethanol	ratio plant:extract	4:1
PS-095	*Equisetum arvense* L.	aerial part	Solid-liquid extraction, water	ratio plant:extract	4:1
PS-096	*Betula pendula* Roth.	leaves	Solid-liquid extraction, 70% ethanol	Flavonoids	4%
PS-097	*Taraxacum officinale* Weber	leaves	Solid-liquid extraction, 70% ethanol	Flavonoids	2%
PS-098	*Punica granatum* L.	fruit and seed	Pulverized fruit and seed without the juice	ratio plant:extract	10:1
PS-099	*Foeniculum vulgare* Mill.	fruit	Solid-liquid extraction, 50% ethanol	Anethol	0.25%
PS-100	*Lepidium meyenii* W.	root	Solid-liquid extraction, 90% ethanol	ratio plant:extract	4:1
PS-101	*Ilex parwaterriensis* A. St. Hill	leaves	Solid-liquid extraction, water	Caffein	8%
PS-102	*Mentha piperita* L.	leaves	Solid-liquid extraction, 80% ethanol	ratio plant:extract	4:1
PS-103	*Vitis vinifera* L.	leaves	Solid-liquid extraction, 60% ethanol	Polyphenols	10%
PS-104	*Paullinia cupana* Kunth.	seed	Solid-liquid extraction, water	Caffeine	4.5%
PS-105	*Polypodium leucotomos* L.	rhizome	Solid-liquid extraction, water	ratio plant:extract	4:1
PS-106	*Eucalyptus globulus* L.	leaves	Solid-liquid extraction, 70% ethanol	Polyphenols	18.1%
PS-107	*Sambucus nigra* L.	fruit	Solid-liquid extraction, 50% ethanol	Anthocyanidins	0.14%
PS-108	*Arctostaphylos uva-ursi* L.	leaves	Solid-liquid extraction, 96% ethanol	Arbutin	21%
				Pentacyclic triterpenes	6.7%
PS-109	*Echinacea purpurea* L. (Moench)	aerial part	Solid-liquid extraction, 70% ethanol	ratio plant:extract	4:1
PS-110	*Thymus vulgaris* L.	leaves	Solid-liquid extraction, 70% ethanol	ratio plant:extract	5:1
PS-111	*Malpighia emarginata* DC.	fruit	Solid-liquid extraction, water	Vitamin C	17%

CODE	PLANT	PLANT PART	EXTRACTION	ANALYTICAL MARKER	CONCENTRATION
PS-112	*Harpagophytum procumbens* DC. and/or *Harpagophytum zeyheri* Decne	root	Solid-liquid extraction, 70% ethanol	Harpagoside	2%
PS-113	*Pimpinella anisum* L.	fruit	Solid-liquid extraction, 70% ethanol	Anethol	1%
PS-114	*Trigonella foenum-graecum* L.	seed	Solid-liquid extraction, 96% ethanol	ratio plant:extract	4:1
PS-115	*Morinda citrifolia* L.	fruit	Pulverized juice	ratio plant:extract	15:1
PS-116	*Rubus* spp.	leaves	Solid-liquid extraction, 50% ethanol	Hydrolysable tannins	15%
PS-117	*Prunus dulcis* (Miller) D.A. Web.	seed	Solid-liquid extraction, water	Proanthocyanidins	30%
PS-118	*Mentha piperita* L.	leaves	Solid-liquid extraction, 60% ethanol	Rosmarinic acid	0.1%
PS-119	*Vitis vinifera* L.	Fruit and leaves	Successive extractions (alcohol, hydroalcoholic mixture and water) of the fruit and leaf followed by purification of the principal groups of active compounds in common grape vine	Full plant profile	
PS-120	*Punica granatum* L.	pericarp	Solid-liquid extraction, 80% ethanol	Punicalagins	20%
				Ellagic acid	20%

3.4. Extractions, fractionations and purifications

3.4.1. Olea europaea L.

3.4.1.1. Fractionation of extracts PS-018 and PS-034

1. PS-033 – 100g of extract PS-034 was dissolved in 200 mL of pure methanol and the solution was sonicated until a complete dissolution of the powder was achieved. 100 mL of ultrapure water was added and the solution was left overnight to precipitate the water insoluble compounds. Applying decantation, the water fraction was separated from the precipitate and subsequently centrifuged at 3000xg, at 4°C, for 30 min. The centrifugation precipitate was added to the overnight precipitate and the powder was dried overnight at 100°C (MA-25). The supernatant was dried using rotary evaporation 50°C until complete removal of the solvent (HT-20).

2. PS-018 – 100g of PS-018 was extracted with 300mL of hexane, producing fraction OLE FR1. The remaining powder was dried and dissolved in 100 mL of methanol. 50 mL of water was added and the

solution was left overnight to precipitate the water insoluble compounds. Applying decantation, the liquid fraction was separated from the precipitate. The precipitate was dried at 100°C producing fraction TT-40. The liquid fraction was further fractionated using chromatographic purification to obtain fraction OPA-80.

3.4.1.2. Purification of hydroxytyrosol

HT-20 was dissolved in water and fractionated using a polymeric adsorbent resin. Fractions were recovered using water as an eluent. The concentration of hydroxytyrosol was calculated in each fraction by HPLC analysis and the fractions with the highest concentration of HT were combined and evaporated to produce a dark coloured viscous liquid – HT60.

3.4.1.3. Purification of oleanolic acid at 97% purity

200g of *O. europaea* L. leaves were immersed in 1L of pure methanol and extraction was performed at 50°C for 4 hours. By slowly cooling the temperature to 4°C during 12 hours oleanolic acid was crystallized and filtered from the extract (OA-97).

3.4.1.4. Production of oleanolic acid salts

5g of pure oleanolic acid was dissolved in 8-10 volumes of either 5M NaOH, 5M KOH or 5M NH_4OH. The solution was stirred for 40 min at ambient temperature and filtered through a Buchner funnel using quantitative filter paper 424. The excess base was removed by washing with 100 volumes of cold water while controlling the pH of the filtrate with a universal pH strip indicator (MColorpHast™, VWR International). The product was dried at 100°C until achieving constant weight.

3.4.1.5. Production of oleanolic acid phospholipid complex

1g pure oleanolic acid and 3g food grade soy lecithin (ratio 1:3) were dissolved in 300 mL dichloromethane. The solution was vigorously shaken and the solvent was evaporated using a rotary evaporator. Upon evaporation of the solvent, a phytophospholipid complex formed between oleanolic acid and soy lecithin. The product was dried at 50°C o/n and its solubility in water was assessed.

3.4.2. Eucalyptus globulus L.

3.4.2.1. Production of new extracts from E. globulus L. – Extracts produced with different solvents

Three new extracts were produced from the leaves of *E. globulus* L., a water extract, a methanol extract and a 70% v/v methanol extract. The ratio of plant to solvent was 1:40, or 2g of plant to 80 mL of solvent. Extraction was carried out at 50°C for three hours with agitation. After extraction, the extracts were filtered using quantitative filter paper 424. Yield of extraction and dry residue were calculated for each extract.

3.4.2.2. Enhancing selectivity – Methanolic extracts and fractions

5g of *E. globulus* leaves were covered with 50 ml of methanol (ratio 1:10) and extraction was performed at ambient temperature for approximately 2-3 hours with agitation. The extract was filtrated using quantitative filter paper 424 and the volume lost to evaporation was adjusted with methanol to achieve 50 mL. 10 mL were put aside in a falcon tube and were labelled EU EXT. The remaining 40 mL were evaporated to a volume of approximately 5-10 mL using a rotary evaporator at a temperature of 40°C and were transferred to a 50 mL falcon tube. Ultrapure water was added until a volume of 40 mL was reached and the tube was centrifuged for 1 hour at 3000 rpm at 4°C. The liquid was separated from the precipitate and was put to evaporation again to remove the excess methanol. Upon removal of the methanol the volume was adjusted with water to 40 mL. The water-soluble fraction was separated from the precipitate and was labelled EU FR1, while the precipitate was dried at 50°C overnight. The dry powder was diluted in 40 mL methanol and labelled EU FR2. The generation of all eucalyptus products is presented schematically in Figure 11 in Section 4.1.3.2. of the Results Section.

3.4.3. Salvia officinalis L.

3.4.3.1. Fractionation of extract PS-024

30 g of extract PS-024 was extracted with 300 mL of hexane for 2h at 45°C with continuous stirring. The liquid solution was filtered from the solid residue and the filtrate – the hexane fraction – was evaporated to dryness by evaporation under reduced pressure at 40°C. The semi-solid product was weighed and labelled SAL FR1. After the extract was degreased, 200 ml of 80% methanol in water v/v was added to the solid residue and the mixture was agitated for 1h at ambient temperature. Precipitation was allowed to occur overnight. The next day, the liquid solution was separated from the precipitate by

vacuum filtration using qualitative grade filter paper. The solid precipitate was dried for 2h at 100°C, weighted and labelled SAL FR2. The filtrate was evaporated under reduced pressure at 40°C to approximately 30 mL and ultrapure water was added to a final volume of 50 mL. During this process, a water-insoluble red-brown residue was separated from the solution sticking to the walls of the round-bottom flask. The water-soluble liquid fraction was transferred to a dark container, sealed and labelled SAL FR3. The residue remained on the wall and was collected with 50 mL of methanol. The fractionation process described is presented schematically in Figure 13. Dry matter was determined in both fractions SAL FR3 and SAL FR4 by drying 10 mL of each fraction in a pre-weighted container for 24 h at 100°C. SAL FR3 contains 23.3 mg/mL dry matter, while SAL FR4 contains 53.0 mg/mL dry matter.

3.4.3.2. Isolation of ursolic acid from S. officinalis leaves

60 g of *Salvia officinalis* leaves were extracted with 1200 mL of methanol at 40°C with agitation. The extract was filtered from the plant residue by vacuum filtration and the filtered extract was concentrated to 100 mL by evaporation under reduced pressure at 40°C. The concentrated extract was left to precipitate for at least 12 hours at 4°C. The precipitate was filtered, washed with ultrapure water and dried for 4h at 100°C. The dry precipitated fraction was weighed and stored for further analysis (Fraction SE1). On the other hand, the filtrate was concentrated to 60 mL by evaporation under reduced pressure at 40°C and ultrapure water was added in a ratio 5:1 v/v. The methanol/water solution was centrifuged for 30 min at 2500 rpm at 4°C. The supernatant was discarded, while the precipitate was dried for 4h at 100°C, weighted and stored for further analysis (Fraction SE2). The first precipitated fraction from the *S. officinalis* extract had a weight of m(Fraction SE1) = 1.41g, while the second centrifuged fraction had a weight of m(Fraction SE2) = 1.43g, with the sum of the dry fractions weight being m(total) = 2.83g. The yield of the extraction was calculated at 4.72% to the weight of the dry plant leaves. Both fractions were analysed by HPLC to determine the concentration of ursolic acid in each one. The results revealed that Fraction SE1 had a high concentration of ursolic acid with 50.43%, while Fraction SE2 had a lower concentration of ursolic acid at 17.18%.

3.4.4. Humulus lupulus L.

3.4.4.1. Fractionation of hops bitter acids (HBAs)

15g of a supercritical CO_2 Hops extract (Flavex Naturexstrakte, Rehlingen, Germany) containing 72.2% HBAs was warmed to 50°C while continuously stirred. 100 mL of equimolar concentration of KOH

was added and the solution was left another 20 min on the plate. A two-phase system was formed consisting of an aqueous phase and an oily phase. The two phases were separated. 10% H_2SO_4 in ethanol was added to aqueous phase at 1:1 volume ratio and the solution was concentrated to half of its volume by rotary evaporation at 35°C. The yellow residue sticking to the wall of the flask was washed with H_2O and recovered with hexane. 3% KOH was slowly added to the oily phase until a pH of 8.6 was reached. The aqueous layer was discarded, while 1N KOH was slowly added to the oily layer at room T with continuous stirring during 30 min. The layer containing the hops oils and resins was discarded and the aqueous layer was acidified to pH 1 with 50% H_2SO_4. After acidification, the beaker was heated to 50°C and hexane was added at 1:1 volume ratio. The solution was stirred for another 15 min at 50°C to dissolve all solids. After cooling, the phases were separated in a separation funnel and the aqueous phase was discarded. The β-acids were allowed to crystalize from the hexane layer at 4°C.

3.4.5. Orthosiphon stamineus Benth.

3.4.5.1. Production of new extracts from O. stamineus leaves

Water extract: 4g of dried leaves of *O. stamineus* Benth. were placed in a 33x80 mm cellulose extraction thimble and were introduced into the Soxhlet extraction apparatus. 160 mL of water were put in a round bottom flask, the flask was combined with the Soxhlet extractor and placed over an electric mantle. The temperature was controlled between 115 – 120°C. 5 cycles were allowed to repeat in the course of 6 hours. After the extraction, the filtrate was separated from residual plant material by filer paper filtration and the volume of the extract was adjusted to 200 mL.

Methanol extract: 4g of dried leaves of *O. stamineus* Benth. were placed in a 33x80 mm cellulose extraction thimble and were introduced into the Soxhlet extraction apparatus. 160 mL of methanol were put in a round bottom flask, the flask was combined with the Soxhlet extractor and was placed in a water bath with the temperature controlled between 75 – 80°C. 6-7 cycles were allowed to repeat in the course of 6 hours. After the extraction, the filtrate was separated from residual plant material by filer paper filtration and the volume of the extract was adjusted to 200 mL.

Dichloromethane extract: 4g of dried leaves of *O. stamineus* Benth. were placed in a 33x80 mm cellulose extraction thimble and were introduced into the Soxhlet extraction apparatus. 160 mL of dichloro-methane were put in a round bottom flask, the flask was combined with the Soxhlet extractor and was placed in a water bath with the temperature controlled between 55 – 60°C. 6-7 cycles were allowed to repeat in the course of 6 hours.

3.4.5.2. Fractionation of the water extract of O. stamineus Benth.

30g of *O. stamineus* roots were extracted with 200 mL water at 65°C for 120 min. The extract was filtered from the plant residue using vacuum filtration. A non-ionic adsorption resin Amberlite® XAD16 (Alfa Aesar, Thermo Fischer Scientific, USA) was conditioned in ultrapure water for 12 h and introduced into a chromatographic column. The extract was added to the column and elution was allowed at a flow rate of 10 mL/min. After the whole extract was eluted, 100 mL of water was added to the column and elution was allowed to continue while maintaining the flow rate. The water eluate was combined with the extract eluate and was labelled OS FR1. Then, 200 mL of 60% ethanol was added to the column and the elution was continued at the same flow rate of 10 mL/min. The eluent was collected and concentrated to 50 mL using evaporation under reduced pressure at 50°C. The concentrated fraction was labelled OS FR2. Finally, 150 mL of 96% ethanol was added to the column and the elution was continued the liquid coming out of the column was transparent. The eluent was collected, evaporated to dryness using evaporation under reduced pressure at 50°C and reconstituted in 50 mL water. The fraction was labelled OS FR3. Total solids were determined in all fractions by evaporating 10 mL of each fraction in pre-weighed containers to constant weight at 100°C.

3.4.6. Punica granatum L.

3.4.6.1. Preparation of new extracts from P. granatum

Dry pomegranate pericarp was milled into a coarse powder and extractions were performed with 4 different solvents: ultrapure H_2O, 60:40 v/v EtOH: H_2O, 50:50 v/v EtOH: H_2O and 50:50 v/v EtOH: H_2O with citric acid added at a concentration of 1g/L. Solid-liquid extractions were performed at 65-70°C during 5h with agitation at a plant:solvent ratio of 1:20. Yield of extraction, concentration of punicalagins, ellagic acid, gallic acid and tannic acid were determined for all extracts.

3.4.6.2. Fractionation of P. granatum extract 50:50

15 g of dry pomegranate peels were milled into a coarse powder and extraction was performed under reflux for 3 hours using 300mL of 50:50 v/v MeOH: H_2O as an extraction solvent (1:20 ratio). The extract was filtered and concentrated using a rotary evaporator at 40°C until the methanol was completely evaporated. 100mL of Amberlite® XAD16 resin (Alfa Aesar, Thermo Fischer Scientific, USA) previously conditioned for 12 hours in ultrapure water was added to a glass chromatography column. 40 mL of the

concentrate was loaded in the column and elution with copious amounts of ultrapure water was performed until the pale-yellow eluate was clear in color. The adsorbed tannins were eluted with pure MeOH, 2x80mL – generating 2 fractions analyzed separately by HPLC). Dry residue, concentration of punicalagins and ellagic acid was determined in the extract, in the concentrate and the two fractions.

3.4.6.3. Acid hydrolysis of pomegranate extract

5g of extract PS-011 were dissolved in 100 mL of 10% H_2SO4. Hydrolysis was left to occur during 5 hours at 105°C. After the hydrolysis, the pH of the solution was brought to neutral using 1M NaOH and the solvent was evaporated. The dry residue was redissolved in 50 mL MeOH and the concentration of ellagic acid was determined.

3.5. High Performance Liquid Chromatography (HPLC)

All HPLC analyses were performed using an Agilent 1200 Infinity (Agilent Technologies) and Agilent 1200 Infinity II (Agilent Technologies) systems equipped with a Diode Array Detector (Infinity 1290 DAD). Two chromatographic columns were used for the analyses, depending on the method: a C18 Symmetry column (250 x 4.6 mm, 5µm, Waters) and a Kinetex EVO C18 column (150 x 4.6 mm, 5µm, Phenomenex). Agilent software was used for the analysis of the chemical compounds.

3.5.1. Determination of oleuropein in *O. europaea* L. samples: method by the European Pharmacopeia 04/2009:2313

3.5.2. Determination of hydroxytyrosol in *O europaea* L. samples: method by Azaizeh *et al*.[198]

3.5.3. Determination of diterpenes and triterpenic acids in samples of *O. europaea* L.*, E. globulus* L. and *S. officinalis* L.:

Isocratic method – 25 min. Column: C18 – 250 mm x 4.6 mm, 5 µm particle size. Mobile phase: ACN/H_2O/H_3PO_4 (85:15:0,05). Flow rate: 1 ml/mL. Column temperature: 25°C. Injection volume: 10 µL. Detection wavelength at 210 nm.

3.5.4. Determination of flavonoids in *E. globulus* L. samples:

Gradient method – 60 min, C18 – 250 mm x 4.6 mm column. Mobile phase A: 0,04% H_3PO_4 in H_20, Mobile phase B: acetonitrile. Gradient as follows: 0 min – 90% A, 12 min 88% A, 17 min – 84% A, 40 min – 75% A, 50 min – 62% A and 60 min – 90% A.

Flow rate 1 ml/min. Column temperature 35°C. injection volume: 10µL. Detection at 280, 330 and 355 nm.

3.5.5. Determination of xanthohumol and hops bitter acids in *H. lupulus* L. samples:
Gradient method – 47 min, C18 – 250 mm x 4.6 mm column. Mobile phase A: 0,25% HCOOH in H_20, Mobile phase B: 0,25% HCOOH in acetonitrile. Gradient as follows: 0 min – 80% A, 3 min 80% A, 33 min – 25% A, 35 min – 0% A, 45 min – 0% A, 47 min – 80% A. Flow rate: 1 ml/mL. Column temperature: 30°C. Injection volume: 10 µL. Detection wavelength at 290 nm (xanthohumol) and 325 nm (HBAs).

3.5.6. Determination of punicalagins and ellagic acid in *P. granatum* L.: method by García-Villalba *et al*. 2015[113]

3.5.7. Determination of gallic and tannic acid in *P. granatum* L: Method developed by KNAUER Wissenschaftliche Geräte GmbH – "Quantitative determination of gallic acid and tannic acid from gallnut extract". Application No.: VPH0063

3.5.8. Determination of phenolic acids in samples of *S. officinalis* L: method by Sinha *et al*.[197]

3.5.9. Determination of rosmarinic acid in samples of *S. officinalis* L., *R. officinalis* L., *M. officinalis* L. and *O. stamineus* Benth.: method by the European Pharmacopoeia 01/2010-2524

3.6. Determination of total phenolic content by Folin-Ciocalteu method

5 mL of extract were diluted 1:10 with a methanol:water (v/v 50:50) solution. 5 mL of this solution were transferred to a 25 mL flask and water was added until the mark was reached. 2 mL of the latest solution were transferred to a 25 mL amber flask and 1 mL of Folin-Ciocalteu's reagent (PanReac AppliChem ITW Reagents, USA) 10 mL of ultrapure water and 12 mL of a Na_2CO_3 solution (290 g/L) were added. The reaction was left to develop during 30 min at room temperature after which the absorbance of the samples was measured at 760 nm using a UV/VIS spectrophotometer against blank, i.e., distilled water. Gallic acid was used as a standard and the results were expressed as (%) of phenolic compounds in the extract per 100% of gallic acid.

3.7. Determination of total flavonoid content

0,1 mL of extract are mixed with 1.9 mL of extraction solvent, 0.1 mL of 10% aluminium chloride, 0.1 mL of 1M potassium acetate and 2.8 mL of ultrapure water. After incubation at room temperature for 30 min, the absorbance of the reaction mixture is measured at 415 nm with a UV/VIS spectrophotometer. The amount of 10% aluminium chloride is substituted by the same amount of distilled water when

preparing the blank. The total flavonoid content was calculated from a standard curve of quercetin prepared in the same conditions at different concentration, 12.5, 25, 50 and 100 µg/mL. Results were expressed as mg of quercetin equivalents per gram of extract (mg quercetin equivalent (QE)/g of EXT).

3.8. Determination of proanthocyanidins in plant extracts

Quantification of condensed tannins (proanthocyanidins) was determined spectrophotometrically using the n-Butan-1-ol method. Briefly, 1 mL of extract was mixed with 1.5 mL of methanol, 15 mL of 5% HCl/n-BuOH and 0.5 mL of 2% ammonium iron (III) sulphate dodecahydrate. The mixture was left to react during 40 min at 100°C and cooled with water during 20 min. The solution was filtered through a 0.45 µm filter and absorbance was measured at 546 nm. The concentration of proanthocyanidins was calculated using a reference standard that contains 95% of proanthocyanins.

MICROBIOLOGY METHODS

3.9. Antibacterial assays

3.9.1. Antibacterial assays using the multimode reader Tecan Spark

The effects of the natural products on the growth of *L. monocytogenes* strain EGD-e were tested by monitoring their proliferation over time using optical density (OD) measurements at 600 nm. The multimode microplate reader Tecan Spark®, an instrument equipped with a temperature-controlled incubator, a High-Speed Monochromator (HMS) with a range from 200-1000 nm, a Fluorometer and a Luminometer was used for this purpose. The Tecan Spark was used in several types of screening assays in this study: measuring the OD_{600} for assessing the antibacterial properties of the natural products in rich growth media; measuring the fluorescence changes over time in order to assess the activation of the SigB in *L. monocytogenes* $P_{lmo2230}$::egfp and measuring the changes over time in luminescence for food-based assays with *L. monocytogenes* EGDe*lux*.

a) OD_{600} based method

The antimicrobial activities of all extracts were assessed *in vitro* in transparent flat bottom 96-well microtiter plates. The optical density OD_{600} of the solutions containing 1 mg/mL of extract dissolved in BHI and an initial bacterial density concentration of 10^6 CFU/mL was read continuously for 24h (measurement points every 15-20 min). Incubation temperature was set at 30°C or 37°C to determine whether there were differences in activity based on the incubation temperature. Assays with both *L. monocytogenes* wt and the isogenic Δ*sigB* mutant were performed. Growth curves were generated for each individual extract for both the wt and the Δ*sigB* mutant strains by plotting the OD_{600} against time in a xy- growth curve. Conclusions of antimicrobial activity of the extracts were derived by comparing each growth curve generated in the experiments to a negative (BHI) and positive (BHI and 10^6 CFU/mL *L. monocytogenes*) controls. The results of all assays are shown as mean values of three biological replicates.

b) Fluorescence based method

The induction of the sigma B general stress response elicited by the presence of sublethal concentrations of selected plant compounds with previously established antibacterial activities and MICs was tested using a reporter strain of *L. monocytogenes* designated *L. monocytogenes* $P_{lmo2230}$::egfp. This strain harbours a reporter engineered with a strong sigma B dependent promoter from the *lmo2230* gene (which encodes a putative arsenate reductase) fused to a gene encoding enhanced green fluorescence

protein (eGFP). The reporter is integrated into the genomes of the wild-type strain *L. monocytogenes* EGD-e, as well as a mutant derivative lacking sigma B. The stress response was evaluated by measuring the fluorescence (every 15 min) in relative fluorescence units (RFU) using the multimode reader Tecan Spark. Black Corning® 96-well with transparent flat bottoms that have low background fluorescence, minimal light scatter and reduced crosstalk microtiter plates (Merck KGaA, Darmstadt, Germany) were used for the purpose. All assays were performed with a bacterial inoculum concentration of 10^6 CFU/mL. All experiments were carried out using three biological replicates.

c) Luminescence based method

The antimicrobial activities of selected extracts were assessed in food matrices and products by measuring the expression of luminescence of a luciferase-tagged *L. monocytogenes* strain called EGDe*lux*. This strain has a chromosomal integration construct named pPL2*lux*P_{help} with a *hlyA* gene promoter cloned into a pPL2*lux* vector as exact translational fusion in front of luxABCDE. The pPL2*lux* vector contains a synthetic *lux* operon derived from *Photorhabdus luminescens* and is optimized for use with gram-positive bacteria to monitor gene expression. The resulting pPL2*lux*P_{help} construct is integrated into a $tRNA^{Arg}$ gene in the chromosome of EGD-e. Luminescence is expressed constitutively given the high activity of the pPL2 promoter. Luminescence was measured in relative light units (RLU) (in photons s^{-1}) and white Thermo Scientific™ Nunc™ flat bottom 96-well microtiter plates (Thermo Fischer Scientific, USA) with maximum reflection and minimal autoluminescence were used. All assays were performed with a bacterial inoculum concentration of 10^6 CFU/mL a temperature of 30°C. The results of all assays are shown as mean values of two or three biological replicates.

3.9.2. Resazurin based antibacterial assay

The high-throughput antimicrobial screening assay incorporating the coloured oxidation–reduction indicator resazurin as an indicator of growth was also used to assess the antibacterial activity of the second batch of 90 extracts against both *L. monocytogenes* EDG-e wt the Δ*sigB* mutant at two temperatures: room temperature (24-27°C) and 37°C. Every conducted assay included a positive (medium + bacterial inoculum + resazurin) and a negative (medium + resazurin) control to account for the sterility of the conditions and the solutions, and the viability of the microorganism, correspondingly. After 24h results summarized as a change in colour were noted and the lowest concentration of plant product for which no change in colour was observed was established as the MIC value. 5 µL of subsequent inoculum from the wells where the colour remained unchanged were plated on standard BHI agar plates and

incubated for additional 24 hours. The lowest concentration for which no growth was observed was taken as the MBC value (corresponding to a reduction in culturability of the initial bacterial inoculum exceeding 99%).

3.9.3. Agar dilution method

For the agar dilution method, a pre-weighted mass of the extract in question was thoroughly mixed with 35 mL of agar medium at 55°C and the obtained homogenous mixture was poured into a 130x13 mm square Petri plate. Each plate was prepared such that it contained a different concentration of the extract under examination. The Petri plate was then divided in 9 equal quadrants and solutions with defined numbers of bacterial cells were spotted directly onto the surface of the nutrient agar plates. Each quadrant was spotted with a one log different bacterial inoculum, starting with an inoculum of 10^1 CFU/mL in the first quadrant and finishing with and inoculum of 10^9 CFU/mL in the last quadrant. After incubation, the presence of bacterial colonies on the plates was examined. An absence of growth in the 10^4 CFU/mL spots indicated growth inhibition by the extract in question, and the concentration in which it was present in the agar medium was read as the MIC. A further assessment was performed for the spots with higher bacterial cell numbers in order to describe the activity of the extract as high, medium or low. The extracts inhibiting the growth up to the 10^9 CFU/mL quadrant were described as highly active, the extracts inhibiting the growth up to the quadrants 10^5 CFU/mL-10^6 CFU/mL were described as extracts with medium antibacterial activities and the extracts inhibiting the growth up to the 10^4 CFU/mL quadrant were described as extracts with low activity.

3.10. Flow cytometry analysis of egfp expressing cells

Bacterial cultures of *L. monocytogenes* wild type $P_{lmo2230}$::egfp and its isogenic derivate Δ*sigB* $P_{lmo2230}$::egfp were grown in BHI to OD_{600}=0.3 (no stress). After reaching OD_{600}=0.3 each bacterial culture was supplemented with sublethal concentrations of selected plant compounds or 0.5M NaCl during 30 min (stress). After the stress treatment, the bacteria were spun down by centrifugation (10,000 x *g*, 18 min, 4°C) and washed twice in PBS, pH 7.4. The cells were fixed with 4% (w/v) paraformaldehyde for 15 min at RT. Fixed cells were harvested by centrifugation and resuspended in 500µL of filtered PBS, pH 7.4. Quantification of single cell fluorescence was achieved by flow cytometry with Beckman Coulter GALLIOS Analyzer with 488nm blue laser excitation and 50,000 events recorded for each sample. The data collected were processed with Kaluza software to plot side and forward scatter values, the percentage of egfp-positive cells and the mean of fluorescence values.

3.11. Fluorescence microscopy

Preparation of the fixed bacterial samples followed the same procedure as the one described for flow cytometry. A solution of poly-L-Lysine (Sigma-Aldrich, Darmstadt, Germany) was prepared at a concentration of 0.01% (w/v) in H_20 MilliQ. Poly-L-Lysine was bound electrostatically to glass cover slip by adding 50 µL of the solution of poly-L-Lysine per slip and incubating for 5 min at room temperature. The excess solution was discarded and the cover slip was left to dry for 15 min. 20 µL of the previously fixed bacterial suspension was incubated with the cover slip during 15 min at room temperature. The cover slip was washed three times with PBS, pH 7.4. A drop of ProLong Gold Antifade (Molecular Probes, Thermo Fiscer Scientific, USA) was applied on the coverslips to mount the microscope slide. Phase-contrast and fluorescence microscopy were performed using an inverted microscope LEICA DMI 6000B. Exposure of eGFP was visualized at fixed exposure time of 400 ms.

3.12. Subcellular fractionation of plant compounds stressed L. monocytogenes

Overnight cultures of *L. monocytogenes* EGD-e wt, *L. monocytogenes* Δ*rsbR1*, *L. monocytogenes rsbR1* T175A and *L. monocytogenes rsbT* N49A were resuspended in BHI and grown with continuous shaking at 37°C and 30°C until reaching mid-exponential phase (OD_{600nm}= 0.3) (no stress). Sublethal concentrations of β-acids (10 µg/mL) and *Eucalyptus globulus* extract (1 µg/mL) were added to separate cultures of the wt strain and all cultures were additionally incubated for 30 min (stress). Around 10^{10} bacterial cells (50 mL culture) were spun down by centrifugation (10,000 x g, 18 min, 4°C) and washed twice in phosphate buffer saline (PBS), pH 7.4. The pellet was kept at -80°C until being subjected to subcellular fractionation. For subcellular fractionation, the pellet of bacteria was washed in 10 ml of TS buffer (10mM Tris HCl pH 6.9, 10mM $MgCl_2$, 0.5M sucrose) and centrifuged at 10,000 x g, 18 min, 4°C. Then, the pellet was resuspended in a lysis solution: TS buffer containing 60 µg/ml mutanolysin from *Streptomyces globisporus* (ATCC 21553, Sigma-Aldrich), 250 µg/ml R-seA and a protease inhibitor cocktail and was incubated for 5 h at 37°C with slow-rotation agitation. The resulting protoplasts were recovered by centrifugation at 15,000 x g, 10 min, 4°C. The supernatant corresponding to the cell wall fraction was discarded and the pellet containing the protoplasts was washed with 1 ml of PBS, pH 7.4 and centrifuged at 10,000 x g, 18min, 4°C. The protoplasts were resuspended into 400 µl PBS, pH 7.4, 1 µg/ml D-seA and a protease inhibitor cocktail and were lysed by low power sonication (3x for 20 sec). Unbroken cells were removed by centrifugation at 20,000 x g, 10 min, 4°C and the supernatant was subjected to ultracentrifugation at 100,000 x g, 1 h, 4°C to separate the cytosolic and membrane fractions. The pellet

containing membrane proteins was washed with PBS, pH7.4 and ultracentrifuged again at 100,000 x g, 1 h, 4°C. A volume of 10 µl of cytosolic and membrane fractions was analysed by SDS-PAGE and Western-Blot using affinity purified polyclonal rabbit antibodies against the stressosome structural protein RsbR1 and SigB.

3.13. SDS-PAGE and Immunoblot analyses of RsbR1 and SigB in L. monocytogenes

Electrophoresis was performed on 12% Tris-Glycine SDS-Polyacrylamide gels under the conditions of 80V for 15 min then increased to 170V for 60 min. Protein concentration was analysed by staining with Coomassie dye and after visualization of the stained gels, adequately adjusted to attain similar concentrations between all samples for a second gel. Western blotting was performed on a Immobilon-P PVDF Membrane (Sigma-Aldrich, Darmstadt, Germany) using a TransBlot SD semi-dry Bio-Rad system. Affinity purified anti-RsbR1 antibodies[128] were used for the detection of free RsbR1 in the cytosolic and membrane fractions. The detection of SigB used non-purified polyclonal rabbit crude serum (Charles River, France) at a dilution 1:10,000. Goat anti-rabbit HRP-conjugated antibodies were used as secondary antibodies (Bio-Rad Laboratories Inc., USA) at a dilution 1:20,000. Purified His-tagged RsbR1 and SigB proteins were used as controls.

3.14. Identification of phosphorylated isoforms of RsbR1 with Phos-Tag SDS-PAGE gel

Phos-tag electrophoresis using a Phos-Tag™ SDS-PAGE acrylamide gel (SuperSep™ Phos-tag™ precast gels, Fujifilm WAKO Chemicals) was used for identification of the phosphorylation state of RsbR1 and in bacterial cells treated with sublethal concentrations of selected antibacterial extracts as a source of environmental stress. The Phos-Tag system contains a Phos-Tag™ functional molecule and divalent Zn^{2+} ions which capture phosphorylated Ser/Thr/Tyr and His/Asp/Lys and is capable of separating phosphorylated and non-phosphorylated forms of protein isoforms was based on their phosphorylation levels. The migration speed of phosphorylated proteins decreases due to the binding of the metallic ion and phosphorylated/non-phosphorylated proteins are separated as different bands. After exposure to the stress or no stress conditions for 30 min, the bacterial cultures were boiled for 20 sec at 100°C to stop the metabolism before harvesting the cells by centrifugation. The cells were washed twice with a killing buffer containing 20mM Tris pH 7.5, 5mM $MgCl_2$, 20mM NaN_3 (10,000 x g, 18 min, 4°C). The pellet was subjected to subcellular fractionation as previously described in Section 3.13.

3.15. LIVE/DEAD BacLight Bacterial Viability Kits

An overnight culture of *L. monocytogenes* EGD-e wt was resuspended in eight flasks of 30 mL BHI (dilution 1:100) and grown at 37°C with agitation until late exponential phase was reached (≈OD_{600}=0.4). Each flask was supplemented with a selected antibacterial plant compound at its previously determined 1xMIC and incubated for one additional hour. After incubation, the bacterial cells were harvested by centrifugation (10,000 x *g*; 15 min, 4ºC), the supernatant was removed and the pellet was resuspended in 2 mL of 0.85% (w/v) NaCl. One mL of the suspension was added to 19 mL of 0.85% NaCl (live bacteria) and the other to 19 mL of 70% isopropyl alcohol (dead bacteria) to reach a 1:20 dilution. The solutions were incubated for 1 h, mixing every 15 min. At the end of the incubation, bacterial cells were harvested by centrifugation (10,000 x *g*; 15 min, 4ºC), the supernatant was discarded, the pellet was washed with 0.85% NaCl and the suspensions were centrifuged again (10,000 x g; 15 min, 4ºC). The supernatant was discarded and the pellets were diluted in 10 mL of 0.85% NaCl. The optical density was determined at 600 nm (OD_{600}) and adjusted to OD_{600}=0.05 for each solution with 0.85% NaCl (≈5 x 10^7 CFU/mL). Different proportions of the live and dead bacteria were mixed to obtain cell suspensions at five different rations, i.e. Ratio of LIVE:DEAD cells: 0:100; 25:75; 50:50; 75:25 and 100:0. This data set was used to plot the standard curve. 100 µL of the prepared suspension mixtures and the treated suspensions were added in triplicate into separate wells of a 96-well black flat-bottom microplate. This kit utilizes a mixture of two stains, green-fluorescent SYTO9 nucleic acid stain and red-fluorescent nucleic acid stain, propidium iodide (PI). These stains differ both in their spectral characteristics and in their ability to penetrate live and dead bacterial cells. SYTO9 is a membrane permeable stain that generally labels all bacteria in a population, whereas PI is impermeable to viable cells and stains only dead or damaged cells with a compromised cell membrane. A 2X stain solution was prepared by mixing equal volumes of Component A (3,34 mmol/L SYTO 9 green dye) and Component B (20 mmol/L propidium iodide) and dissolving the mixture in sterile ultrapure water at 6:1000 dilution. When both stains are present inside the cell, PI is capable of displacing SYTO9 causing a reduction in SYTO9 fluorescence. Thereby, when SYTO9 and PI are used simultaneously, viable bacteria with intact cell membranes will be stained green by SYTO9, whereas damaged or dead cells with a compromised membrane will be stained red by PI. Using a new tip for each well, 100 µL of the 2X staining solution were added to each well and mixed thoroughly by pipetting up and down several times. The plate was incubated for 15 min in the dark at room temperature. At the end of the incubation period, fluorescence intensity was measured in each well of the microplate for both dyes (SYTO-9 excitation wavelength at 485 nm, emission at 530 nm, Green; propidium iodide excitation wavelength still centered

at 485 nm, emission at 630 nm, Red). The Green ⁄ Red (G ⁄ R) ratio was calculated by dividing the fluorescence intensity of the stained bacterial suspensions (F_{cell}) at emission 1 (green) by the fluorescence intensity at emission 2 (red). A standard curve was plotted from the ratio of LIVE:DEAD cells and the percentage of live cells after treatment with plant compounds was calculated in GraphPad Prism version 6 (San Diego, USA) by standard curve interpolation analysis using a 95% confidence interval.

3.16. Food matrices

3.16.1. Commercial product food matrices

Three commercial food matrices were used in the food assays in Chapter III: reconstituted skimmed milk powder (30% w/v - Nestlé), 20% fat cooking cream (Hacendado, Spain) and Béarnaise sauce (a slightly viscous sauce made of clarified butter emulsified in egg yolks and white wine vinegar, flavoured with herbs – Nestlé). All food matrices used were provided as aseptic, commercially sterile recipes. Skimmed milk and Béarnaise sauce were produced by Nesté and a gift from Dr. Nicholas Johnson from Nestlé Research & Development in Konolfingen, Switzerland.

3.16.2. Preparation of KonoMatrix

The basic ingredients in the food recipe and their individual proportions in the matrix are provided in Table 3. The produced food matrix was named KonoMatrix (KM) and was prepared by the following procedure:

Table 3. KonoMatrix (KM) recipe

Mass [g]	5 % Oil		8,3 % Oil	
Final solution	100 g	350 g	100 g	350 g
Stock solution	70	245	70	245
Starch in stock	1	3,5	1	3.5
NaCl in stock	1.5	5.25	1.5	5.25
Glucose in stock	3	10.5	3	10.5
WPI in stock	1	3.5	1	3.5
Addition Oil/Lecithin mix	6	21	12	35
Oil in O/L mix	5	17.5	8.3	29.05
Lecithin in O/L mix	1	3.5	1.7	5.1
Addition H_2O	24	84	18	70

Each KonoMatrix was prepared in two phases – first, by separately preparing an aqueous stock solution and an oil-lecithin stock solution, and second, by adding each natural product to the oil-lecithin solution and mixing with the aqueous stock solution. Reverse osmosis (RO) purified water was used for the preparation of the food matrices. The aqueous stock solution was prepared by adding starch, salt, glucose and whey protein in RO water and heating the solution to 95°C with continuous stirring until a homogeneous solution was achieved. The oil-lecithin stock solution was prepared in a 5:1 ratio and heated to 50°C with continuous stirring. After the lecithin was completely dissolved, the oil stock solution was divided into separate containers where each natural product was added at different concentrations. Three increasing concentrations were used for each natural product: 50 µg/mL, 100 µg/mL and 500 µg/mL for *S. officinalis* L. extract PS-024; 10 µg/mL, 100 µg/mL and 500 µg/mL for oleanolic acid and 10 µg/mL, 50 µg/mL and 100 µg/mL for β-hops bitter acids. All solutions were continuously stirred until the extract was completely dissolved in the oil fraction of the matrix and kept in a water bath at 50°C until their posterior mixing with the aqueous phase. After obtaining a homogeneous aqueous solution, the solution was allowed to cool down to 60°C and was added to each oil-extract fraction at the appropriate ratio to achieve 5% and 8,3% final oil concentrations. The 5% and 8,3% oil concentrations were chosen after a food matrix trial experiment was completed with 5%, 10% and 25% oil concentrations. The results of this experiment showed that the resulting food matrices were not stable at higher oil concentrations and succumb to water-oil separation at oil concentrations higher than 10%. Each solution was thoroughly mixed for 1-2 minutes with an IKA T25 Ultra-Turrax high performance dispersing instrument at high velocity – approximately 20 000 rpm. A GEA Niro Soravi Laboratory High-Pressure Homogenizer was used for the emulsification of the food matrices. Homogenization technology uses efficient high-pressure energy to break particles present in fluids to the smallest possible size, down to nanometre range. The pressure settings used for the preparation of the KM food matrices in this study were 100/400 bars for the first and second valve of the instrument correspondingly. Each sample was collected in a previously marked container as a milky white emulsion. The final volume of each sample was approximately 300 mL. After collection, all samples were pasteurized in a water bath with a low temperature long time (LTLT) pasteurization method – 62.8°C for 30 min, immediately cooled down and kept at 4-5°C until the end of the study. All samples produced in this manner are presented in Table 4.

Solutions with a pH range from pH 4.0 to pH 7.0 with 0.25 increments were produced by adjusting the pH of the 500 µg/mL *S. officinalis* KM samples with 10% citric acid and 1N sodium hydroxide (NaOH). A total of 44 samples were produced.

Table 4. Description and characteristics of the KonoMatrix samples

	Name	Oil (%)	Extract	Concentration	Appearance
1	KM 5% Control	5	/	/	Milky-white liquid
2	KM 5% 1-1	5	*Salvia officinalis*	50 µg/mL	Milky-white liquid
3	KM 5% 1-2	5	*Salvia officinalis*	100 µg/mL	Milky-white liquid
4	KM 5% 1-3	5	*Salvia officinalis*	500 µg/mL	White-greenish liquid
5	KM 5% 2-1	5	Oleanolic acid	10 µg/mL	Milky-white liquid
6	KM 5% 2-2	5	Oleanolic acid	100 µg/mL	Milky-white liquid
7	KM 5% 2-3	5	Oleanolic acid	500 µg/mL	Milky-white liquid
8	KM 5% 3-1	5	β-Hops bitter acids	10 µg/mL	Milky-white liquid
9	KM 5% 3-2	5	β-Hops bitter acids	50 µg/mL	Milky-white liquid
10	KM 5% 3-3	5	β-Hops bitter acids	100 µg/mL	Milky-white liquid
11	KM 8,3% Control	8,3	/	/	Milky-white liquid
12	KM 8,3% 1-1	8,3	*Salvia officinalis*	50 µg/mL	Milky-white liquid
13	KM 8,3% 1-2	8,3	*Salvia officinalis*	100 µg/mL	Milky-white liquid
14	KM 8,3% 1-3	8,3	*Salvia officinalis*	500 µg/mL	White-greenish liquid
15	KM 8,3% 2-1	8,3	Oleanolic acid	10 µg/mL	Milky-white liquid
16	KM 8,3% 2-2	8,3	Oleanolic acid	100 µg/mL	Milky-white liquid
17	KM 8,3% 2-3	8,3	Oleanolic acid	500 µg/mL	Milky-white liquid
18	KM 8,3% 3-1	8,3	β-Hops bitter acids	10 µg/mL	Milky-white liquid
19	KM 8,3% 3-2	8,3	β-Hops bitter acids	50 µg/mL	Milky-white liquid
20	KM 8,3% 3-3	8,3	β-Hops bitter acids	100 µg/mL	Milky-white liquid

3.17. Measurement of microbial growth in KM matrices

The antibacterial properties of the control and antimicrobial plant enriched KM food matrices were assessed by luminescence measurements. Plate counts were performed for the KM food matrices with the highest concentrations of the plant antimicrobial agents. The 5.0; 5.5 and 6.0 pH solutions of *S. officinalis* PS-024 – 500 µg/mL were also tested by the plate count method.

Plate Count Growth Curves. 20 mL of KM media control and KM media enriched with plant antimicrobial agents were inoculated with an overnight culture of *L. monocytogenes* EGD-e diluted 1:100 or 1:1000. The solutions were incubated at 30°C with agitation at 120 rpm. The incubation temperature was the same as the one for luminescence measurements. Samples were taken at pre-set time intervals,

at the time of inoculation designated as t_0, and at $t_{0+30min}$; t_{0+1h}; t_{0+2h}, t_{0+3h}; t_{0+4h} and t_{0+5h}.The samples were diluted in ¼ strength Ringers' solution (LabM, Lancashire, UK), plated on BHI agar and incubated at 30°C for 24 h after. Colonies were counted manually after incubation. Plates that had between 30 and 300 colonies were chosen for counting. Growth rates were calculated using the Online DMFit version provided by ComBase. Samples where the log CFU did not change more than 0.25 between the initial and final cell population number were considered as no growth. All experiments were carried out using two biological replicates.

3.17.1. Primary model

Growth curves were obtained by plotting the log RLU or the log CFU signal against time and fitting the data in a primary model (logistic growth model[141]). The growth kinetics parameters were fitted using Excel Solver to calculate: $\log_{10}N_0$ initial (N_0) and the $\log_{10}N$(max) final cell population (N_{max}), the maximum specific growth rate within the conditions of the experiments (h^{-1}) (μ_{max}) and the duration of the lag phase (λ). Both a growth curve fitting and a time-to-detection approach[142] (fixed at log RLU = 2,4 as the time point threshold) were used to estimate the growth parameters. The quality of the fits was expressed through the coefficient of determination (R^2) and the root mean square error (RMSE). All experiments were carried out using three technical replicates.

3.17.2. Dose-response secondary fitting

Linear regression. The growth rates of the bacteria calculated by the primary model fitting for each concentration of the antimicrobial plant agent were averaged and then standardized to 1 for growth levels without the added antimicrobial. Triplicate values for each concentration of the antimicrobial using the log RLU growth rate and time-to-detection growth rate were used. The standardized data was analysed in GraphPad Prism version 6 (San Diego, USA) using the linear regression fitting to establish the dose-response of growth in the presence or absence of the antimicrobial agent. The predicted MIC value (y-intercept), R^2 coefficient and standard deviation of the residuals (Sy.x) were calculated for each model.
Hill Plots. The growth rates of the bacteria calculated by the primary model fitting for each concentration of the antimicrobial plant agent were averaged and then normalized to growth levels without the added antimicrobial. The normalized data was analysed in GraphPad Prism version 6 (San Diego, USA) using the log(inhibitor) vs. response -- Variable slope (four parameters) non-linear fitting to establish the Hill Plot dose-response of growth in the presence or absence of the antimicrobial agent. The IC50 value, R^2 coefficient and absolute sum of squares (SS) were calculated for each model.

RESULTS

4.1. Chapter I: Screening of plant extracts for antibacterial activities against *Listeria monocytogenes*

4.1.1. Introduction – Initial screening

Bioassay-guided fractionation of natural products entails the combination of chemical separations with screening for biological activities. This method can be shortly described as a step-by step process that includes: i) the extraction of phytochemicals from plants; ii) a bioassay screening of the crude extracts; iii) fractionation of the bioactive extracts and bioactivity screenings of each fraction; iv) further fractionation step or isolation of the active molecule(s) from the bioactive fractions; v) a bioassay screening of each isolate; and, vi) identification of the isolated molecule(s). Following this concept, an initial screening was stared, designed to evaluate the antibacterial properties of 120 plant extracts against *L. monocytogenes* strain EDG-e, wild-type (wt) and its isogenic mutant Δ*sigB.* As SigB is responsible for the activation of the general stress response in *L. monocytogenes*, we reasoned that a mutant lacking one of the most important alternative sigma factors that controls more than 300 genes associated with stress resistance mechanisms[67,134] will be more sensitive to antibacterial compounds. In this context, the term "stress" could mean any environmental perturbation that reduces the growth rate (a mild stress) or negatively impacts cell survival (a more severe stress). Studies done by Begley *at al*.[95] showed that SigB is important for growth and survival upon treatment with the bacteriocins lacticin 3147 and nisin, or the antibiotics of choice in the treatment of listeriosis, penicillin G and ampicillin. In this study, detailed growth curves and survival assays showed that the Δ*sigB* mutant was impaired in growth in the presence of sublethal levels of each antibiotic and was killed more rapidly at lethal levels.

For our study, an initial group of plant extracts was selected, described in detail in Table 2 – Materials & Methods. The 120 chosen extracts originate from 86 different plants and are representative of all the major and most minor phytochemical groups portraying a myriad of bioactive compounds. Most initial extracts were previously obtained at pilot or industrial scale for the use in food supplements, herbal medicinal products, feed or cosmetics. In the primary phase of the screening, all extracts were in the form of dry powders except for extracts PS-014 and PS-086 which were obtained as concentrated viscous liquids.

Sample preparation

As a first step, most extracts were analysed by HPLC or by other phytochemical characterization methods in order to determine the concentration of an analytical marker characteristic for that extract, or the dominant phytochemical group of compounds. A list of all extracts used in the initial screening together with the results from the determination of their specific markers is also provided in Table 2 –

Materials & Methods. After the early characterization of the extracts, solubility trials were performed. The extracts are normally stored in non-sterile sealed containers at a controlled temperature of 23°C with adequate protection from direct sunlight. Accordingly, environmental microorganisms are present in the extracts in low concentrations. Therefore, the first step was dissolving the dry extracts in 100% dimethyl sulfoxide (DMSO) and allowing the organic solvent to act as a chemical sterilizing agent. Apart from the purpose of killing the innate microflora, DMSO was chosen as the solvent because it is an important polar aprotic solvent that dissolves both polar and nonpolar compounds, and is miscible in a wide range of organic solvents, as well as water. After dissolving in DMSO, all 120 extracts were further diluted to 1 mg/mL starting extract concentration in the working antibacterial assays, while not exceeding 0.5% (v/v) DMSO. Concentrations above 1% DMSO can have deleterious effects on the bacterial cell membrane, and concentrations higher than 5% DMSO can denature proteins by virtue of rupturing hydrogen bonds.

4.1.2. Antibacterial properties of the plant extracts in the initial screening

The initial 120 extracts were tested for antibacterial activities against *L. monocytogenes* EGD-e wild-type (wt) and its Δ*sigB* mutant at two different temperatures, 30°C and 37°C to determine if differences occurred in tolerance to the plant antimicrobial agents between the two temperatures. All extracts were firstly tested at a concentration of 1 mg/mL, then extracts that showed growth inhibition properties at this concentration were additionally tested in a serial dilution assay to determine the minimum inhibitory concentration (MIC). Two broth microdilution methods (Section 3.10 – Material & Methods) were employed to assess the antibacterial properties of each extract: i) following the growth of the bacteria by continuously measuring the optical density at 600 nm in a multimode reader – **OD_{600} measurements** – (columns 2 and 3 in Table 5); and,

ii) using a modified version of the microtiter plate-based antimicrobial assay described by Sarker *et al*.[199] that incorporates the coloured oxidation-reduction indicator resazurin – **MIC determination method** (column 3, 4, 5 and 6 in Table 5).

The growth and survival of *L. monocytogenes* were examined using an inoculum concentration of 10^6 CFU/mL in both assays. A total of 40 extracts were additionally tested with the **agar dilution method** (agar Dilution, Table 5). These extracts, including PS-010, PS-011, PS-014, PS-031, PS-073, PS-106 among others, showed inconclusive results with the broth dilution methods. Our interpretation is that it was due to the presence of hydrophobic compounds in the extracts unable to dissolve and homogenize in the broth culture medium. To better assess the antibacterial properties of these extracts, the agar dilution

method was included in the screening. The results of the initial screening of the 120 plant extracts with all methods against both *L. monocytogenes* strains at both temperatures are presented in Table 5 below.

Table 5. Antibacterial activities of the initially screened 120 plant extracts against *L. monocytogenes* wt and Δ*sigB*.

CODE	OD_{600} measurements		MIC determination method				Agar dilution
	OD_{600} EGDe wt	OD_{600} EGDe Δ*sigB*	MIC EGDe wt 30°C	MIC EGDe Δ*sigB* 30°C	MIC EGDe wt 37°C	MIC EGDe Δ*sigB* 37°C	
PS-001	-	-	-	-	-	-	NT
PS-002	-	not conclusive	-	-	-	-	NT
PS-003	not conclusive	not conclusive	-	-	-	-	NT
PS-004	-	-	-	-	-	-	NT
PS-005	-	-	-	-	-	-	NT
PS-006	-	-	-	-	-	-	NT
PS-007	-	-	-	-	-	-	NT
PS-008	-	-	-	-	-	-	NT
PS-009	-	-	-	-	-	-	NT
PS-010	moderate activity	moderate activity	-	-	-	-	low activity
PS-011	high activity	high activity	1 mg/mL	1 mg/mL	1 mg/mL	1 mg/mL	medium activity
PS-012	-	-	0.5 mg/mL	0.5 mg/mL	0.5 mg/mL	0.5 mg/mL	NT
PS-013	-	-	-	-	-	-	NT
PS-014	high activity	high activity	0.063 mg/mL	0.063 mg/mL	0.063 mg/mL	0.063 mg/mL	NT
PS-015	-	-	-	-	-	-	NT
PS-016	-	-	-	-	-	-	NT
PS-017	high activity	high activity	0.25 mg/mL	0.25 mg/mL	0.125 mg/mL	0.125 mg/mL	high activity
PS-018	-	-	1 mg/mL	1 mg/mL	0.5 mg/mL	0.5 mg/mL	-
PS-019	-	-	-	-	-	-	NT
PS-020	-	-	0.125 mg/mL	0.125 mg/mL	0.125 mg/mL	0.125 mg/mL	NT
PS-021	-	-	-	-	-	1 mg/mL	NT
PS-022	moderate activity	moderate activity	0.5 mg/mL	0.5 mg/mL	0.5 mg/mL	0.5 mg/mL	-
PS-023	-	-	-	-	-	1 mg/mL	NT
PS-024	moderate activity	moderate activity	0.25 mg/mL	0.25 mg/mL	0.125 mg/mL	0.125 mg/mL	high activity
PS-025	-	-	-	-	-	-	-
PS-026	moderate activity	moderate activity	0.25 mg/mL	0.25 mg/mL	0.25 mg/mL	0.25 mg/mL	medium-high activity
PS-027	not conclusive	not conclusive	-	-	-	-	NT

CODE	OD_{600} measurements		MIC determination method				Agar dilution
	OD_{600} EGDe wt	OD_{600} EGDe Δ*sigB*	MIC EGDe wt 30°C	MIC EGDe Δ*sigB* 30°C	MIC EGDe wt 37°C	MIC EGDe Δ*sigB* *37*°C	
PS-028	not conclusive	not conclusive	-	-	-	-	NT
PS-029	not conclusive	not conclusive	0.25 mg/mL	0.25 mg/mL	0.25 mg/mL	0.25 mg/mL	low activity
PS-030	not conclusive	not conclusive	0.25 mg/mL	0.25 mg/mL	0.25 mg/mL	0.25 mg/mL	medium activity
PS-031	not conclusive	not conclusive	0.063 mg/mL	0.063 mg/mL	0.031 mg/mL	0.031 mg/mL	high activity
PS-032	not conclusive	not conclusive	0.125 mg/mL	0.125 mg/mL	0.125 mg/mL	0.125 mg/mL	medium-high activity
PS-033	-	-	0.5 mg/mL	0.5 mg/mL	0.25 mg/mL	0.25 mg/mL	-
PS-034	-	-	1 mg/mL	1 mg/mL	1 mg/mL	1 mg/mL	-
PS-035	-	-	-	-	-	-	NT
PS-036	-	-	-	-	-	-	NT
PS-037	-	-	-	-	-	-	NT
PS-038	-	-	-	-	-	-	NT
PS-039	-	-	-	-	-	-	NT
PS-040	-	-	-	-	-	-	NT
PS-041	-	-	-	-	-	-	NT
PS-042	-	-	-	-	-	-	NT
PS-043	-	-	-	-	-	-	NT
PS-044	-	-	-	-	-	-	NT
PS-045	-	-	-	-	-	-	-
PS-046	-	-	-	-	-	-	NT
PS-047	-	-	-	-	-	-	NT
PS-048	-	-	-	-	-	-	NT
PS-049	low activity	low activity	-	-	-	-	-
PS-050	-	-	-	-	-	-	NT
PS-051	-	-	-	-	-	-	NT
PS-052	-	-	0.5 mg/mL	0.5 mg/mL	0.5 mg/mL	0.5 mg/mL	-
PS-053	-	-	-	-	-	-	NT
PS-054	-	-	-	-	-	-	NT
PS-055	-	-	-	-	-	-	NT
PS-056	-	-	-	-	-	-	NT
PS-057	low activity	low activity	0.5 mg/mL	0.5 mg/mL	0.5 mg/mL	0.5 mg/mL	-
PS-058	-	-	-	-	-	-	NT
PS-059	-	-	-	-	-	-	NT
PS-060	low activity	low activity	-	-	-	-	-
PS-061	not conclusive	not conclusive	1 mg/mL	1 mg/mL	1 mg/mL	1 mg/mL	low activity
PS-062	low activity	low activity	-	-	-	-	-
PS-063	-	-	-	-	-	-	NT

CODE	OD_{600} measurements		MIC determination method				Agar dilution
	OD_{600} EGDe wt	OD_{600} EGDe Δ*sigB*	MIC EGDe wt 30°C	MIC EGDe Δ*sigB* 30°C	MIC EGDe wt 37°C	MIC EGDe Δ*sigB* *37*°C	
PS-064	not conclusive	not conclusive	1 mg/mL	1 mg/mL	0.5 mg/mL	0.5 mg/mL	low activity
PS-065	moderate activity	moderate activity	-	-	-	-	-
PS-066	-	-	-	-	-	-	NT
PS-067	-	-	-	-	-	-	NT
PS-068	-	-	-	-	-	-	NT
PS-069	-	-	-	-	-	-	NT
PS-070	-	-	-	-	-	-	NT
PS-071	-	-	-	-	-	-	NT
PS-072	-	-	-	-	-	-	NT
PS-073	-	-	1 mg/mL	1 mg/mL	1 mg/mL	1 mg/mL	low activity
PS-074	-	-	-	-	-	-	NT
PS-075	-	-	-	-	-	-	NT
PS-076	-	-	-	-	-	-	NT
PS-077	-	-	-	-	-	-	NT
PS-078	-	-	-	-	-	-	NT
PS-079	moderate activity	moderate activity	1 mg/mL	1 mg/mL	1 mg/mL	1 mg/mL	high activity
PS-080	-	-	-	-	-	-	NT
PS-081	-	-	-	-	-	-	NT
PS-082	-	-	-	-	-	-	NT
PS-083	-	-	-	-	-	-	NT
PS-084	-	-	-	-	-	-	NT
PS-085	-	-	-	-	-	-	NT
PS-086	-	-	-	-	-	-	NT
PS-087	-	-	-	-	-	-	NT
PS-088	-	-	-	-	-	-	NT
PS-089	-	-	-	-	-	-	-
PS-090	-	-	-	-	-	-	-
PS-091	-	-	-	-	-	-	NT
PS-092	-	-	-	-	-	-	NT
PS-093	-	-	-	-	-	-	NT
PS-094	-	-	-	-	-	-	NT
PS-095	low activity	low activity	-	-	-	-	-
PS-096	moderate activity	moderate activity	-	-	1 mg/mL	1 mg/mL	low activity
PS-097	low activity	low activity	-	-	-	-	-
PS-098	low activity	low activity	-	-	-	-	-
PS-099	-	-	-	-	-	-	NT
PS-100	-	-	-	-	-	-	NT

CODE	OD_{600} measurements		MIC determination method				Agar dilution
	OD_{600} EGDe wt	OD_{600} EGDe Δ*sigB*	MIC EGDe wt 30°C	MIC EGDe Δ*sigB* 30°C	MIC EGDe wt 37°C	MIC EGDe Δ*sigB* *37*°C	
PS-101	-	-	-	-	-	-	NT
PS-102	low activity	low activity	1 mg/mL	1 mg/mL	0.5 mg/mL	0.5 mg/mL	-
PS-103	-	-	-	-	-	-	NT
PS-104	-	-	-	-	-	-	NT
PS-105	-	-	-	-	-	-	NT
PS-106	high activity	high activity	1 mg/mL	1 mg/mL	0.5 mg/mL	0.5 mg/mL	Medium activity
PS-107	-	-	-	-	-	-	-
PS-108	low activity	low activity	-	-	-	-	moderate activity
PS-109	moderate activity	moderate activity	-	-	-	-	-
PS-110	-	-	-	-	-	-	NT
PS-111	-	-	-	-	-	-	NT
PS-112	-	-	-	-	-	-	NT
PS-113	-	-	-	-	-	-	NT
PS-114	-	-	-	-	-	-	-
PS-115	-	-	-	-	-	-	NT
PS-116	-	-	-	-	-	-	-
PS-117	-	-	-	-	-	-	NT
PS-118	-	-	-	-	-	-	NT
PS-119	-	-	-	-	1 mg/mL	1 mg/mL	-
PS-120	not conclusive	not conclusive	-	1 mg/mL	1mg/mL	1 mg/mL	Low activity

Three methods were used to assess the antibacterial properties of the extracts (Table 2 – Section 3.4 Materials and Methods) – OD_{600} measurements, a MIC determination method using resazurin as an indicator of growth and agar dilution. The antibacterial potential of each extract was evaluated against a 10^6 CFU/mL inoculum of *L. monocytogenes* EGD-e wild-type or its isogenic derivate Δ*sigB*. The MIC of each extract was determined at two temperatures, 30°C and 37°C by serial dilutions. Results interpretation – OD_{600} measurements. The interpretation of the data was: low activity, delay in lag phase, medium activity, lag delay and growth curve slope changed; and high activity – no changes in OD_{600}, not conclusive – initial OD_{600} value too high. Agar dilution assay: low activity – growth impairment of an inoculum up to 10^4 CFU/mL, medium activity – growth impairment up to 10^6 CFU/mL and high activity – growth impairment of an inoculum concentration higher than 10^7 CFU/mL. NT – not tested. (-) – no activity. Each result represents a mean value of at least three biological replicates.

From the results presented in Table 5 and Figure 6 below, it can be noted that all extracts that displayed activities against the wt strain were also active against the Δ*sigB* mutant with no discrimination in activity towards either of the two strains. Although subtle differences could be observed, the Δ*sigB* mutant was not more sensitive to the antibacterial effects of the bioactive extracts, since a "one well" difference is generally accepted as an assay variation error. Taking into account the results from all three methods (Figure 6), four categories of antilisterial activity were assigned: not-active, low, medium and

high. For the MIC method, low antilisterial activities were attributed to extracts that displayed inhibition properties only at the highest concentration of 1 mg/mL, medium antilisterial activities implied MICs between 0.25 – 0.5 mg/mL, while high antilisterial activities were considered MICs at 0.125 mg/mL and below. In the agar dilution assay, low activity was regarded when the extract impaired the growth of an inoculum of 10^4 CFU/mL, medium activity for growth impairment of inocula up to 10^6 CFU/mL and high activity when the extract could impair the growth of bacterial inocula higher than 10^7 CFU/mL. Finally, in the OD_{600} method, low activity implied that the extract delayed the lag phase, medium activity when additional to the lag delay, the growth curve slope was notably changed and high activity when no changes in turbidity were observed during 24 hours.

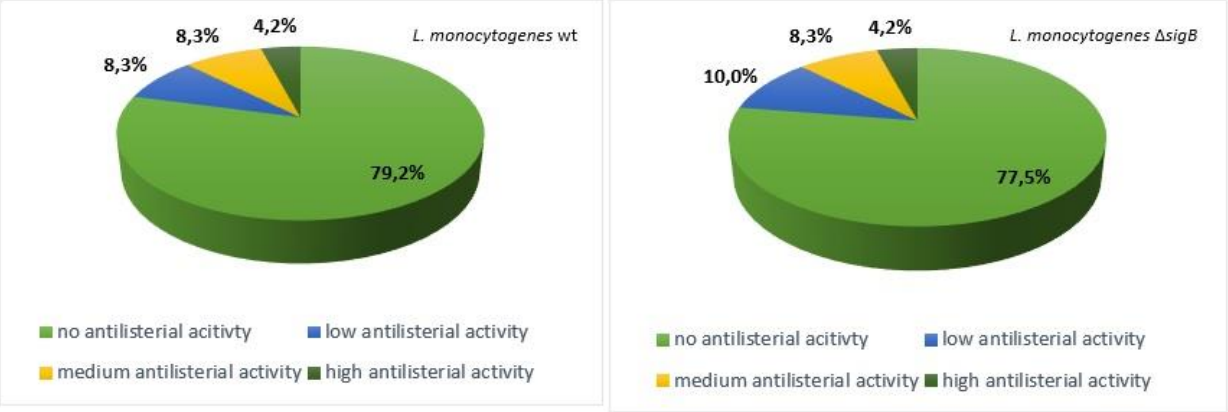


Figure 6. Overall results of the antilisterial activities of the initial 120 plant extracts against *L. monocytogenes* wt and Δ*sigB*. The results from all three antibacterial assessment methods were considered to categorise the antibacterial properties of the extracts in 4 groups: no activity, low, medium and high antibacterial activity.

Altogether, 95 extracts did not present growth inhibition properties against *L. monocyto*genes wt, while this number was 93 for the Δ*sigB* mutant. The difference can be explained by minor inoculum variations in the assays, since the antibacterial effects of plant extracts are highly dependent on the initial bacterial cell population density. Additionally, it is also probable that these two extracts had a MIC slightly above, but very close to 1 mg/mL, which was the highest concentration used for the screening of the extracts in this study. At the same time, no variations were observed for the extracts considered with medium, 10 extracts, or high antibacterial activities, 5 extracts. It could be observed that some of the active extracts, albeit with high, medium or low activity originated from the same plant, suggesting that they might contain the same active phytochemicals in a lesser or greater degree. These plants included *Olea europaea* L. and *Humulus lupulus* L.

In next steps, the abovementioned two plants along with seven other plant species that produced the most active extracts were selected for the subsequent phases of the bioassay-guided fractionation.

These plants were subjected to *de novo* extractions and different fractionation and purification approaches in order to isolate and identify the active phytochemical(s). More detailed information considering all the processes of fractionation, purification, chemical identification, as well as the results of the antibacterial activities of each newly

produced fraction or purified compound is given in Section 4.1.3 of Chapter 4.1.

Information about the selected 11 extracts identified as the most active in the initial screening, the plant they originate from, their analytical markers and the MICs observed in the antibacterial assays are specified in Table 6. These extracts are all rich in different bioactive molecules, representing various classes of phytochemicals, from more simple phenolic compounds like oleuropein and rosmarinic acid, to complex polyphenols like punicalagins, together with flavonoids, stilbenes, pentacyclic triterpenes and others.

Table 6. Plant origin, concentration of analytical marker(s) and MICs of the extracts selected for further characterization and bioactivity studies from the initial screening.

	CODE	PLANT	ANALYTICAL MARKER	PURITY	MIC
1	PS-018	*Olea europaea L.*	Oleuropein	20%	0.5 mg/mL
2	PS-033	*Olea europaea L.*	Hydroxytyrosol Oleanolic acid Maslinic acid Tocopherols	3.2% 11.7% 3.0% 3.1%	0.5 mg/mL
3	PS-014	*Humulus lupulus* L.	Hop bitter acids	35%	0.063 mg/mL
4	PS-079	*Humulus lupulus* L.	Hop bitter acids Xanthohumol	10% 2.5%	1 mg/mL
5	PS-011	*Punica granatum* L.	Punicalagins	40%	1 mg/mL
6	PS-022	*Rosmarinus officinalis* L.	Rosmarinic acid	6%	0.25-0.5 mg/mL
7	PS-024	*Salvia ofiicinalis* L.	Ursolic acid	15.1%	0.125-0.25 mg/mL
8	PS-026	*Camelia sinensis L.*	Catechins	64%	0.25-0.5 mg/mL
9	PS-052	*Melissa officinalis* L.	Rosmarinic acid	5%	0.5 mg/mL
10	PS-057	*Orthosiphon stamineus Benth.*	Sinensetin	0.2%	0.5 mg/mL
11	PS-106	*Eucalyptus globulus L.*	Polyphenols	18.1%	0.5 mg/mL

4.1.3. Selected plants with distinguished antibacterial activities against L. monocytogenes

4.1.3.1. Olea europaea L.

Olea europaea L. (family Oleaceae) is an essential tree crop in the Mediterranean basin. According to data from 2016, the worldwide harvested area of olive trees was about 10.6 million hectares, with >96% of this area concentrated in the Mediterranean region[96]. In parallel, Spain is the main producer of olive oil worldwide, and the production of olive oil and olive fruit is of paramount economic importance in this region. The popularity of these foods is mostly due to the health benefits associated with their culinary uses, which in turn are a result of the phytochemical profile of *O. europaea* L. Phenolic compounds constitute one of the major classes of phytochemicals, with several different families including flavonoids, secoiridoids, flavanones and simple phenols, while other phytochemicals of importance include triterpenes, lignans, xylitol, sterols and others. Polyphenols are present in almost all parts of the olive tree, but their nature and concentration vary greatly among the tissues. Many of these compounds have been fully identified: flavonols (quercetin 3-rutinoside, luteolin 7-O-glucoside, apigenin 7-O-glucoside), phenolic acids (chlorogenic acid, caffeic acid, p-hydroxybenzoic acid, protocatechuic acid, vanillic acid, syringic acid, p-coumaric acid, o-coumaric acid, ferulic acid, sinapic acid, benzoic acid, cinnamic acid, gallic acid), phenolic alcohols (3,4-DHPEA, p-HPEA) and secoiridoids (oleuropein, demethyloleuropein, ligstroside, nuzhenide)[97]. The main hydroxycinnamic acid derivative of importance in olive is verbascoside, while hydroxytyrosol and tyrosol are the principal phenolic compounds of the olive fruit, present at 76.7 and 19.5 mg/100 g olives, respectively [98]. On the other hand, the leaves of *O. europaea* L. can contain up to 5% triterpenic acids[99].

Initial screening

In the initial screening, several extracts of *O. europaea* L. were evaluated for their antibacterial properties, including extract PS-018 and extract PS-033. PS-018 is an extract obtained from the leaves of the olive tree, and among the most important phytochemical compounds that can be detected in this extract are the secoiridoid oleuropein (20%) and pentacyclic triterpenoids (4%). On the other hand, extract PS-033 is a mixture of bioactive compounds originating from the olive fruit biomass after pressing the virgin olive oil. Among the most important phytochemicals contained in this extract are the triterpenic acids oleanolic acid and maslinic acid (11.7% and 3%), and the phenylethanoid hydroxytyrosol (3.1%). The antibacterial activities of both extracts were evaluated using the two dilution broth methods and the agar dilution method. The results are presented in Table 7.

Table 7. Antibacterial activities of *O. europaea* L. extracts PS-018 and PS-033 against *L. monocytogenes* EGD-e wt and Δ*sigB*.

Plant	Extract	Conc. [mg/ml]	OD_{600} wt [mg/ml]	OD_{600} Δ*sigB* [mg/ml]	Micro-dilution assay	*L. monocytogenes* wt 30°C [mg/ml]	*L. monocytogenes* Δ*sigB* 30°C [mg/ml]	*L. monocytogenes* wt 37°C [mg/ml]	*L. monocytogenes* Δ*sigB* 37°C [mg/ml]	Agar dilution assay
Olea europaea	PS-018	1.00	1.00	1.00	MIC	1.00	1.00	0.50	0.50	-
					MBC	1.00	1.00	1.00	1.00	
	PS-033	1.00	1.00	1.00	MIC	0.50	0.50	0.25	0.25	Low 10^4
					MBC	0.50	0.50	0.50	0.50	

The MICs (minimum inhibitory concentrations) and MBCs (minimum bactericidal concentrations) of the extracts were determined by broth serial dilution against exponentially grown cells at 10^6 CFU/mL inoculum concentration. In the OD_{600} assay the optical density was continuously measured at 600 nm. (-) – no activity at the tested concentration.

Even though the initial results of the antibacterial activities of these two extracts were not exceptionally high, it was noteworthy that both distinct *O. europaea* L. extracts regarding their compositions, however, possessed antibacterial activities. These activities can be potentiated by identifying and concentrating the bioactive compounds and testing the separate fractions.

Primary O. europaea L. olive fruit and olive leaf fractions

By chemical composition, the dominant phytochemical compounds of extract PS-033 – the olive fruit extract (OFE) are 11.7% maslinic acid and 3.1% hydroxytyrosol. Utilizing the differences in solubility in water of these phytocompounds, two fractions were produced:

- OLE FR1 – a water insoluble fraction that contained 25% of maslinic acid; and
- OFE FR2 – a water-soluble fraction that contained hydroxytyrosol at 20%.

On the other hand, PS-018 – the olive leaf extract (OLE) contains 20% of oleuropein as a major phytochemical and 4% of triterpenoids. From this extract, the following fractions were produced:

- OLE FR1 – a lipophilic fraction that was isolated by treating the extract with hexane;
- OLE FR2 – produced by water precipitation and concentration of the triterpenic acids to 40%;
- OPA-80 – a water-soluble fraction that was subsequently fractionated by chromatographic purification to produce an isolate that contained 80% of oleuropein.

All newly produced olive fractions were tested for their antibacterial potential in order to identify the phytochemical group of compounds responsible for the bioactivities of the initial extracts. The results are presented in Table 7. Details about the fractionations are provided in Section 3.5.1 – Materials & Methods.

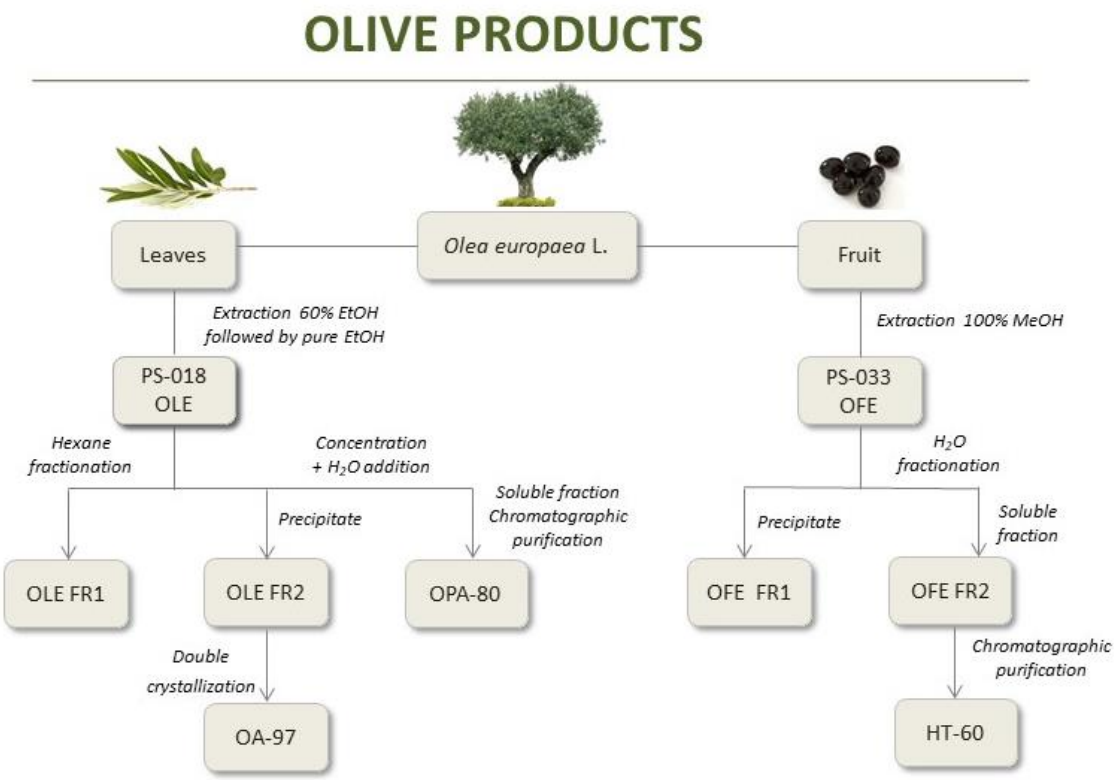


Figure 7. Fractionation and purification scheme of olive-derived products

Table 8. Antibacterial activities of the newly produced *O. europaea* L. fractions against *L. monocytogenes* EGD-e wt and Δ*sigB*.

						L. monocytogenes				
			OD_{600}		**Micro-dilution assay**	wt 30°C [mg/ml]	Δ*sigB* 30°C [mg/ml]	wt 37°C [mg/ml]	Δ*sigB* 37°C [mg/ml]	**Agar dilution assay**
Plant	Fraction	Conc. [mg/ml]	wt [mg/ml]	Δ*sigB* [mg/ml]						
Olive fruit	OFE FR1	1.00	-	-	MIC	-	-	-	-	-
					MBC	-	-	-	-	
	OFE FR2	1.00	NC	NC	MIC	0.125	0.125	0.125	0.125	High 10^7
					MBC	0.25	0.25	0.25	0.25	
Olive leaf	OLE FR1	1.00	-	-	MIC	-	-	-	-	-
					MBC	-	-	-	-	
	OLE FR2	1.00	NC	NC	MIC	0.25	0.25	0.125	0.125	-
					MBC	0.25	0.25	0.125	0.125	
	OPA-80	1.00	-	-	MIC	1.00	1.00	0.50	0.50	Low 10^5
					MBC	1.00	1.00	1.00	1.00	

The MICs and MBCs of the plant agents were determined by broth serial dilution against exponentially grown cells at 10^6 CFU/mL inoculum concentration. In the OD_{600} assay the optical density was continuously measured at 600 nm. (-) – no activity at the tested concentration. NC – not conclusive.

The results presented in Table 8 suggest that the phytochemicals oleuropein and maslinic acid, as well as the non-polar compounds present in OLE FR1 do not have strong antibacterial activities and were probably not carriers of the antibacterial properties of the initial extracts, the olive leaf extract PS-018 and the olive fruit extract PS-034. On the other hand, the fraction that contains 20% hydroxytyrosol, OFE FR2, and the fraction concentrated in pentacyclic triterpenes, OLE FR2, were more active and further investigated.

Purification of oleanolic acid and hydroxytyrosol from O. europaea L.

Hydroxytyrosol was purified from OFE FR2 using an adsorption resin that retains aromatic compounds and was eluted with a polar solvent. Fractions of equal volume were recovered every 10 minutes and the percent concentration of hydroxytyrosol was calculated in each fraction by HPLC. The fraction with the highest concentration was evaporated to produce a dark coloured viscous liquid (see Section 3.5.1). This product had a concentration of 60% hydroxytyrosol and was named HT-60 – Figure 8.

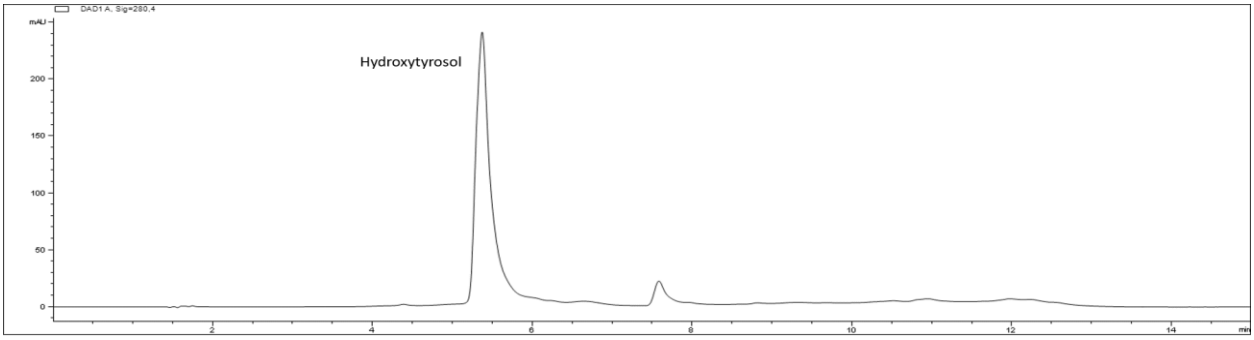


Figure 8. HPLC chromatogram of fraction HT-60 – a fraction that contains 60% hydroxytyrosol. Retention time – 5,3 min.

In contrast, oleanolic acid (OA) was purified from OLE FR2 following an industrial method developed by Natac Biotech. Namely, by taking advantage of the distinct temperature solubilities of different triterpenic acids, after several rounds of solvent fractionation, pure oleanolic acid was separated from maslinic and ursolic acid. HPLC analysis revealed 97% of oleanolic acid in the product that was labelled OA-97.

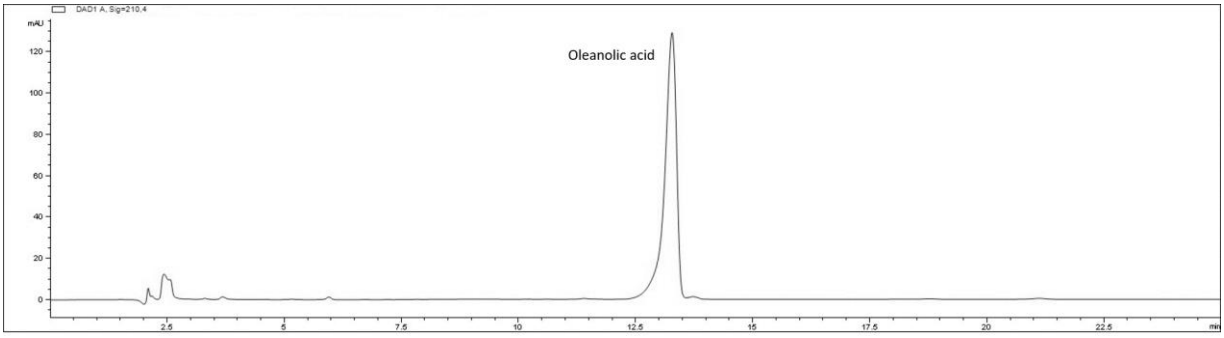


Figure 9. HPLC chromatogram of purified oleanolic acid. Retention time – 14,5 min.

The concentrated hydroxytyrosol fraction, HT-60, was active at the same concentration of hydroxytyrosol present in OFE FR2, implying that mainly hydroxytyrosol was responsible for the observed antibacterial activities. To confirm, pure hydroxytyrosol was also included in the next phase of the antibacterial assays. As expected, the MIC of pure hydroxytyrosol coincided with the MICs of OFE FR2 and HT-60 calculated according to the concentration of hydroxytyrosol. Furthermore, OA-97, or pure oleanolic acid, was one of the most active compounds in the study. The MIC of oleanolic acid against *L. monocytogenes* EGD-e was placed between 5-10 μg/mL and it coincides as a MBC as well.

Table 9. Antibacterial properties of purified *O. europaea* L. products – hyroxytyrosol (HT-60) and oleanolic acid (OA-97) against *L. monocytogenes* EGD-e wt and Δ*sigB*.

						L. monocytogenes				
			OD_{600}		**Micro-dilution assay**	wt 30°C [μg/ml]	Δ*sigB* 30°C [μg/ml]	wt 37°C [μg/ml]	Δ*sigB* 37°C [μg/ml]	**Agar dilution assay**
Plant	Fraction	Conc. [μg/ml]	wt [μg/ml]	Δ*sigB* [μg/ml]						
Olea europaea	HT-60	1000	200	200	MIC	250	250	125	125	High - 10^7
					MBC	1000	1000	1000	1000	
	OA-97	1000	5-10	5-10	MIC	10	10	5-10	5-10	High - 10^8
					MBC	10	10	5-10	5-10	

The MICs and MBCs of the plant agents were determined by broth serial dilution against exponentially grown cells at 10^6 CFU/mL inoculum concentration. In the OD_{600} assay the optical density was continuously measured at 600 nm at various concentrations of the isolated phytocompounds until the MIC was detected.

At the same time, oleanolic acid is a difficult compound to work with because of its hydrophobicity and insolubility in water. The antibacterial properties of this compound were only observable when it was first solubilized in DMSO, and then introduced into the growth medium, not exceeding 0.5% final DMSO concentration in the assay. Several other solvents like methanol and ethanol were used for solubilization of oleanolic acid in order to exclude DMSO interactions from the observed bioactivity and the compound demonstrated identical antibacterial activity in all solvents. To improve the solubility of oleanolic acid in water and try to eliminate the use of an organic solvent for solubilization two approaches were taken: first, a sodium salt (Na^+OA), a potassium salt (K^+OA) and an ammonium salt (NH_4^+OA) of oleanolic acid were produced by neutralization with sodium hydroxide, potassium hydroxide and ammonia, and second, an oleanolic acid phytophospholipid complex was produced by complexation with soy lecithin. A phytophospholipid complex is a complex of a natural active ingredient or an isolated active principle and a phospholipid. The water solubility of the complex was significantly improved to 3600 ppm/mL from

practically zero for oleanolic acid itself. Additionally, the newly produced sodium and potassium salts, but not the ammonium salt of oleanolic acid, also had improved solubilities in the growth medium.

Nonetheless, while the sodium and potassium salt retained the same antibacterial properties as pure oleanolic acid, the ammonium salt had a higher MIC, an expected result since its solubility was not improved. On the contrary, the OA-phytophospholipid complex did not show any antibacterial activities, indicating that, the engulfment of oleanolic acid in the phospholipid micelle impaired the contact between the active molecule and the bacterial cells. This data might also suggest that oleanolic acid probably exerts its activity on the external surfaces of the bacterial cells, either on the cell wall or the bacterial membrane, since the possible absorption of the complex inside the cell did not produce observable cell damage.

Table 10. Antibacterial properties of oleanolic acid derivatives against *L. monocytogenes* EGD-e wt and Δ*sigB*.

				L. monocytogenes				
Plant	Compound	Conc. [μg/ml]	**Micro-dilution assay**	wt 30°C [μg/ml]	Δ*sigB* 30°C [μg/ml]	wt 37°C [μg/ml]	Δ*sigB* 37°C [μg/ml]	**Agar dilution assay**
Olea europaea	OA-97	1000	MIC	10	10	5-10	5-10	High 10^7-10^8
			MBC	10	10	5-10	5-10	
	Na^+OA	1000	MIC	10	10	5-10	5-10	High 10^7-10^8
			MBC	10	10	5-10	5-10	
	K^+OA	1000	MIC	10	10	5-10	5-10	High 10^7-10^8
			MBC	10	10	5-10	5-10	
	NH_4^+OA	1000	MIC	-	-	500-1000	500-1000	-
			MBC	-	-	500-1000	500-1000	
	Phyto-phospholipid	1000	MIC	-	-	-	-	-
			MBC	-	-	-	-	

The MICs and MBCs of the plant agents were determined by broth serial dilution against exponentially grown cells at 10^6 CFU/mL inoculum concentration. (-) – no activity at the tested concentration.

Altogether, *O. europaea* L. produces several phytocompounds capable of impairing the growth of *L. monocytogenes*, the most potent being the pentacyclic triterpenic acid oleanolic acid and the simple phenol hydroxytyrosol. Weaker antibacterial affects were also observed for the secoiridoid oleuropein.

4.1.3.2. Eucalyptus globulus L.

Eucalyptus globulus L. (family Myrtaceae) is one of the world's most important and widely planted species, providing high quality pulp for the paper industry, as well as fuelwood and charcoal[100]. As one of the most cultivated species, it generates a substantial amount of biomass residues, particularly woody residues like bark and branches, large amounts of leaves and fruits. A part of the leaf biomass has been recycled for the production of the most required product of *E. globulus*, eucalyptus essential oil (EO)[100]. This EO, characterized by pleasant odour and established bioactivities has generated interest for use as a natural product in several industries including the food industry and cosmetics. Apart from essential oil components, the leaves of *E. globulus* contain other phytocompounds such as flavonoids and proantho-cyanidins, as well as various bioactive compounds with the potential to be converted in high value-added products. These include several triterpenic acids with lupane, ursane and oleanane skeletons, namely, betulonic, betulinic, 3-acetylbetulinic, ursolic, 3-acetylursolic, oleanolic and 3-acetyloleanolic acids[101].

Initial screening

Only one plant extract originating from the genus *Eucalyptus* was included in the initial screening. This extract was coded PS-106 and it is a 70% ethanol extract of the leaves of *E. globulus* L.. This extract was rich in polyphenolic compounds which were present at 18,1% total content.

Table 11. Antibacterial activity of *E. globulus* L. extract PS-106 against *L. monocytogenes* EGD-e wt and Δ*sigB*.

						L. monocytogenes				
			OD_{600}		**Micro-dilution assay**	wt 30°C	Δ*sigB* 30°C	wt 37°C	Δ*sigB* 37°C	**Agar dilution assay**
Plant	Extract	Conc. [mg/ml]	wt [mg/ml]	Δ*sigB* [mg/ml]		[mg/ml]	[mg/ml]	[mg/ml]	[mg/ml]	
Eucalyptus globulus	PS-106	1.00	1.00	1.00	MIC	1.00	1.00	0.50	0.50	medium activity 10^5
					MBC	1.00	1.00	1.00	1.00	

The MICs and MBCs of all extracts were determined by broth serial dilution against exponentially grown cells at 10^6 CFU/mL inoculum concentration. In the OD_{600} assay the optical density was continuously measured at 600 nm.

PS-106 was one of the few initial extracts that completely inhibited the growth of *L. monocytogenes* in the OD_{600} microdilution method carried out in the multimode reader (Figure 10).

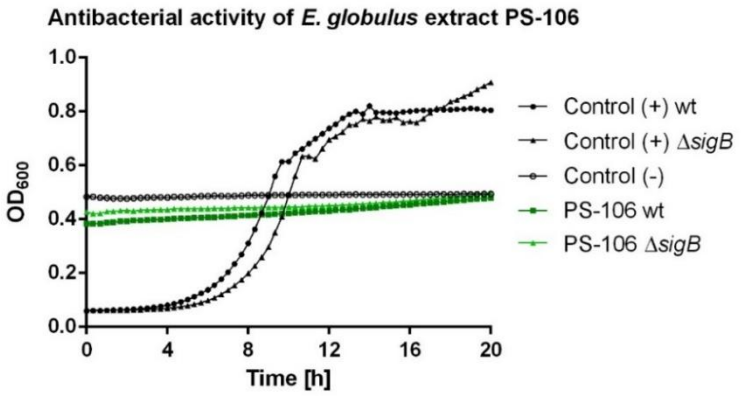


Figure 10. Growth curve of *L. monocytogenes* wt and Δ*sigB* in the presence of extract PS-106. An inoculum of 10^6 CFU/mL *L. monocytogenes* EGD-e wild-type or *L. monocytogenes* EGD-e Δ*sigB* were introduced in BHI supplemented with 1 mg/mL of extract PS-106. OD at 600 nm was continuously measured during 20h. Control (-) – extract PS-106 in BHI; Control (+) *L. monocytogenes* wt or Δ*sigB* in BHI. The results are representative of three technical replicates.

Since this extract is not completely soluble in water, an emulsion forms from the dispersion of the insoluble compounds in the water-based medium that increases the opacity of the solution and results in a higher initial value for OD_{600}, between 0.4 – 0.5. This effect is clearly shown in the negative control growth curve in Figure 10 above, Control (-), that consists only of the extract dissolved in BHI.

Production of new extracts and fractions from E. globulus leaves

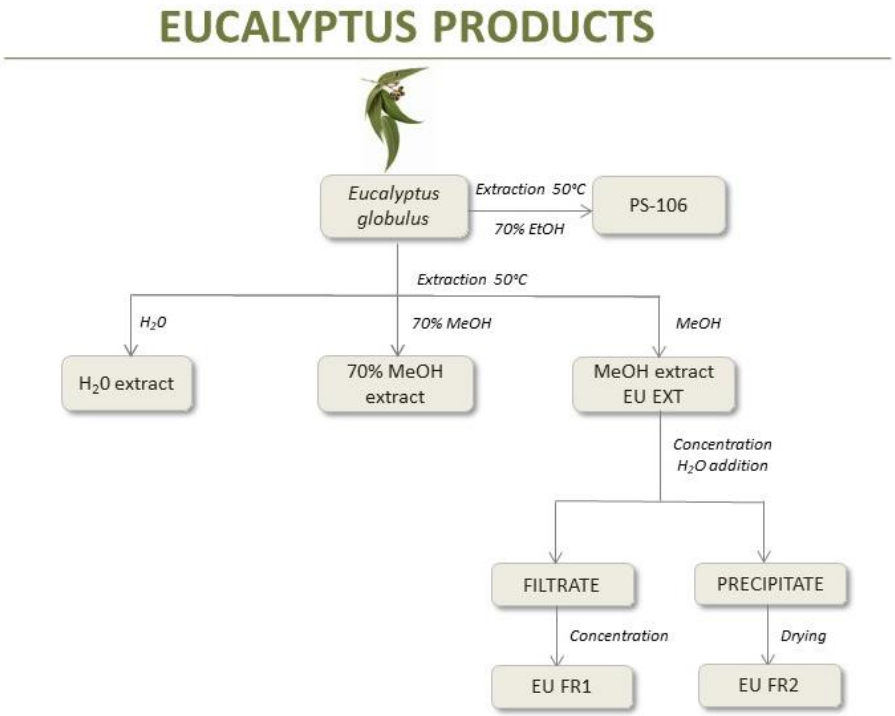


Figure 11. Schematic representation of the production process of various *E. globulus* leaves products

Upon confirming the activity of extract PS-106, three new extracts were freshly produced from the leaves of *E. globulus*, a water extract, a methanolic extract and a 70% v/v methanolic extract. The methanolic extract had the highest yield of extraction, 31.4%, while the water extract had the lowest yield, 18.3%. The 70% methanol extract had a similar yield to the pure methanol extract, 28.8%. The newly obtained *E. globulus* extracts were tested for their antibacterial activities and the results are presented in Table 12. The water extract had very limited antibacterial properties, showing inhibitory effects only at 37°C at the highest tested concentration, while the 70% methanol extract was more active in comparison, with MICs of 0.45 mg/mL and MBCs of 0.9 mg/mL. On the other hand, the methanolic extract was distinctively more active, with MICs 31 µg/mL at 37°C and 62 µg/mL at 30°C, and MBCs of 123 µg/mL.

Table 12. Antibacterial activities of newly produced *E. globulus* L. extracts against *L. monocytogenes* EGD-e wt and Δ*sigB*.

				L. monocytogenes				
Plant	Extract	Conc. [mg/mL]	Microdilution method	wt 30°C [mg/ml]	Δ*sigB* 30°C [mg/ml]	wt 37°C [mg/ml]	Δ*sigB* 37°C [mg/ml]	Agar dilution method
	H_2O extract	2.29	MIC	-	-	2.29	2.29	-
			MBC	-	-	-	-	
Eucalyptus globulus	MeOH extract	3.92	MIC	0.062	0.062	0.031	0.031	high activity 10^9
			MBC	0.123	0.123	0.123	0.123	
	70% MeOH extract	3.60	MIC	0.45	0.45	0.45	0.45	high activity 10^6
			MBC	0.90	0.90	0.90	0.90	

In the second round of bioassay-guided fractionation three new *E. globulus* L. leaves extracts, a water extract, a methanolic extract and a 70% v/v methanolic extract were produced. The MICs and MBCs of the plant agents were determined by broth serial dilution against exponentially grown cells at 10^6 CFU/mL inoculum concentration. In the OD_{600} assay the optical density was continuously measured at 600 nm. (-) – no activity.

Once the methanolic extract was established as the most active, an extraction approach using a higher plant to solvent ratio was applied to increase the concentration of active compound in the extract. The newly produced more concentrated methanolic extract (EU EXT) was further subjected to solvent-solvent fractionation and two fractions were produced, EU FR1 and EU FR2. The fractionation process was carried out by first concentrating the extract to a fifth of its volume, after which water was added to precipitate the water-insoluble compounds. The remaining methanol was evaporated and the water-insoluble precipitate (EU FR2) was separated from the water-soluble fraction (EU FR1). Dry matter, total polyphenolic and total flavonoid content were calculated for all newly produced *E. globulus* extracts.

Table 13. Phytochemical analyses of the methanolic extract and its fractions of *E. globulus* L.. Dry matter, total phenolics and total flavonoid content were determined in the methanolic extract EU EXT and its fractions EU FR1 and EU FR2.

Plant	Sample	Dry matter (mg/mL)	Total polyphenols in extract (%)	mg of RE per 1 g of extract
Eucalyptus globulus	EU EXT	21.54	12.8%	16.24
	EU FR1	12.74	21.3%	34.08
	EU FR2	8.10	5.1%	11.09

The EU EXT had a yield of extraction of 21.5% and a total phenolics content of 12.8%. Calculated to the leaves, approximately 2.5% of phenolics were present in the leaves of *E. globulus* L. Upon fractionation, the phenolic compounds preferentially distribute in the water-soluble fraction (EU FR1), while the precipitate fraction (EU FR2) showed a low amount of total polyphenols. Very similar distribution could be observed regarding the total flavonoids content. Among the flavonoid family, quercetin was detected by HPLC in low concentrations in the EU EXT – 0.02% and EU FR1 – 0.05%.

A fairly high concentration of pentacyclic triterpenic acids was detected in the EU EXT. The concentration of these compounds was 9.3% in total in the extract. Upon fractionation, the triterpenoids distribute in the precipitate fraction, since their solubility in water is almost nil. This was further confirmed by the fact that they were not detected in EU FR1. Accordingly, their preferential distribution in the water insoluble fraction EU FR2 increased the final concentrations of triterpenic acids in EU FR2 to 18.9%.

Table 14. Concentration of the most common triterpenoid acids, ursolic acid, oleanolic acid and betulinic acid in *E. globulus* L. products.

Plant	Sample	Ursolic acid		Oleanolic acid		Betulinic acid	
		mg/mL	Content (%) Extract	mg/mL	Content (%) Extract	mg/mL	Content (%) Extract
Eucalyptus globulus	EU EXT	0.780	4.59%	0.551	3.24%	0.256	1.50%
	EU FR1	0	0	0	0	0	0
	EU FR2	0.913	11.44%	0.259	3.25%	0.332	4.16%

The methanolic extract EU EXT and its fractions EU FR1 and EU FR2 were analysed by HPLC to determine the concentration of the triterpenoids in each product. Analytical standards of the three triterpenoid acids were used as a reference.

As before, all newly produced *E. globulus* products were tested for their antibacterial activities against *L. monocytogenes* wild-type and the Δ*sigB* mutant.

Table 15. Antibacterial properties of the final *E. globulus* L. products against *L. monocytogenes* EGD-e wt and Δ*sigB*.

Plant	Extract / Fraction	Conc. [mg/ml]	Micro-dilution assay	*L. monocytogenes* wt 30°C [mg/ml]	*L. monocytogenes* Δ*sigB* 30°C [mg/ml]	*L. monocytogenes* wt 37°C [mg/ml]	*L. monocytogenes* Δ*sigB* 37°C [mg/ml]	Agar dilution assay
Eucalyptus globulus	EU EXT	2.15	MIC	0.22	0.22	0.11	0.11	high activity 10^9
			MBC	0.43	0.43	0.22	0.22	
	EU FR1	1.27	MIC	-	-	0.64	0.64	low activity 10^5
			MBC	-	-	1.27	1.27	
	EU FR2	0.81	MIC	0.05	0.05	0.025	0.025	high activity 10^9
			MBC	0.10	0.10	0.05	0.05	

The methanolic extract of *E. globulus* L. (EU EXT) was fractionated to produce EU FR1 and EU FR2. The MICs and MBCs of all extracts were determined by broth serial dilution against exponentially grown cells at 10^6 CFU/mL inoculum concentration. (-) – no activity.

It can be observed that the most active *E. globulus* L. products are EU FR1 and the EU EXT. This result implies that the inhibition properties of *E. globulus* L. against *L. monocytogenes* mostly come from water insoluble compounds that precipitate upon addition of water, such as, ursolic acid among others. The inhibitory activities of these extract can also be observed in Figure 12 below. This fraction had one of the lowest MICs and MBCs in the screening, and detailed studies placed the MIC at 25 μg/mL.

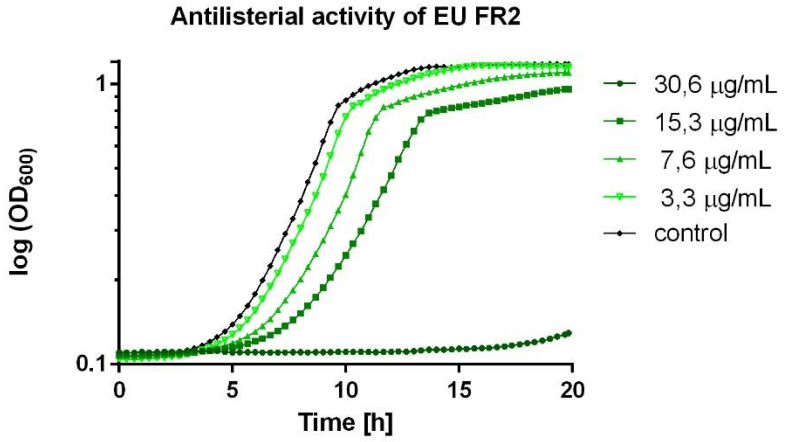


Figure 12. **Growth curve of *L. monocytogenes* EGD-e wild-type in the presence of various concentrations of fraction EU FR2.** A serial dilution of EU FR2 was performed in BHI after which an inoculum of 10^6 CFU/mL *L. monocytogenes* was introduced. OD at 600 nm was continuously measured for 20h. Control – *L. monocytogenes* EGD-e wt in BHI. The results are representative of three technical replicates.

4.1.3.3. Salvia officinalis L.

Salvia officinalis L. (family Lamiaceae), or common sage, is considered the queen of herbs. Also known as the "Salvation Plant", is has a long history of use both in culinary and medicinal preparations. It has been used to reduce perspiration, as a gargle for sore throat, to fight gastroenteritis, to improve lipid status and liver function in general, to improve appetite and digestion, and to improve mental capacity. The most commonly used and tested sage natural product is its essential oil, but recent studies, show that other sage products offer a great potential. The biologically active compounds present in *S. officinalis* L. belong to several phytochemical classes and families, including monoterpenes, diterpenes, triterpenes, and phenolic components. The most abundant phenolic compounds in this plant can be divided into two groups: phenolic acids (caffeic, vanillic, ferulic, and rosmarinic acids) and flavonoids (luteolin, apigenin, and quercetin). On the other hand, highly abundant monoterpenes include: α- and β-thujone, 1,8-cineole, and camphor, while the most common diterpenes include: carnosic acid, carnosol, rosmadial, and manool. Triterpenes present in sage include oleanolic and ursolic acids[102].

Initial screening

In the initial screening, two *S. officinalis* L. extracts were included – PS-024 and PS-025. PS-024 is a methanolic extract of *S. officinalis* L. with a high concentration of triterpenes, especially triterpenoid pentacyclic acids like ursolic acid, present at a concentration of 15.1% in the extract. On the other hand, extract PS-025 is a water-soluble sage extract abundant in polyphenolic phytochemicals, flavonoids in particular. Since the beginning of the study, extract PS-024 was singled out for its distinguished antibacterial activities, and until the end it remained one of the best extracts for the purposes of this screening. The initial screening results are presented in Table 16.

Table 16. Antibacterial activities of *S. officinalis* extracts PS-024 and PS-025 against *L. monocytogenes* EGDe wt and Δ*sigB*.

			OD_{600}		**Micro-dilution assay**	*L. monocytogenes*				
						wt	Δ*sigB*	wt	Δ*sigB*	**Agar dilution assay**
Plant	Extract	Conc. [mg/ml]	wt [mg/ml]	Δ*sigB* [mg/ml]		30°C [mg/ml]	30°C [mg/ml]	37°C [mg/ml]	37°C [mg/ml]	
Salvia officinalis	PS-024	1.00	0.25	0.25	MIC	0.25	0.25	0.125	0.125	high activity 10^9
					MBC	0.25	0.25	0.125	0.125	
	PS-025	1.00	-	-	MIC	-	-	-	-	-
					MBC	-	-	-	-	

The MICs and MBCs of the plant agents were determined by broth serial dilution against exponentially grown cells at 10^6 CFU/mL inoculum concentration. In the OD_{600} assay the optical density was continuously measured at 600 nm. (-) – no activity.

According to the results presented in Table 16, extract PS-024 has a MIC and MBC that vary between 125-250 µg/mL depending on the method used and the incubation temperature. This extract has a low solubility in water, so DMSO at a final concentration of 0.5% v/v was used to increase its solubility in the culture medium. This step was omitted in the agar dilution assay since the 1.5% agar solution acts as an emulsifying agent in the assay. The results obtained with the agar dilution method confirm the excellent antibacterial properties of the extract itself, with a total inhibition of the growth of 10^9 CFU/mL bacterial cell population at extract concentration of 1 mg/mL. On the contrary, antibacterial properties were not detected by any of the assays for extract PS-025 at the highest tested concentration of 1 mg extract/mL.

Based on the initial results, the following step was fractionation of extract PS-024 in order to determine the fractions with antibacterial activities and discard the inactive ones. By separating the compounds into different fractions, conclusions can be drawn about their polarity and possibly the phytochemical family to which they belong.

Fractionation of extract PS-024 and antibacterial properties of the obtained fractions

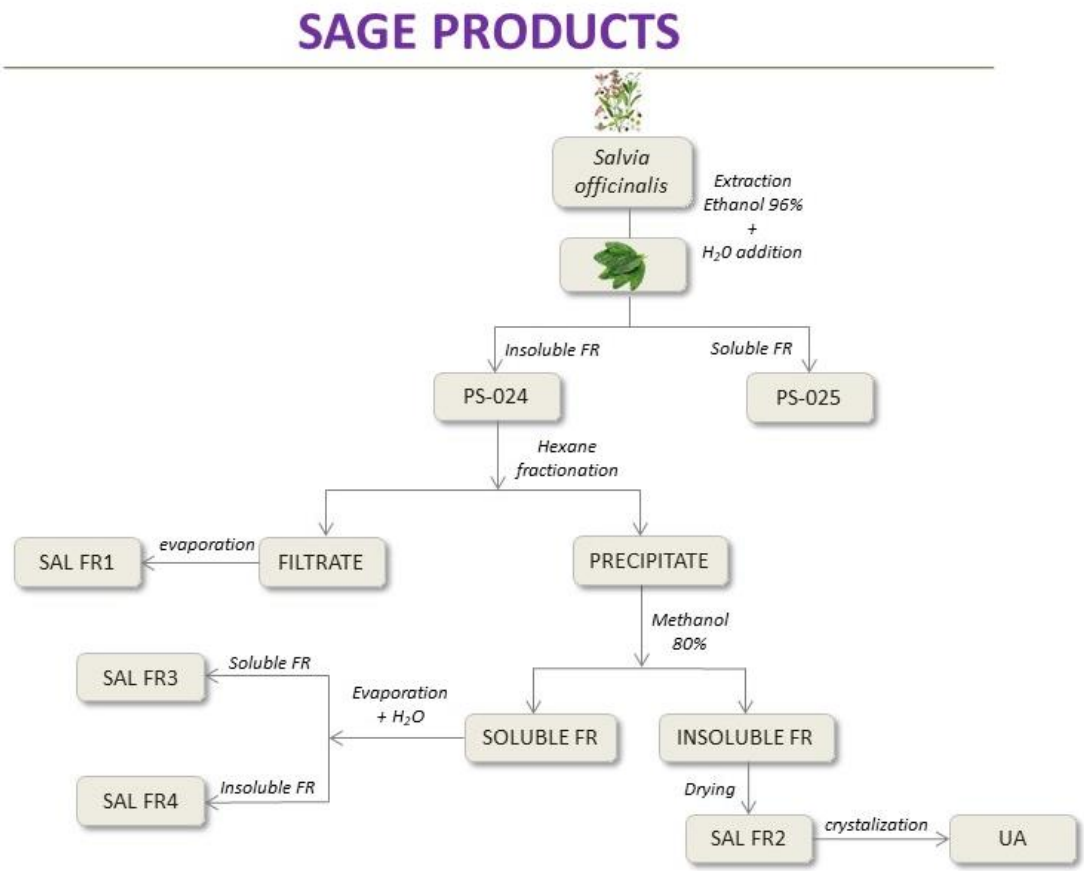


Figure 13. Fractionation diagram of *Salvia officinalis* products

Extract PS-024 was fractionated into four different fractions – SAL FR1 to SAL FR4. Primarily, the extract was suspended in hexane to separate the lipophilic compounds and later on converted to powder form by adding maltodextrin, resulting in SAL FR1. After separating the lipophilic fraction, the solid residue was dissolved in 80% methanol and left overnight to allow precipitation of the insoluble compounds, resulting in SAL FR2. To finish, the filtrate was divided into two fractions, a water-soluble fraction labelled SAL FR3, and a water-insoluble red-brown fraction that separated from the filtrate upon removal of methanol. The fractionation process is described in detail in Section 3.5.3 and presented schematically in Figure 13.

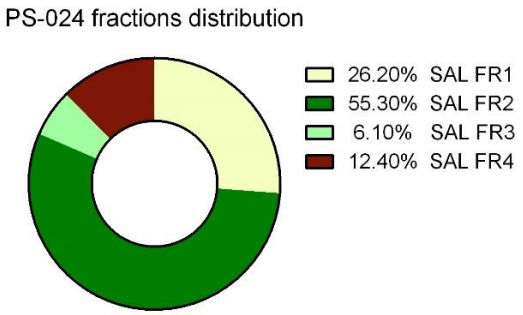


Figure 14. Percent distribution of fractions SAL FR1, SAL FR2, SAL FR3 and SAL FR4 within extract PS-024.

Fraction SAL FR2 was the dominating fraction separated from extract PS-024, with 55.3% distribution within the extract, followed by the hexane fraction SAL FR1 with 26.2%. The water-soluble and insoluble fractions form together 18.5% of the extract, with 6.1% belonging to SAL FR3 and 12.4% to SAL FR4 (Figure 14). Dry matter (DM) results showed 23.3 mg/mL DM in SAL FR3 and 53.0 mg/mL DM in SAL FR4.

SAL FR2 was analysed by HPLC for the presence of triterpenic compounds. 23.5% of ursolic acid and 9.6% of oleanolic acid were detected, making a total of 33.1% of pentacyclic triterpenic acids in SAL FR2. On the other hand, SAL FR4 was analysed for several phytochemical compounds based on literature findings. The fraction was tested for phenolic compounds – rosmarinic acid, chlorogenic acid, caffeic acid, vanillic acid, ferulic acid and p-coumaric acids and diterpenic compounds – carnosic acid, carnosol and methyl carnosoate. The results of these analyses confirmed the presence of 3.8% rosmarinic acid, and vanillic acid, caffeic acid, ferulic acid and p-coumaric acid in minute concentrations. Chlorogenic acid and caffeic acid were not detected. From the diterpenoids, SAL FR4 had a considerable amount of diterpenoids which were present at 33% in the fraction, and the ones identified were carnosol at 16.7%, carnosic acid at a 10.6% and methyl carnosoate at 0.7%. In contrast, the triterpenic acids identified in SAL FR2 were not present in SAL FR4.

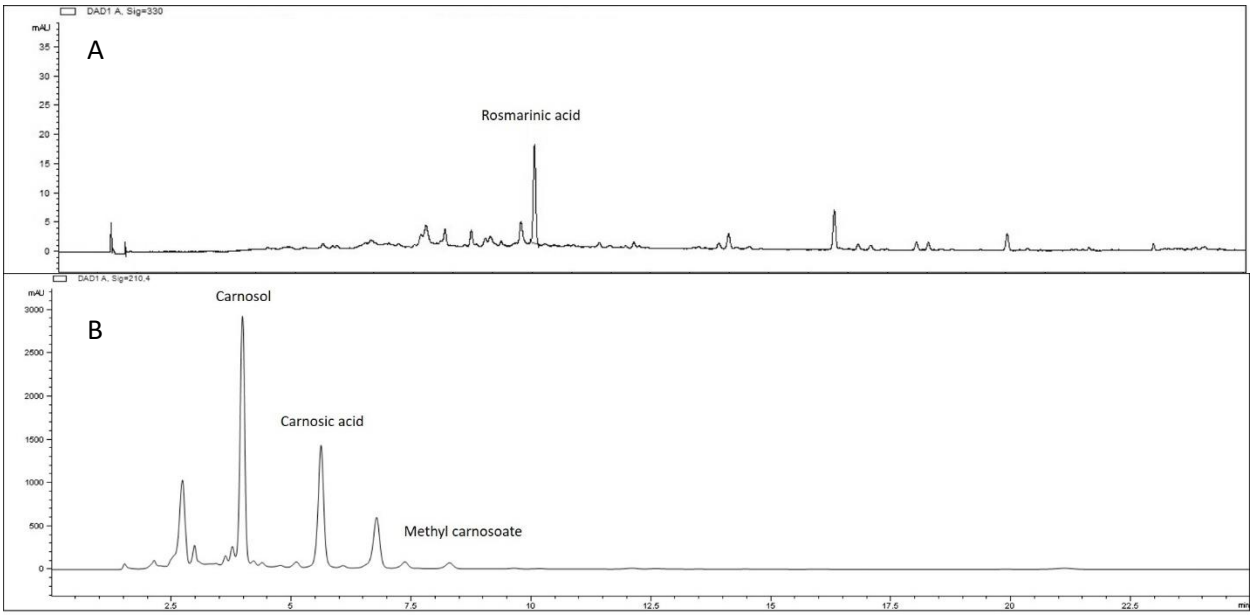


Figure 15. HPLC chromatograms of SAL FR4 analysed for (A) phenolic acids and (B) diterpenic compounds. Retention times: (A) Peak 24,1 min – rosmarinic acid. (B) Peak 3,98 min – carnosol; peak 5,6 min – carnosic acid; peak 7,4 min – methyl carnosoate.

The obtained fractions were assessed for their antibacterial properties using the same methods as before. In the initial screening, PS-024 was tested at a starting concentration of 1 mg/mL. Further on, in order to be able to directly compare the activity of each fraction to the activity of the extract, each fraction was prepared at a starting concentration equal to its fractional presence in the extract while treating the whole extract as 1.00 or 100%. For example, as SAL FR1 corresponds to 26.2% of the extract, the initial testing concentration was 0.262 mg/mL, and so on. The results from the antibacterial screenings of the fractions are presented in Table 17. Since SAL FR3 did not show any antibacterial effects at a concentration corresponding to its fractional presence of 0.061 mg/mL, it was additionally tested at higher concentrations in order to see whether some of the bioactive compounds have separated in this fraction. It was found that the MIC/MBC for this fraction is 1.5 mg/mL at 37°C, and 3 – 6 mg/mL at 30°C.

SAL FR1 was also effective at stopping the growth of *L. monocytogenes* at concentrations as low as 65 – 131 µg/mL. Since this fraction is almost insoluble in water, it was not possible to exclude DMSO in the assays. An assay was performed where this fraction was dissolved in culture media supplemented with 1% Tween or 0.2% agar, but antibacterial properties were not detected due to the failure of the selected emulsifiers to substantially solubilize the bioactive compounds of this fraction in broth media. On the other hand, when tested with the agar dilution assay, the antibacterial properties were evident, but lower in comparison to the extract and the other fractions.

Table 17. Antibacterial properties of the fractions of extract PS-024 against *L. monocytogenes* EGDe wt and Δ*sigB*.

Plant	Extract / Fraction	Conc. [mg/ml]	Microdilution method	*L. monocytogenes* wt 30°C [mg/ml]	*L. monocytogenes* Δ*sigB* 30°C [mg/ml]	*L. monocytogenes* wt 37°C [mg/ml]	*L. monocytogenes* Δ*sigB* 37°C [mg/ml]	Agar dilution method
Salvia officinalis	Extract PS-024	1.000	MIC	0.250	0.250	0.125	0.125	high activity 10^9
			MBC	0.250	0.250	0.125	0.125	
	SAL FR1	0.262	MIC	0.131	0.131	0.065	0.065	high activity 10^7
			MBC	0.131	0.131	0.065	0.065	
	SAL FR2	0.553	MIC	0.030	0.030	0.030	0.030	high activity 10^7
			MBC	0.060	0.060	0.030	0.030	
	SAL FR3	0.061	MIC	-	-	-	-	-
			MBC	-	-	-	-	
	SAL FR4	0.125	MIC	0.125	0.125	0.125	0.125	medium activity 10^5
			MBC	0.125	0.125	0.125	0.125	

The MICs and MBCs of the plant agents were determined by broth serial dilution against exponentially grown cells at 10^6 CFU/mL inoculum concentration. In the OD_{600} assay the optical density was continuously measured at 600 nm. (-) – no activity.

It can be noted that almost all fractions of extract PS-024 are active against *L. monocytogenes* at different concentrations. When directly compared to the whole extract, fraction SAL FR2 was the most effective for stopping the growth of *L. monocytogenes* with a MIC of 30 µg/mL.

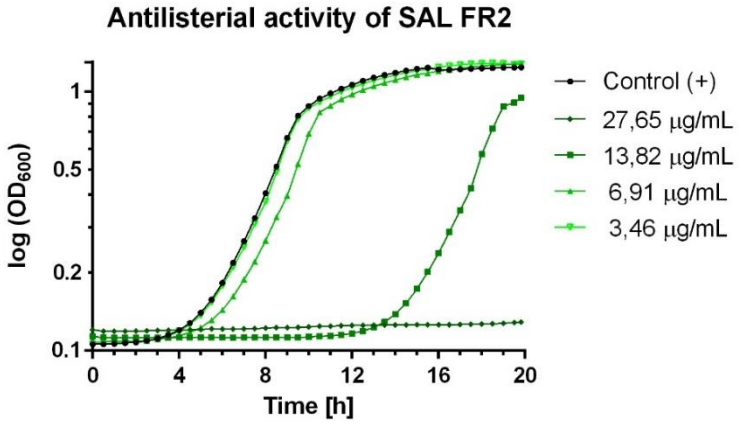


Figure 16. Growth curve of *L. monocytogenes* EGD-e wild-type in the presence of various concentrations of fraction SAL FR2. A serial dilution of SAL FR2 was performed in BHI after which an inoculum of 10^6 CFU/mL *L. monocytogenes* was introduced. OD at 600 nm was continuously measured for 20h. Control (+) – *L. monocytogenes* EGD-e wt in BHI. The results are representative of three technical replicates.

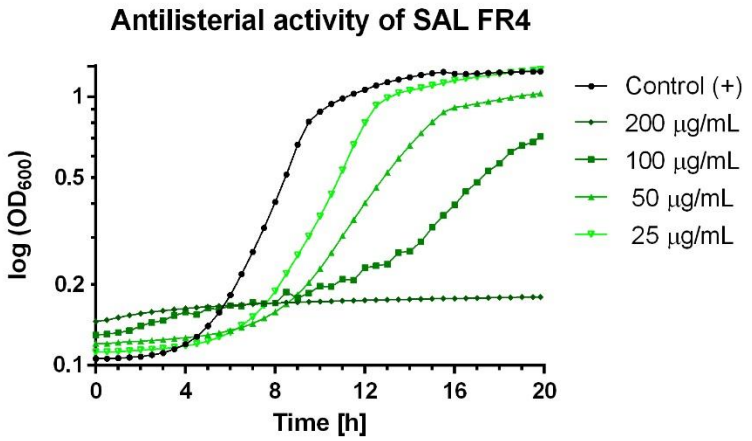


Figure 17. Growth curve of *L. monocytogenes* EGD-e wild-type in the presence of various concentrations of fraction SAL FR4. A serial dilution of SAL FR4 was performed in BHI after which an inoculum of 10^6 CFU/mL *L. monocytogenes* was introduced. OD at 600 nm was continuously measured for 20h. Control – *L. monocytogenes* EGD-e wt in BHI. The results are representative of three technical replicates.

In another experiment with a larger range of concentrations it was confirmed that the MIC for this fraction is even lower than 30 μg/mL and lies somewhere between 14-28 μg/mL (Figure 16). SAL FR4 was also characterized with excellent antilisterial properties. From the data shown in Figure 17, it can be noticed that the degree of inhibition of bacterial growth for this fraction is proportional to the concentration of the plant agent, with a maximum inhibition between 100 – 200 μg/mL.

Extraction of triterpenes from Salvia officinalis L.

Since SAL FR2 showed exceptional antibacterial activities and it is concentrated in ursolic acid, further investigations were done in order to evaluate the antibacterial activity of pure ursolic acid and to compare the results to extract PS-024. Purification of ursolic acid was performed from *S. officinalis* L. leaves. 1.41g of a product containing 50.4% of ursolic acid was obtained. The yield was calculated at 4.7% to the weight of dry plant leaves. Having previously observed that oleanolic acid has great antibacterial effects and that ursolic acid is one of the major compounds contained in SAL FR2, with 23.5%, the data suggested that this phytocompound could be the carrier of the observed bioactivity. To confirm this hypothesis, pure ursolic acid was acquired. Since ursolic acid, just like oleanolic acid, has very low solubility in water, apart from the acidic from, a sodium salt was obtained by neutralization with NaOH in order to produce a more soluble product for the testing. Additionally, a third triterpenic acid, glycyrrhizic acid was added in the final screening following the same logic of potential antibacterial activity of triterpenic acids.

Table 18. Antibacterial activities of triterpenic acids: pure ursolic acid (UA), a sodium salt of ursolic acid (Na^+OA), oleanolic acid (OA) and glycyrrhizic acid (GA).

Plant	Compound	Conc. [μg/ml]	Microdilution method	*L. monocytogenes* wt 30°C [μg/ml]	*L. monocytogenes* Δ*sigB* 30°C [μg/ml]	*L. monocytogenes* wt 37°C [μg/ml]	*L. monocytogenes* Δ*sigB* 37°C [μg/ml]	Agar dilution method
Salvia officinalis	UA	100	MIC	2.5 – 5	2.5 – 5	2.5 – 5	2.5 – 5	high activity 10^8
			MBC	2.5 – 5	2.5 – 5	2.5 – 5	2.5 – 5	
	Na^+UA	100	MIC	1.2 – 2.5	1.2 – 2.5	1.25	1.25	high activity 10^8
			MBC	1.2 – 2.5	1.2 – 2.5	1.25	1.25	
	OA	100	MIC	5 – 10	5 – 10	5	5	high activity 10^8
			MBC	5 – 10	5 – 10	5	5	
	GA	5000	MIC	-	-	-	-	-
			MBC	-	-	-	-	

The MICs and MBCs of the plant antimicrobial agents were determined by broth serial dilution against exponentially grown cells at 10^6 CFU/mL inoculum concentration. In the OD_{600} assay the optical density was continuously measured at 600 nm. (-) – no activity.

The results shown in Table 18 reveal that ursolic acid, just like oleanolic acid, is highly effective at inhibiting the growth of *L. monocytogenes,* with some of the lowest observed MICs in the study, between 2.5 – 5 μg/mL. It seems that the sodium salt is slightly more active than ursolic acid itself, suggesting that higher solubility was achieved for this compound. On the other hand, glycyrrhizic acid, even though it shares structural similarities with both ursolic and oleanolic acid, was not able to inhibit the growth of *L. monocytogenes* even at concentrations up to 5 mg/mL.

4.1.3.4. Humulus lupulus L.

Humulus lupulus L. (family Cannabaceae), or common hops, is a dioecious vine widely grown around the world for its use in the brewing industry to confer bitterness, aroma, and flavour to beer. Only the mature female cones of hops contain glandular trichomes in which secondary metabolites such as prenylated flavonoids, essential oils (mono- and sesquiterpenes) and bitter acids are produced. Hops bitter acids (HBAs) are a natural major constituent of hops cones and are present in amounts ranging from 2 – 24% depending on the hop variety. HBAs are soluble in organic solvents and are readily extracted by liquid or supercritical CO_2 extraction. The most important representatives of α-acids are humulone (35–70% of total α-acids), cohumulone (20–65% of total α-acids), and adhumulone (10–15% of total α-acids). As their chemical structures are very similar, the names and quantities of respective β-acids are analogous – lupulone (30–55% of total β-acids), colupulone, and adlupulone[103]. Hop cones are also rich in prenylated phenols including chalcones and flavanones. Xanthohumol is the most highly concentrated constituent in this class of compounds, while other prenylated flavanones and chalcones include 8-prenylnarigenin, 6-prenylnarigenin and isoxanthohumol. Apart from their widely known antimicrobial properties, recent studies have revealed that hops compounds possess other biological effects, such as, strong antioxidative action, estrogenic activity, anti-inflammatory action, and several anticarcinogenic features[104].

Initial screening

During the study, three initial extracts originating from *H. lupulus* L. were tested: PS-014, PS-073 and PS-079. All three extracts are products of Natac Biotech with adjusted concentrations of the bioactive compounds according to their market demand and suggested use as food supplements. Extract PS-014 is a supercritical CO_2 extract with 35% hops bitter acids (HBAs), PS-073 is an extract concentrated in prenylated flavonoids and contains 25% xanthohumol and PS-079 is a mixture containing 10% hops bitter acids and 3.5% prenylated flavonoids, of which 2.5% is xanthohumol.

As before, the antibacterial properties of all three extracts were assessed against both *L. monocytogenes* wild-type and the Δ*sigB* mutant. All three extracts demonstrated inhibition properties against the strains in different extents. The results are shown in Table 19.

Table 19. Antibacterial activities of extracts PS-014, PS-073 and PS-079 against *L. monocytogenes* EGD-e wt and Δ*sigB*.

Plant	Extract	Conc. [mg/ml]	OD_{600} wt [mg/ml]	OD_{600} Δ*sigB* [mg/ml]	Micro-dilution assay	*L. monocytogenes* wt 30°C [mg/ml]	*L. monocytogenes* Δ*sigB* 30°C [mg/ml]	*L. monocytogenes* wt 37°C [mg/ml]	*L. monocytogenes* Δ*sigB* 37°C [mg/ml]	Agar dilution assay
Humulus lupulus	PS-014	1.00	0.25	0.25	MIC	0.063	0.063	0.063	0.063	high activity 10^9
					MBC	0.25	0.25	0.25	0.25	
	PS-073	1.00	1.00	1.00	MIC	1.00	1.00	1.00	1.00	low activity 10^4
					MBC	1.00	1.00	1.00	1.00	
	PS-079	1.00	1.00	1.00	MIC	1.00	1.00	0.50	0.50	high activity 10^9
					MBC	1.00	1.00	1.00	1.00	

The MICs and MBCs of the plant agents were determined by broth serial dilution against exponentially grown cells at 10^6 CFU/mL inoculum concentration. In the OD_{600} assay the optical density was continuously measured at 600 nm. (-) – no activity.

Extracts PS-073 and PS-079 showed limited antimicrobial activities with MICs of 1 mg/mL, and induced a longer lag phase and a lower final OD_{600} in the growth inhibition assay compared to the positive control. Extract PS-014 was the most active initial extract, with a MIC of 63 µg/mL, and complete inhibition of bacterial growth with no change in the OD at 600 nm during 24 hours – Figure 18.

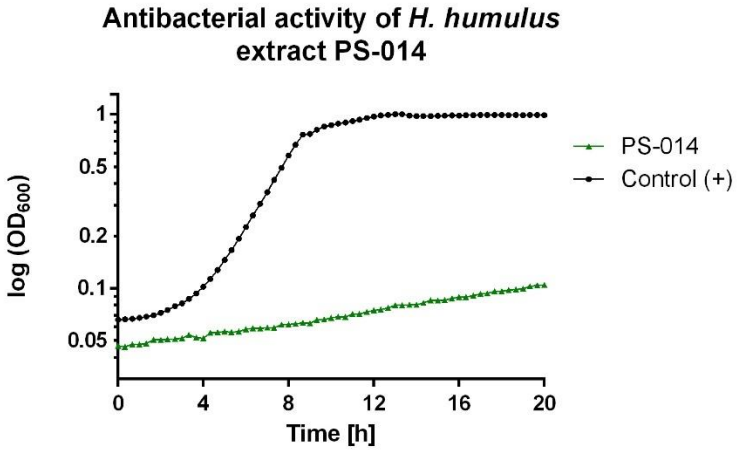


Figure 18. Growth curve of *L. monocytogenes* EGD-e wild-type in the presence of PS-014. An inoculum of 10^6 CFU/mL *L. monocytogenes* EGD-e wild-type was introduced in BHI supplemented PS-014 at 1 mg/mL concentration. OD at 600 nm was continuously measured for 24h. Control – *L. monocytogenes* EGD-e wt in BHI. The results are representative of three technical replicates.

In order to reveal the identity of compounds responsible for the antibacterial activity, a second round of bio-activity guided screening was started using a supercritical CO_2 hops extract highly concentrated in HBAs and a sample of pure xanthohumol. Both the HBAs extract and pure xanthohumol were acquired by

commercial suppliers. The concentration of HBAs in the supercritical CO_2 hops extract was determined by HPLC and 71.2% of HBAs were detected, out of which 53.6% were α-acids and 176% β-acids.

Table 20. Antibacterial properties of a supercritical CO_2 *H. lupulus* L. extract concentrated in HBAs and xanthohumol against *L. monocytogenes* EGDe wt and Δ*sigB*.

						L. monocytogenes				
			OD_{600}		**Micro-dilution assay**	wt 30°C [μg/ml]	Δ*sigB* 30°C [μg/ml]	wt 37°C [μg/ml]	Δ*sigB* 37°C [μg/ml]	**Agar dilution assay**
Plant	Compound	Conc. [μg/ml]	wt [μg/ml]	Δ*sigB* [μg/ml]						
Humulus lupulus	HBAs	100	25	25	MIC	6.3-12.5	6.3-12.5	6.3-12.5	6.3-12.5	High 10^9
					MBC	50	50	50	50	
	XU	25	-	-	MIC	-	-	-	-	Low 10^4
					MBC	-	-	-	-	

The MICs and MBCs of the plant agents were determined by broth serial dilution against exponentially grown cells at 10^6 CFU/mL inoculum concentration. In the OD_{600} assay the optical density was continuously measured at 600 nm. (-) – no activity. HBAs – Hops bitter acids; XU – xanthohumol.

The results in Table 20 suggest that xanthohumol possesses mild antibacterial activities, detected only by the agar dilution assay. Further tests revealed that a concentration of 100 μg/mL of xanthohumol inhibits the growth of up to 10^9 CFU/mL of *L. monocytogenes*. On the other hand, the hops bitter acids were highly more efficient, with MICs lower than 12.5 μg/mL. The results suggested that the bitter acids were the main phytochemicals responsible for the antibacterial activities of the initial extracts. In the next phase, separation and purification processes were undertaken in order to test each group of hops bitter acids separately.

Isolation of α and β acids from a supercritical CO_2 extract

The isolation of α and β acids was mainly achieved by exploiting the different pH solubilities that α and β acids have. In this regard, the α acids were first isolated from the extract by treating with a mild inorganic base, up to pH=8.6, then the β acids were separated from the remaining hops oils and resins by treating with a stronger inorganic base. The solubility differences between the β acids and the hops oils and resins in basic aqueous solutions was utilized to remove the oily contaminants and to subsequently crystalize the β acids from oversaturated hexane solution. On the other hand, the α acids were hydrolysed from their neutralized salt form with sulphuric acid and were later eluted with hexane and concentrated. The obtained products had a purity of 83.9% for the α acids and 89.4% for the β acids. Details about the

isolation of α and β acids from a supercritical CO_2 extract are provided in Section 3.5.4 of Materials & Methods. The chromatograms of the initial extract and purified products are shown in Figure 20.

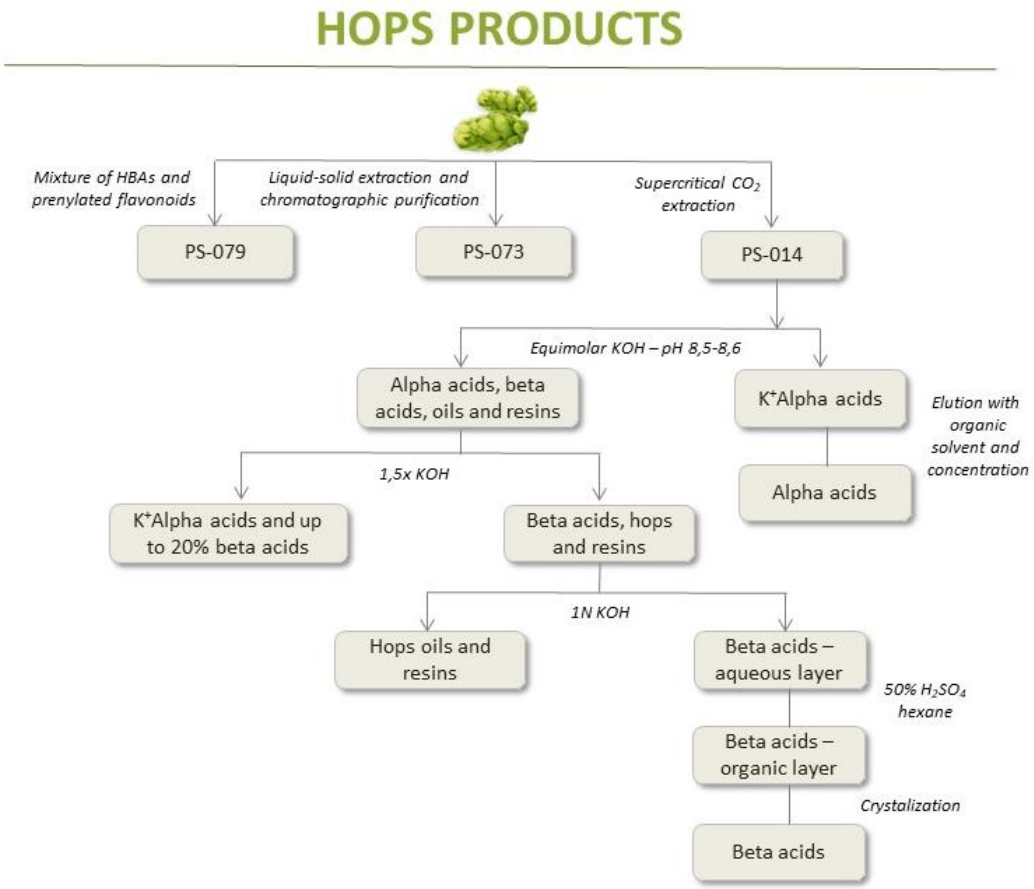


Figure 19. Schematic representation of the extraction, fractionation and purification processes of *H. lupulus* products.

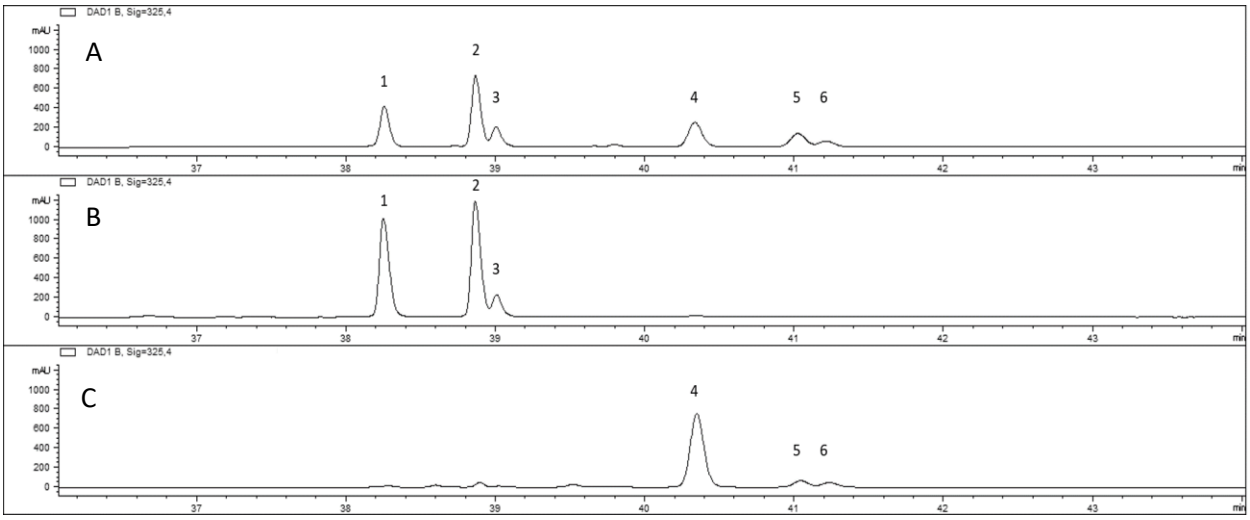


Figure 20. HPLC chromatograms showing the separation of alpha and beta acids from a supercritical CO_2 extract of *H. lupulus* L. Chromatogram **A)** - *H. lupulus* L. CO_2 extract – Alpha acids: 1 – humulone, 2 – cohumulone and 3 – adhumulone. Retention

times of peaks: 1 - 38,25 min; 2 - 38,86 min and 3 - 39,00 min; Beta acids: 4 – lupulone, 5 – colupulone and 6 – adlupulone. Retention times of peaks 4 – 40,33 min; 5 – 41,02 min and 6 – 41,21 min. Chromatogram **B)** – only alpha acids are present: 1 – humulone, 2 – cohumulone and 3 – adhumulone. Chromatogram **C)** – only beta acids are present: 4 – lupulone, 5 – colupulone and 6 – adlupulone.

Finally, the purified α and β acids were tested for their antibacterial propertied against *L. monocytogenes* wt and the Δ*sigB* mutant. The results revealed that β acids have a MIC lower than 6.25 μg/mL, and were more active than the α acids that had a MIC between 25 and 50 μg/mL. A subsequent analysis was performed to more accurately pinpoint the MICs of both groups of compounds and it was determined that α acids have a MIC of 35 μg/mL, while β acids have a MIC of 5 μg/mL.

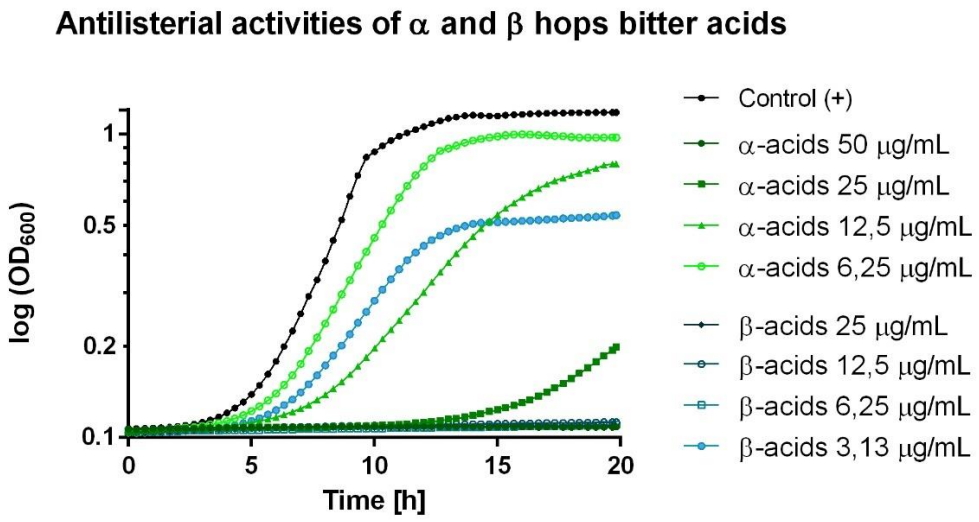


Figure 21. Growth curve of *L. monocytogenes* in the presence of α and β acids isolated from *H. lupulus* L.. An inoculum of 10^6 CFU/mL *L. monocytogenes* EGD-e wild-type was introduced in BHI supplemented with various concentrations of α-acids or β-acids. OD at 600 nm was continuously measured for 20h. Control – *L. monocytogenes* EGD-e wt in BHI. The results are representative of three technical replicates.

4.1.3.5. Orthosiphon stamineus Benth.

Orthosiphon stamineus Benth. is a widely distributed plant in South-eastern Asia and Africa. *O. stamineus* is well known for its strong diuretic effects and is extensively used in traditional Asian medicine to improve general health and for the treatment of bladder inflammation, gout and diabetes. Various phytochemical compounds have been identified in this plant such as triterpenes, diterpenes, flavonoids and phenolic acids. Among them sinensetin, eupatorin and rosmarinic acid are the most well-known[105].

Initial screening

Two *O. stamineus* Benth. extracts were included in the initial group of 120 extracts under the codes of PS-056 and PS-057. Extract PS-056 is a extract that contains 3% total flavonoids, while extract PS-057 is an extract of the leaves of the Asian plant that contains 0.2% of the methylated flavone sinensetin. Accordingly, both have excellent solubility in water. In the initial screening, extract PS-056 did not exhibit any antibacterial activities by all antibacterial assays, while PS-057 was successful at inhibiting the growth of *L. monocytogenes* at a concentration of 0.5 mg/mL (Table 21).

Table 21. Antibacterial properties of *O. stamineus* extracts against *L. monocytogenes* EGDe wt and Δ*sigB*.

						L. monocytogenes				
			OD_{600}		**Micro-dilution assay**	wt 30°C [mg/ml]	Δ*sigB* 30°C [mg/ml]	wt 37°C [mg/ml]	Δ*sigB* 37°C [mg/ml]	**Agar dilution assay**
Plant	Extract	Conc. [mg/ml]	wt	Δ*sigB*						
Orthosiphon stamineus	PS-056	1.00	-	-	MIC	-	-	-	-	not tested
					MBC	-	-	-	-	
	PS-057	1.00	1.00	1.00	MIC	0.50	0.50	0.50	0.50	low 10^5
					MBC	1.00	1.00	0.50	0.50	

The MICs and MBCs of the plant agents were determined by broth serial dilution against exponentially grown cells at 10^6 CFU/mL inoculum concentration. In the OD_{600} assay the optical density was continuously measured at 600 nm. (-) – no activity.

In the OD_{600} assay it could be observed that extract PS-057 slowed the growth of the bacteria by affecting the length of the lag phase and lowering the final OD_{600} value at the 20-hour end point. Better results were achieved with the resazurin microdilution method where the extract showed inhibitory effects at a concentration of 0.50 mg/mL at both 30°C and 37°C. When a sample from the wells with no visible growth was re-plated on fresh solid media there was no subsequent bacterial growth for the assay performed at 37°C, so the minimum inhibitory concentration coincided as a minimum bactericidal

concentration at this temperature. At 30°C around 20-30 colonies could be counted after re-plating, but altogether the bacterial population was severely reduced in comparison to the positive control. Since this extract has excellent solubility in water, no unexpected results were obtained by the agar dilution method. 1 mg/mL of extract PS-057 inhibited the growth of *L. monocytogenes* colonies up to the quadrant containing 10^5 CFU/mL confirming the medium-low activity of the extract.

Extractions and antibacterial activities of the new extracts

In the next step *de novo* extractions were carried out from the leaves of *O. stamineus* Benth. with three extraction solvents with decreasing polarity: water, methanol and dichloromethane. The newly prepared extracts were tested for antibacterial activity against *L. monocytogenes*. It was found that the pure water extract was the most active, with MIC of 0.32 mg/mL. The methanol extract showed inhibitory activities only at the highest concentration tested, and the dichloromethane extract was not inhibitory to the growth of the bacteria. While the methanol extract showed growth inhibition, it was not very efficient at killing the bacterial cells. Since methanol is a fairly polar solvent, it is possible that the same bioactive ingredient was extracted with methanol as was with water, but to a lesser extent, and hence the lower antibacterial capacity observed for this extract. It was also noticed that the bioactive phytochemicals did not remain very stable in the water solution and with time the activity of the extract decreased greatly.

Table 22. Antibacterial activities of newly prepared *O. stamineus* Benth. extracts against *L. monocytogenes* EGDe wt and Δ*sigB*.

				L. monocytogenes				
					Δ*sigB*		Δ*sigB*	Agar
Plant	Extract	V [mg/ml]	Microdilution method	wt 24°C [mg/ml]	24°C [mg/ml]	wt 37°C [mg/ml]	37°C [mg/ml]	dilution method
Orthosiphon stamineus	H_2O extract	0.64	MIC	0.32	0.32	0.32	0.32	low activity 10^5
			MBC	0.64	0.64	0.32	0.32	
	MeOH extract	0.55	MIC	0.55	0.55	0.55	0.55	-
			MBC	-	-	-	-	
	CH_2Cl_2 extract	0.21	MBC	-	-	-	-	-
			MBC	-	-	-	-	

The MICs and MBCs of the plant agents were determined by broth serial dilution against exponential growth-phase cells at 10^6 CFU/mL inoculum concentration. (-) – no activity.

After concluding that the water extract of *O. stamineus* Benth. was the most active, the next step was to fractionate the compounds of this extract and test each fraction to further characterize the bioactive compounds present in this plant.

Fractionation of the water extract and antibacterial activities of the fractions

A new water extract was prepared from the dried leaves of *O. stamineus* Benth. by using a higher plant to solvent ratio to increase the concentration of the active compound. Once obtained, the extract was fractionated using a non-ionic adsorption resin. The extract was added to the column and the eluent was allowed to pass. Fractions were recovered with three solvents with decreasing polarity, namely, water (OS FR1), 60% ethanol (OS FR2) and 96% ethanol (OS FR3). Ethanol was removed by evaporation in both OS FR2 and OS FR3 and the dry residue was redissolved in water. More details about the fractionation process are provided in Section 3.5.5 of Materials & Methods. Total solids and total polyphenols were determined in all fractions (Table 23).

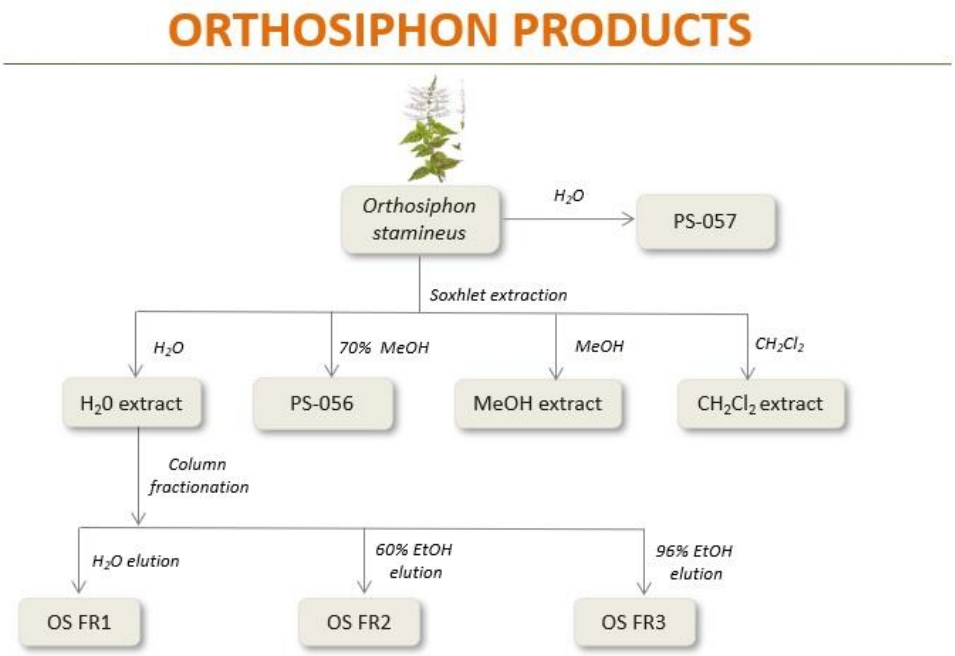


Figure 22. Schematic representation of the extraction and fractionation processes of all *O. stamineus* Benth. products. H_2O – water; MeOH – methanol; EtOH – ethanol; CH_2Cl_2 – dichloromethane; OS FR – *O. stamineus* fraction.

The total yield of extraction was calculated at 10.3%. Upon fractionation, OS FR2 was the most abundant with 10.29 mg/mL of dry residue, while OS FR3 was the poorest with a dry residue of only 0.91 mg/mL. Since the solvent used for extraction was a polar solvent and the plant part used were leaves, the extract and its fractions were expected to contain a high concentration of phenolic compounds. Indeed, 29.1% of total phenolic compounds were quantified in the extract by the Folin-Ciocalteu method. Considering the fractions, the highest portion of the phenolics partition in OS FR2, with 37% total phenolics detected.

Table 23. Total solids, total polyphenols, rosmarinic acid and caffeic acid in *O. stamineus* Benth. water extract and its fractions.

Sample	Dry residue (mg/mL)	Total polyphenols	% rosmarinic acid	% caffeic acid
H_2O Extract	15.46	29.06%	0.55	0.124
OS FR1	5.12	8.89%	0.00	0.038
OS FR2	10.29	36.97%	2.13	0.340
OS FR3	0.91	17.76%	5.29	1.071

Examining the phytochemical profile of the aqueous extract of *O. stamineus* Benth and its corresponding fractions the following compounds were identified. Rosmarinic acid was present in fractions OS FR2 and OS FR3, as well as the extract. Upon fractionation, none of the rosmarinic acid was eluted from the column with H_2O, leading to no detection of this compound in OS FR1. The majority of rosmarinic acid was recovered with 60% ethanol, resulting in 0.28 mg/mL concentration of this compound in OS FR2. Since rosmarinic acid has very weak potential to inhibit the growth of *L. monocytogenes*, this compound was probably not the active antibacterial agent. Caffeic acid was present in all fractions and the extract itself. The highest concentration of caffeic acid was detected in the extract at 0.12 mg/mL, while percentage wise, OS FR3 had the highest percent concentration.

All fractions were tested for their antibacterial potential against *L. monocytogenes* wt and the mutant Δ*sigB*. In order to do so, a precalculated volume of each fraction was evaporated to achieve 1 mg of dry residue of initial concentration. It was found that only OS FR2 was able to inhibit the growth of the bacteria. These results suggest that during column fractionation, the active antibacterial phytocompounds extracted from *O. stamineus* Benth. form stronger intermolecular bonds with the column than with water and can be eluted only when ethanol is added, suggesting an aromatic character of the compound(s).

Table 24. Antibacterial activities of *O. stamineus* Benth fractions against *L. monocytogenes* EGDe wt and Δ*sigB*.

				L. monocytogenes				Agar dilution method
Plant	Fraction	Conc. [mg/mL]	Microdilution method	wt 30°C [mg/ml]	Δ*sigB* 30°C [mg/ml]	wt 37°C [mg/ml]	Δ*sigB* 37°C [mg/ml]	
Orthosiphon stamineus	H_2O fraction	1.00	MIC	-	-	-	-	-
			MBC	-	-	-	-	
	60% EtOH	1.00	MIC	0.40	0.40	0.40	0.40	-
			MBC	0.40	0.40	0.40	0.40	
	96% EtOH	1.00	MBC	-	-	-	-	-
			MBC	-	-	-	-	

The MICs and MBCs of the plant agents were determined by broth serial dilution against exponential growth-phase cells at 10^6 CFU/mL inoculum concentration. (-) – no activity.

4.1.3.6. *Punica granatum* L.

Pomegranate (*Punica granatum* L., family Lythraceae) has recaptured consumer interest globally due to its health promoting benefits. Pomegranate is a good source of micro- and macro-nutrients, organic acids and bioactive compounds. The most valuable phytochemicals of pomegranate are its polyphenols, such as the hydrolysable tannins (punicalagins and punicalins), condensed tannins, anthocyanins and phenolic (gallic acid and ellagic acid) acids. Other identified compounds of importance are the organic acid, malic acid and the polyunsaturated fatty acid, punicic acid. Both pomegranate peel and pulp are rich in bioactive compounds and have demonstrated significant antioxidant capacity. Pomegranate peel is an invaluable source of punicalagin, an ellagitannin with an antioxidant capacity that is unrivalled and unique to this plant[106]. On the other hand, pomegranate pulp contains highly-active anti-atherogenic compounds. The biological activities of pomegranate extracts are varied and their current health promoting benefits include superior antioxidant and anti-inflammatory properties, promoting cardiovascular and joint protection and anti-aging effects. Moreover, due to their long-known antimicrobial effects, pomegranate extracts are also being investigated for their potential application in the food industry as biopreservatives.

Initial screening

In the initial group of extracts two *P. granatum* L. extracts were included: PS-010 and PS-011. Both extracts are derived from the peel of the pomegranate fruit. Extract PS-011 contains 40% punicalagins, the most abundant ellagitannins of pomegranate with the highest molecular weight discovered so far, while extract PS-010 is derived from PS-011 by acid hydrolysis, and it contains 40% ellagic acid, the monomeric unit of punicalagins.

Table 25. Antibacterial activities of *P. granatum* extracts against *L. monocytogenes* EGDe wt and Δ*sigB*.

Plant	Extract	Conc. [mg/ml]	OD_{600} wt	OD_{600} Δ*sigB*	Micro-dilution assay	*L. monocytogenes* wt 30°C [mg/ml]	*L. monocytogenes* Δ*sigB* 30°C [mg/ml]	*L. monocytogenes* wt 37°C [mg/ml]	*L. monocytogenes* Δ*sigB* 37°C [mg/ml]	Agar dilution assay
Punica granatum	PS-010	1.00	not conclusive		MIC	-	-	-	-	low 10^4
					MBC	-	-	-	-	
	PS-011	1.00	not conclusive		MIC	1.00	1.00	1.00	1.00	low 10^6
					MBC	1.00	1.00	1.00	1.00	

The MICs and MBCs of the plant agents were determined by broth serial dilution against exponentially grown cells at 10^6 CFU/mL inoculum concentration. In the OD_{600} assay the optical density was continuously measured at 600 nm. (-) – no activity.

The pomegranate extracts had limited antibacterial activities against *L. monocytogenes*, especially extract PS-010 which at the tested concentration of 1 mg/mL showed weak inhibitory properties only by the agar dilution method. Extract PS-011 was more active, and inhibited the growth of 10^6 CFU/mL of *L. monocytogenes* at a concentration of 1 mg/mL in both broth microdilution and agar dilution methods. Since both these extracts form opaque solutions in water with an OD_{600} reading of 1.2, conclusions about their activities by continuously measuring the optical density at 600 nm could not be reached.

Fractionation of P. granatum L. extracts

After evaluating the effects of the extracts, the next step was purifying the dominant compounds of the extracts, namely, punicalagins from extract PS-011 and ellagic acid from extract PS-010. Additional to these two abundantly present compounds, two other phytochemicals normally present in pomegranate were also included in the screening. These compounds are tannic acid and gallic acid. Tannic acid is a plant polyphenol, a specific form of tannin. It is also characterized as a hydrolysable tannin which consists of gallic acid molecules and glucose.

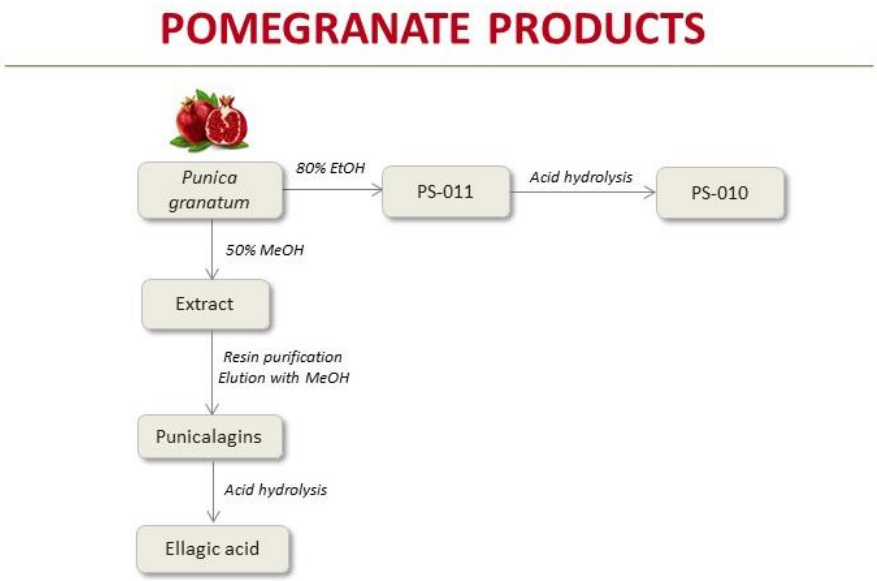


Figure 23. Schematic representation of the extraction, fractionation and purification processes of *P. granatum* L. products.

For the purification of punicalagins and ellagic acid, a 50% methanol:water (v/v) extract was produced from pomegranate pericarp. This extract had 29.1% punicalagins determined by HPLC and 2% ellagic acid. After evaporating the methanol from the extract, chromatographic purification was used to purify the punicalagins. The eluted fraction contained 74.6% of punicalagins. Since punicalagin is a

hydrolysable tannin, strong acid hydrolysis was used to produce its hydrolysis product, ellagic acid. Upon hydrolysis, the reaction mixture was neutralized with NaOH, the solvent was evaporated from the eluent and dissolved in methanol. HPLC analysis revealed 44.8% of ellagic acid in the product. Details about the extraction and fractionation processes are described in Section 3.5.6 in Materials & Methods.

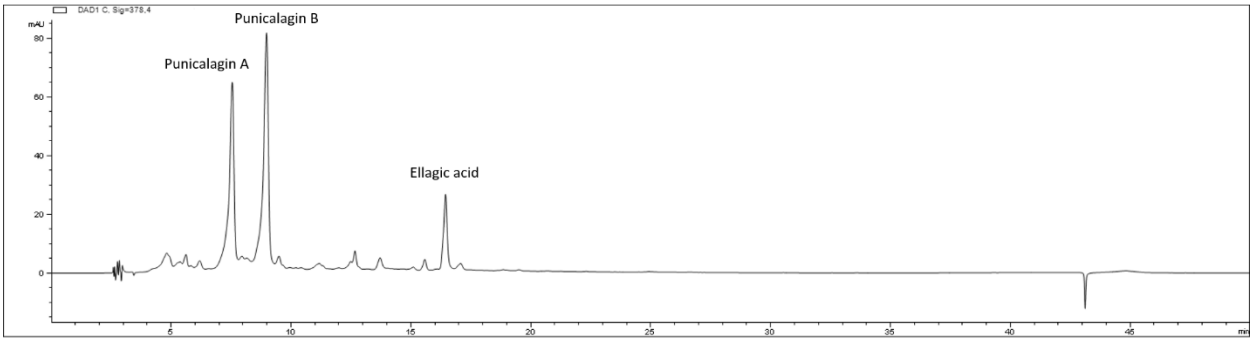


Figure 24. HPLC chromatogram displaying the phytochemicals of interest in the chromatographic fraction of the extract of *P. granatum*. Retention times: Punicalagin A (7,54 min), Punicalagin B (8,97 min) and ellagic acid (16,44 min).

On the other hand, pomegranate peel contains a low concentration of free gallic acid and tannic acid and only 0.4% and of 0.5% of these compounds were recovered in the extract. Accordingly, the antimicrobial activities of gallic acid and tannic acid, as well as ellagic acid because of insufficient purity (44.8%) were tested using analytical standards. Almost all phenolic compounds, except for ellagic acid, showed weak antibacterial activities against *L. monocytogenes*, and the most effective pomegranate phytochemical was found to be gallic acid with a MIC of 0.1 mg/mL and MBC of 0.2 mg/mL (Table 26).

Table 26. Antibacterial activities of main *P. granatum* L. phytochemicals against *L. monocytogenes* EGDe wt and Δ*sigB*.

				L. monocytogenes				
Plant	Compound	Conc. [mg/ml]	Microdilution method	wt 30°C [mg/ml]	Δ*sigB* 30°C [mg/ml]	wt 37°C [mg/ml]	Δ*sigB* 37°C [mg/ml]	Agar dilution method
Punica granatum	Punicalagins	0.40	MIC	0.40	0.40	0.40	0.40	medium activity 10^5
			MBC	-	-	0.40	0.40	
	Ellagic acid	0.40	MIC	-	-	-	-	no activity
			MBC	-	-	-	-	
	Tannic acid	0.40	MIC	0.40	0.40	0.40	0.40	medium activity 10^5
			MBC	0.40	0.40	0.40	0.40	
	Gallic acid	0.40	MIC	0.10	0.10	0.10	0.10	medium activity 10^5
			MBC	0.20	0.20	0.2-0.4	0.2-0.4	

The MICs and MBCs of the plant agents were determined by broth serial dilution against exponentially grown cells at 10^6 CFU/mL inoculum concentration. (-) – no activity.

4.1.3.7. Melissa officinalis L. *and Rosmarinus officinalis* L.

Three extracts originating from the plants *Melissa officinalis* L. and *Rosmarinus officinalis* L. were included in the initial screening of 120 extracts. Extract PS-021 and PS-022 are *R. officinalis* L. extracts; whilst *M. officinalis* L. was represented by extract PS-052. Extract PS-021 is characterized by containing 20% of diterpenoids, with carnosol, carnosic acid and methyl carnosoate among others, while extract PS-022 is an extract abundant in polyphenolic compounds, especially hydroxycinnamic acid derivatives, 6% of which is rosmarinic acid. Furthermore, extract PS-052 has a similar phytochemical profile to extract PS-022, it is an extract also rich in polyphenols and hydroxycinnamic acid derivatives, 5% of which is rosmarinic acid. Initially, the antibacterial properties of all three extracts were determined – Table 27.

Table 27. Antibacterial activities of extracts PS-021, PS-022 and PS-052 against *L. monocytogenes* EGDe wt and Δ*sigB*.

				L. monocytogenes				
				wt	Δ*sigB*	wt	Δ*sigB*	Agar
		Conc.	Microdilution	30°C	30°C	37°C	37°C	dilution
Plant	Extract	[mg/ml]	method	[mg/ml]	[mg/ml]	[mg/ml]	[mg/ml]	method
Rosmarinus officinalis	PS-021	1.00	MIC	-	-	-	-	no activity
			MBC	-	-	-	-	
	PS-022	1.00	MIC	0.50	0.50	0.50	0.50	no activity
			MBC	0.50	0.50	0.50	0.50	
Melissa officinalis	PS-052	1.00	MBC	0.50	0.50	0.50	0.50	no activity
			MBC	1.00	1.00	0.50	0.50	

The MICs and MBCs of the plant agents were determined by broth serial dilution against exponential growth-phase cells at 10^6 CFU/mL inoculum concentration. In the OD_{600} assay the optical density was continuously measured at 600 nm. (-) – no activity.

Extract PS-021 did not display any antibacterial activities against *L. monocytogenes* at the maximum tested concentration of 1 mg/mL. On the other hand, extracts PS-022 and PS-052 had very similar antibacterial properties. The two extracts had MICs of 0.5 mg/mL at the temperatures of 30°C and 37°C, MBC of 0.5 mg/mL at 37°C and 1 mg/mL at 30°C. Strangely, neither one was able to inhibit the growth of the bacterial colonies in the agar dilution method.

Since both extracts are characterized by their similar concentration of rosmarinic acid, it was assumed that this phenolic acid could be responsible for the antibacterial effects demonstrated by the extracts. To test this hypothesis, a pure rosmarinic acid (98% purity) analytical standard was acquired. The compound was diluted to the same concentration present in the extracts, 0.06 mg/mL and antibacterial potential was compared to the activities of the two active extracts, PS-022 and PS-052 – Table 28.

Table 28. Antibacterial activities of extract PS-022, PS-052 and rosmarinic acid against *L. monocytogenes* EGDe wt and Δ*sigB*.

Plant / Compound	Extract	Conc. [mg/ml]	Microdilution method	*L. monocytogenes* wt 30°C [mg/ml]	*L. monocytogenes* Δ*sigB* 30°C [mg/ml]	*L. monocytogenes* wt 37°C [mg/ml]	*L. monocytogenes* Δ*sigB* 37°C [mg/ml]	Agar dilution method
Rosmarinus officinalis	PS-022	1.00	MIC	0.50	0.50	0.50	0.50	no activity
			MBC	1.00	1.00	1.00	1.00	
Melissa officinalis	PS-052	1.00	MIC	0.50	0.50	0.50	0.50	no activity
			MBC	1.00	1.00	1.00	1.00	
Rosmarinic acid		0.06	MBC	-	-	-	-	no activity
			MBC	-	-	-	-	

The MICs and MBCs of the plant agents were determined by broth serial dilution against exponentially grown cells at 10^6 CFU/mL inoculum concentration. (-) – no activity.

The results presented in Table 28 suggest that rosmarinic acid was not the molecule responsible for the medium-low antibacterial properties displayed by extracts PS-022 and PS-052. Further evaluation was performed at a higher concentration of 0.1 mg/mL rosmarinic acid and the result was the same, no antibacterial properties. Since the antibacterial effects of these extracts were medium-low to begin with, further characterization of their phytochemical composition was not pursued.

4.1.3.8. Camellia sinensis L.

Camellia sinensis L.*,* or the tea plant, is a species of evergreen shrubs whose leaves and leaf buds are used to produce tea. While all teas originate from *C. sinensis*, the amount of oxidation that a tea leaf undergoes following harvesting dictates the type of tea produced. During oxidation, chlorophyll is broken down and tannins are released, making the leaves turn darker. The oxidation process can be stopped at different times by deactivating the enzymes responsible for breaking down chlorophyll, resulting in three types of tea: black tea, that is fully oxidized, oolong tea, semi-oxidized, and green tea is unoxidized. Apart from tannins and the alkaloid caffeine, the tea catechins, or flavan-3-ols, are the most important group of phytochemicals in tea, known to affect its colour and flavour and contribute to its bitterness. Particular attention focuses on epigallocatechin gallate (EGCG), as the most abundant polyphenol in tea extracts.

Initial screening

In this study, the green tea plant, *C. sinensis* L., was represented by only one extract, extract PS-026. This extract is highly concentrated in polyphenols, up to 81,3% of the extract's contents determined by UV spectroscopy are phenolic compounds. More specifically, the extract is characterized by 64% catechins, out of which 34% is identified as epigallocatechin-3-gallate (EGCG) by HPLC. When evaluated for its antibacterial activities, extract PS-026 was able to inhibit the growth of *L. monocytogenes* at a MIC of 0.5 mg/mL at both 30°C and 37°C. Interestingly enough, when samples from the inhibitory wells were re-plated on fresh media there was no further bacterial growth, distinguishing the antibacterial properties of this extract as bactericidal.

Table 29. Antibacterial activity of extract PS-026 against *L. monocytogenes* EGDe wt and Δ*sigB*.

						L. monocytogenes				
			OD_{600}		**Micro-dilution assay**	wt 30°C [mg/ml]	Δ*sigB* 30°C [mg/ml]	wt 37°C [mg/ml]	Δ*sigB* 37°C [mg/ml]	**Agar dilution assay**
Plant	Extract	C [mg/ml]	wt	Δ*sigB*						
Camellia sinensis	PS-026	1.00	1.00	1.00	MIC	0.50	0.50	0.25	0.25	high
					MBC	0.50	0.50	0.50	0.50	10^7-10^8

The MICs and MBCs of the plant agents were determined by broth serial dilution against exponentially grown cells at 10^6 CFU/mL bacterial inoculum concentration. In the OD_{600} assay the optical density was continuously measured at 600 nm. (-) – no activity.

Given that catechins are the predominant phytochemicals of extract PS-026, pure catechin, epicatechin, epicatechin gallate (ECG) and epigallocatechin-3-gallate (EGCG) were acquired with the aim of evaluating

the antibacterial properties of each flavonoid. Solutions were prepared at concentrations identical to the ones in the extract and antibacterial assays were carried out in parallel with extract PS-026.

Table 30. Antibacterial activity of *C. sinensis* L. catechins against *L. monocytogenes* EGDe wt and Δ*sigB*.

Plant	Natural compound	C [mg/ml]	Microdilution method	*L. monocytogenes* wt 30°C [mg/ml]	*L. monocytogenes* Δ*sigB* 30°C [mg/ml]	*L. monocytogenes* wt 37°C [mg/ml]	*L. monocytogenes* Δ*sigB* 37°C [mg/ml]	Agar dilution method
Camellia sinensis	Extract PS-026	1.00	MIC	0.25	0.25	0.25	0.25	high activity 10^7-10^8
			MBC	0.50	0.50	0.25	0.25	
	Catechin	0.30	MIC	-	-	-	-	no activity
			MBC	-	-	-	-	
	Epicatechin + Epicatechin gallate	0.30	MIC	0.15	0.15	0.15	0.15	low activity
			MBC	-	-	0.30	0.30	
	EGCG	0.30	MIC	0.15	0.15	0.15	0.15	low activity
			MBC	-	-	0.30	0.30	

The MICs and MBCs of the plant agents were determined by broth serial dilution against exponentially grown cells at 10^6 CFU/mL bacterial inoculum concentration. (-) – no activity.

Every catechin compound has a different extent of antilisterial effects. The most effective was EGCG with a MIC of 0.15 mg/mL at both temperatures of 37°C and 30°C, and bactericidal properties only at 37°C at a concentration of 0.30 mg/mL. Catechin did not demonstrate antibacterial effects at the maximal tested concentration of 0.30 mg/mL, while epicatechin and epicatechin gallate were tested in combination from a solution containing 0.15 mg/mL of each, 0.30 mg/mL total. The effects of the solution containing these two compounds were comparable to the effects of EGCG. A laboratory mixture of all four catechins was produced at their concentrations comprised in the extract. The antibacterial activity of this mixture was tested and the results were compared to the activity of extract PS-026. Interestingly, the antibacterial potential of the laboratory mixture was identical to the one of the extract, suggesting that only these four phytochemicals or their combination thereof were responsible for the observed effects of extract PS-026.

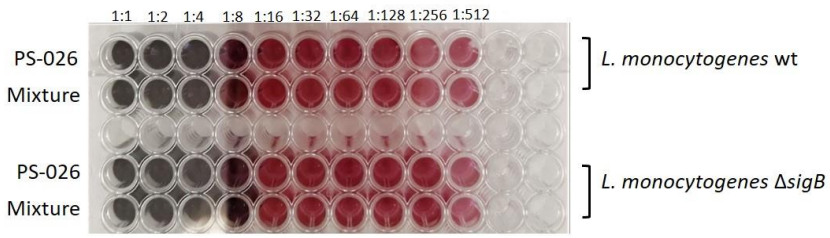


Figure 25. Antibacterial activities of extract PS-026 and the laboratory catechin mixture by the serial dilution (1:2) using the resazurin based broth dilution test. Dark blue – no growth; Pink – bacterial growth. Starting concentration in first well 1 mg/mL.

4.1.4. Final selection of the best plant-derived antimicrobials

After evaluating the antibacterial activities of all extracts from the initial screening and the newly prepared ones, their respective fractions and purified phytocompounds, eight final candidates were appointed as the best plant-derived compounds in the study. These can be categorized as pure phytochemicals and extractive fractions.

Table 31. Final selection of the best plan-derived antilisterial agents.

PLANT	FRACTION COMPOUND	Analytical marker	Purity	MIC	MBC
Purified phytochemicals					
***Humulus lupulus* L.**	purified α-acids	humulone, adhumulone, cohumulone	78%	35 µg/mL	100 µg/mL
	purified β-acids	lupulone, adlupulone, colupulone	86%	5 µg/mL	50 µg/mL
***Olea europaea* L.**	OA-97	oleanolic acid	97%	5-10 µg/mL	5-10 µg/mL
	HT-60	hydroxytyrosol	60%	125 µg/mL	1000 µg/mL
Extractive fractions					
***Salvia officinalis* L.**	SAL FR2	triterpenic acids	33%	30 µg/mL	30 µg/mL
	SAL FR4	diterpenoids	33%	125 µg/mL	125 µg/mL
***Orthosiphon stamineus* Benth.**	OS FR2	polyphenols	37%	400 µg/mL	400 µg/mL
***Eucalyptus globulus* L.**	EU FR2	triterpenic acids	18.9%	25 µg/mL	50 µg/mL

These eight samples, were further used for evaluating the activation of the general stress response in *L. monocytogenes* (Chapter 4.2) and their potential as food preservatives in simple food systems (Chapter 4.3).

4.2. Chapter II: Effects of selected plant extracts on the activation of the general stress response in *L. monocytogenes*

Plants synthesize various secondary metabolites in response to environmental triggers, including potent antimicrobial phytochemicals that serve as defence agents against bacterial pathogens[107]. As plants and *L. monocytogenes* share their natural habitat, it stands to reason that this bacterium needs to be able to sense the presence of antibacterial compounds synthesized by various plants in order to survive and persevere in the environment. Even though considerable literature exists on the antimicrobial abilities of various plant compounds on a wide range of microorganisms[10,12,23,107], relatively limited information is available about the effect plant compounds have on bacterial gene expression and pathogenesis. In *L. monocytogenes*, a large number of genes involved in environmental sensing are under the control of the alternative sigma factor sigma B, SigB (σ^B) (up to 304 according to recent studies[67]), collectively known as the General Stress Response (GSR) regulon. This stress activated sigma factor plays a key role in *L. monocytogenes* in sensing detrimental environmental conditions, circumventing cell injury and ensuring survival by reprogramming gene expression, expressing stress tolerance factors and activating stress resistance mechanisms (including responses to acidic, osmotic, oxidative and temperature stress[70,73,74,75,84]). The protective role of σ^B on bacterial survival during exposure to antibiotics such as ampicillin, penicillin and vancomycin and antimicrobial peptides like nisin and lacticin 3147 has also been reported[95,108,109]. Therefore, it is reasonable to assume that one of the possible responses of *L. monocytogenes* to the presence of plant antibacterial agents could as well be the activation of the GSR generated by σ^B. To test this theory, the level of σ^B activity reached after exposure to the final eight selected plant products (Section 4.1.4 – Chapter 1) exhibiting antibacterial activities against *L. monocytogenes*, was tested. In particular, each plant-derived antibacterial agent was introduced at a subinhibitory concentration (SIC, concentration not inhibiting bacterial growth) in a culture of a reporter strain of *L. monocytogenes* EGD-e during exponential growth phase. This reporter strain, designated $P_{lmo2230}$*::egfp* fuses a strong σ^B -dependent promoter from the *lmo2230* gene to a gene encoding eGFP, allowing the real-time activity of σ^B to be monitored by measuring the fluorescence intensity emitted by eGFP[110]. Apart from the wild-type eGFP reporter strain of *L. monocytogenes*, a mutant derivative lacking *sigB*, designated Δ*sigB* $P_{lmo2230}$*::egfp* was also included in the study as a negative control.

4.2.1. SigB activation in L. monocytogenes by antimicrobial plant compounds

When utilizing bacterial sensors to investigate biological responses, a good correlation must be established between the wild type and the reporter strain. The strains must show identical responses to the stress imposed, which in this case is the presence of plant-derived antimicrobial compounds. To this end, the growth characteristics of the reporter strains wild type $P_{lmo2230}$*::egfp* and Δ*sigB* $P_{lmo2230}$*::egfp* in

rich media, as well as the antibacterial effects of the selected eight plant antimicrobial agents on these strains, were examined. Favourably, no differences between the strains were observed in both analyses. The growth curves of the reporter stains were comparable to the growth curves of *L. monocytogenes* wild-type (wt) and its isogenic mutant *L. monocytogenes* Δ*sigB*. Furthermore, the MICs of the selected plant products reported for the wt and Δ*sigB* strains in Chapter 4.1 were identical the MICs observed for the eGFP reporter strains (Table 32). Additionally, a luciferase-tagged *L. monocytogenes* EGD-e strain named EGDe*lux* also showed an identical response to the activity of the selected plant antimicrobial agents. This last strain was used further along in the study, in studying the effect of the plant agents in food systems.

Table 32. MICs of selected plant-derived products against *L. monocytogenes* wild-type (wt), Δ*sigB*, $P_{lmo2230}$*::egfp* wt and $P_{lmo2230}$*::egfp* Δ*sigB*. MIC – minimal inhibitory concentration.

PLANT	FRACTION COMPOUND	wt MIC	Δ*sigB* MIC	egfp wt MIC	egfp Δ*sigB* MIC	EDGe*lux*
Humulus lupulus	α-acids	35 µg/mL	35 µg/mL	35 µg/mL	35 µg/mL	35 µg/mL
	β-acids	5 µg/mL	5 µg/mL	5 µg/mL	5 µg/mL	5 µg/mL
Olea europaea	Oleanolic acid	5-10 µg/mL	5-10 µg/mL	5-10 µg/mL	5-10 µg/mL	5-10 µg/mL
	Hydroxytyrosol	125 µg/mL	125 µg/mL	125 µg/mL	125 µg/mL	125 µg/mL
Salvia officinalis	SAL FR2	30 µg/mL	30 µg/mL	30 µg/mL	30 µg/mL	30 µg/mL
	SAL FR4	125 µg/mL	125 µg/mL	125 µg/mL	125 µg/mL	125 µg/mL
Orthosiphon stamineus	OS FR2	400 µg/mL	400 µg/mL	400 µg/mL	400 µg/mL	400 µg/mL
Eucalyptus globulus	EU FR2	25 µg/mL	25 µg/mL	25 µg/mL	25 µg/mL	25 µg/mL

The fluorescence characteristics of the *L. monocytogenes* EGD-e wt and Δ*sigB* strains bearing the reporter fusion p*lmo2230::egfp* integrated in the chromosome were then studied by fluorescence microscopy (Figure 26). The fluorescence intensity of the cells was assessed at OD_{600nm}=0.3 designated as t_0, at one hour time points during three hours after t_0 – t_{0+1h}, t_{0+2h} and t_{0+3h} and, in stationary phase (24h). The wild type $P_{lmo2230}$*::egfp* strain showed low fluorescence intensity during early exponential phase, which increased over time as cells entered stationary phase. Since σ^B is activated intrinsically in *L. monocytogenes* in stationary phase, the bacterial cells in this growth phase were highly fluorescent even without the presence of environmental stress signals, as it can be clearly seen Figure 26. On the other hand, as expected, the Δ*sigB* $P_{lmo2230}$*::egfp* cells were not fluorescent in the microscope images even in stationary phase.

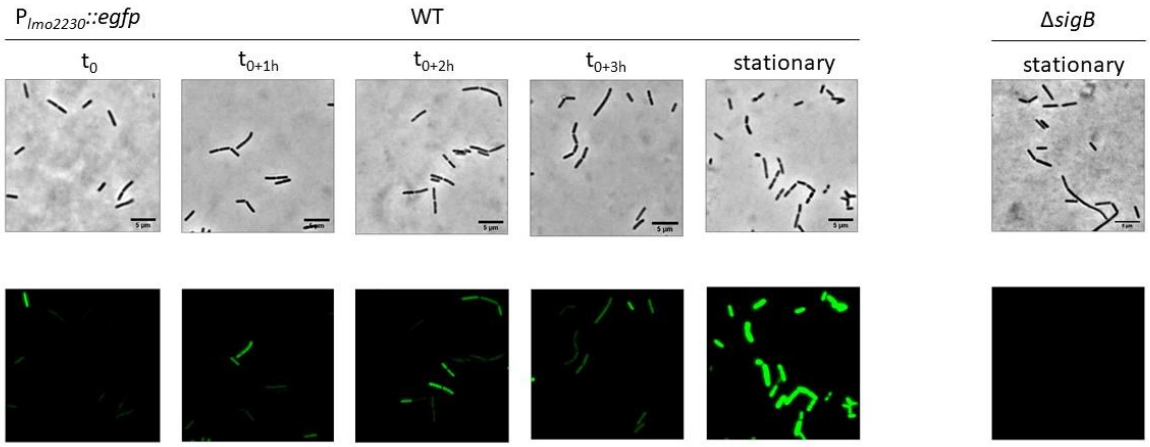


Figure 26. Fluorescence intensity of *L. monocytogenes* wild-type $P_{lmo2230}$*::egfp* and Δ*sigB* $P_{lmo2230}$*::egfp*. Bacteria were grown in BHI until reaching OD_{600nm}=0.3, time designated as t_0. Samples were drawn at t_{0+1h}, t_{0+2h}, t_{0+3h} and in stationary phase (24h). The cells were fixed with 4% (w/v) paraformaldehyde and analysed by fluorescence microscopy. Bar = 5 µm. Representative of three independent experiments.

4.2.2. SigB activation in response to stress cannot be accurately measured by a 96-well fluorescence- assay based on the $P_{lmo2230}$-eGFP reporter

Further on, an attempt was made to test the potential activation of σ^B by the selected extracts or purified compounds using the fluorescence module of the multimode reader. For this purpose, a 96-well plate fluorescence-based assay was developed where the stress response was evaluated by measuring the fluorescence intensity of the system in relative fluorescence units (RFU). The potential activation of σ^B was studied at several sub-inhibitory concentrations of the antibacterial plant agents: 2 times lower, at 4 times lower and at 8 times lower than the MIC. The assay included a simultaneous measurement of the growth patterns of *L. monocytogenes* $P_{lmo2230}$*::egfp* in time (log OD_{600}) and the fluorescence signal of eGFP (excitation wavelength 485 nm and emission wavelength 510nm). A standard curve of the strain grown in BHI without the addition of plant products is given in Figure 27.

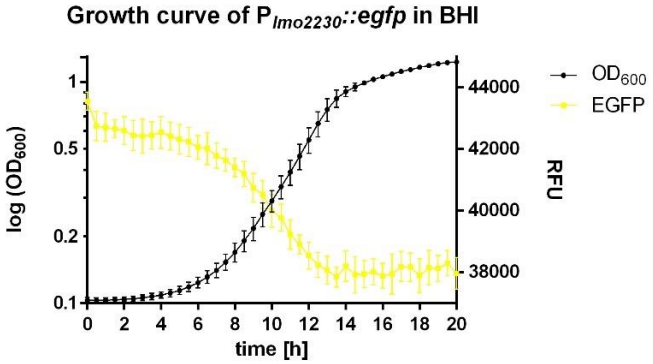


Figure 27. Growth curve (log OD_{600nm} – black) and fluorescence signal (RFU – relative fluorescence units – yellow) of *L. monocytogenes* $P_{lmo2230}$*::egfp* in BHI medium without antimicrobials. Error bars show results from three technical replicates.

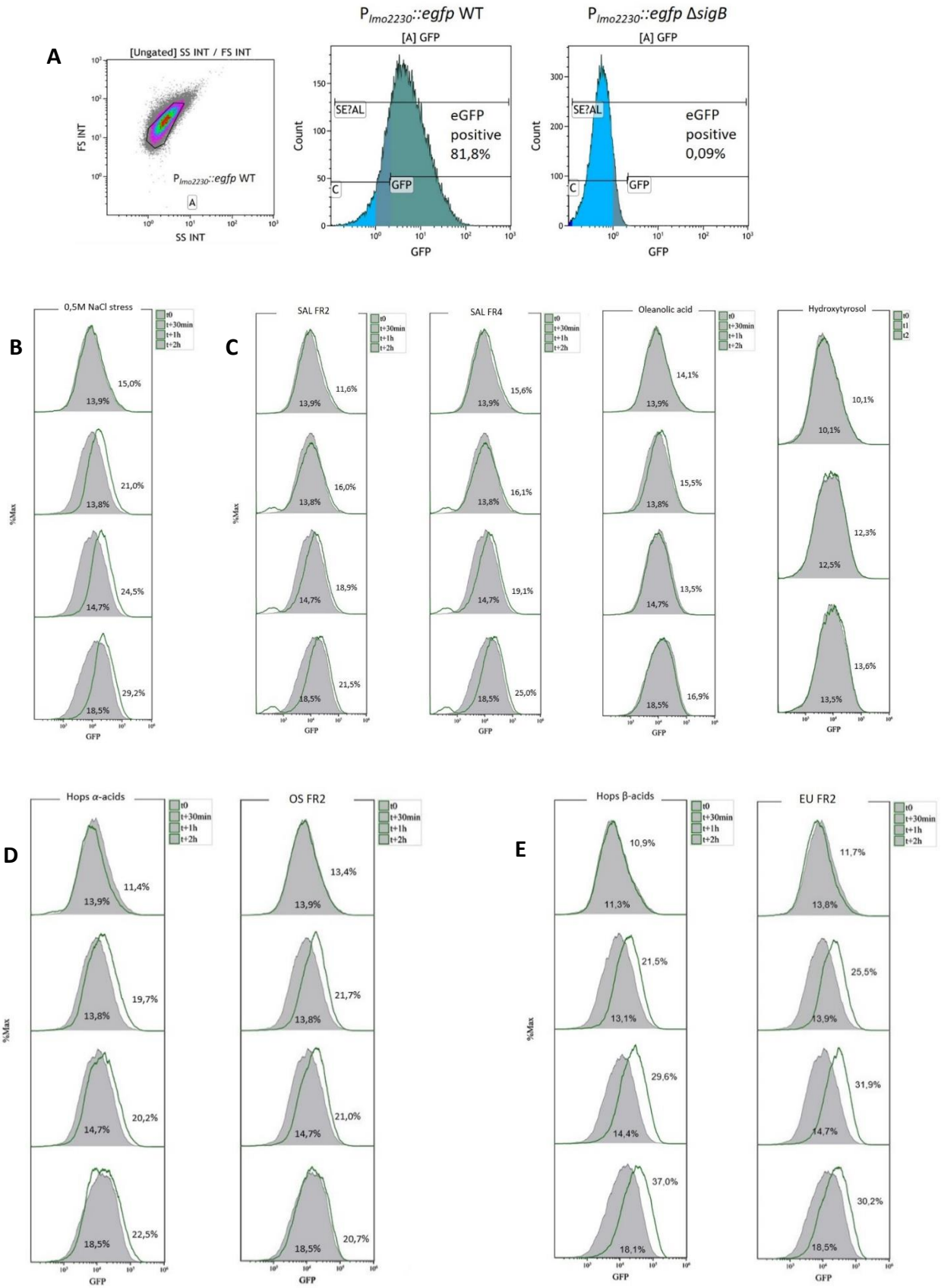
Unfortunately, a lot of intracellular molecules, as well as compounds suspended in the liquid media have an intrinsic autofluorescence in the green region of the visible spectrum. The background fluorescence emitted by the media, the extracts and the intracellular components of unstained cells (pyridines, flavins, etc.) was initially very high (≥40,000 RFU) and close to the limit of detection of the instrument, rendering the added fluorescence emitted by the produced eGFP negligible and insufficient to be able to draw any conclusions. As shown in Figure 27, the relative fluorescence signal was stable during lag phase, dropped continuously during log phase (possibly indicating the depletion of a fluorescent compound from the growth medium as the cell number increased) and stabilized again as the cells entered in stationary phase. Contrary to the obtained results, a rise in fluorescence was expected at least from cells in stationary phase, since σ^B is activated intrinsically in all cells in stationary phase.

4.2.3. Measurement of SigB activation by antimicrobial stress using flow cytometry

To study whether exposure to plant antibacterial agents causes σ^B activation in *L. monocytogenes* a flow cytometry approach was used by measuring eGFP expressing cells. Flow cytometry is a rapid, high-throughput, quantitative method able to analyse individual cells and provide information at a population level. This method has been used to monitor σ^B activity in populations of intracellular *L. monocytogenes*[165]. In the analysis, exponentially grown cells of the reporter strain wild type $P_{lmo2230}$*::egfp* were stressed with subinhibitory concentrations of the plant antibacterial agents – between ½ and ¼ of the MICs – during 30 min ($t_{0+30min}$), 1 hour (t_{0+1h}) and 2 hours (t_{0+2h}) (stress). The subinhibitory concentrations for each agent were selected on the basis of previous experiments where the concentrations of each plant agent that caused no significant bacterial growth inhibition in comparison to the control were determined (Table 33). The following plant antibacterial agents were used: α-acids, β-acids, oleanolic acid, hydroxytyrosol, FR2 and FR4 from *S. officinalis* L., FR2 from *E. globulus* L. and the polyphenols fraction, OS FR2, of *O. stamineus* Benth. Exposure to 0.5 M NaCl serving as osmotic stress was included as a positive control of a known σ^B inducer. Intrinsic fluorescence was excluded by using the reporter fusion strain $P_{lmo2230}$*::egfp* Δ*sigB.* Since approximately 81,8% of the exponentially growing bacteria not exposed to antimicrobial stresses were identified as eGFP-positive, the mean of florescence intensity was used to compare σ^B activation.

Table 33. Sublethal concentration of the plant agents used in the flow cytometry analysis.

Plant Agent	α-acids	β-acids	Oleanolic acid	Hydroxy-tyrosol	SAL FR2	SAL FR4	OS FR2	EU FR2
MIC fraction	½ MIC	½ MIC	¼ MIC	½ MIC	⅓ MIC	⅓ MIC	¼ MIC	⅓ MIC
Concentration (µg/mL)	20	5	2,5	75	10	50	100	10


A
[Ungated] SS INT / FS INT
FS INT
SS INT
P lmo2230::egfp WT
A
P lmo2230::egfp WT
[A] GFP
SE?AL
eGFP positive 81,8%
C
GFP
Count
GFP
P lmo2230::egfp ΔsigB
[A] GFP
SE?AL
eGFP positive 0,09%
C
GFP
Count
GFP
B
0,5M NaCl stress
t0
t+30min
t+1h
t+2h
15,0%
13,9%
21,0%
13,8%
24,5%
14,7%
29,2%
18,5%
%Max
GFP
C
SAL FR2
11,6%
13,9%
16,0%
13,8%
18,9%
14,7%
21,5%
18,5%
SAL FR4
15,6%
13,9%
16,1%
13,8%
19,1%
14,7%
25,0%
18,5%
Oleanolic acid
14,1%
13,9%
15,5%
13,8%
13,5%
14,7%
16,9%
18,5%
Hydroxytyrosol
t0
t1
t2
10,1%
10,1%
12,3%
12,5%
13,6%
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11,4%
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22,5%
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OS FR2
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Hops β-acids
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EU FR2
11,7%
13,8%
25,5%
13,9%
31,9%
14,7%
30,2%
18,5%
GFP

Figure 28. Flow cytometry (FCM) analysis of SigB activation in response to plant antimicrobial stress. (Instrument Beckman Coulter's Gallios). Fluorescence was quantified for *L. monocytogenes* wild type (EGD-e) bearing a $P_{lmo2230}$*::egfp* reporter fusion integrated in the chromosome. Bacteria were grown in BHI until reaching OD_{600nm}=0.3 (untreated) or after the addition of plant antibacterial agents at SICs during 30 min, 1h and 2h (antimicrobial stress). The cells were fixed with 4% (w/v) paraformaldehyde and analysed by FCM: **A)** left to right: $P_{lmo2230}$*::egfp* gate; eGFP $P_{lmo2230}$*::egfp* positive cells at t_0; $P_{lmo2230}$*::egfp* Δ*sigB* eGFP positive cells at t_0. Overlap of the histograms between $P_{lmo2230}$*::egfp* untreated/antimicrobial stress and comparison of the mean of eGFP fluorescence **B)** SigB activation by osmotic stress (0.5M NaCl) **C)** Non-inducers: *S. officinalis* L. SAL FR2 and SAL FR4, oleanolic acid and hydroxytyrosol **D)** Mild SigB inducers: hops α-acids and *O. stamineus* Benth. OS FR2 E) Strong SIgB inducers: hops β-acids and *E. globulus* L. EU FR2.

When the cells were exposed to osmotic stress (0.5 M NaCl), the mean of eGFP fluorescence intensity increased gradually from 15% at t_0 to 21% after 30 min, 24.5% after 1 hour and 29.2% after 2 hours, clearly indicating increased σ^B activity compared to the untreated sample whose mean of fluorescence reached a maximum of 18.5% at t_0+2 h. Comparing these results to the results of σ^B activation induced by the plant agents, three groups of samples emerged: plant antibacterial agents that did not cause σ^B activation, mild σ^B inducers and strong σ^B inducers.

- No σ^B activation: The plant antimicrobial agents that were not capable of activating σ^B included the *S. officinalis* L. fractions, FR2 and FR4 and the *O. europaea* L. phytocompounds, oleanolic acid and hydroxytyrosol.
- Mild σ^B inducers: The agents that were appointed mild inducers had comparable σ^B activation results to the one caused by osmotic stress, and these samples were the hops α-acids and the *O. stamineus* Benth. fraction OS FR2. Exposure to the hops α-acids induced significant σ^B activation in the first 30 min evident from the change from 11,4% to 19,7% fluorescence intensity mean, that continued to increase slowly during time and reached its maximum of 22,5% intensity mean at 2 hours after exposure. On the other hand, the OS FR2 reached its maximum of 21,7% at 30 min after exposure and then stabilized until the end of the measurement.
- Strong σ^B inducers: Exposure of *L. monocytogenes* $P_{lmo2230}$*::egfp* to SICs of hops β- acids and EU FR2 from *E. globulus* L. caused a strong and proportional increase in fluorescence with a significant difference in the mean of fluorescence intensity of the eGFP positive cells that increased more than two-fold after 30min, almost 3-fold after 1h and continued to increase to more than a 3-fold difference after 2h of exposure to these plant antibacterial agents. The highest measured mean of fluorescence intensity was triggered by the hops β- acids 2 hours after exposure, 37%.

This increase in fluorescence intensity of the *L. monocytogenes* eGFP positive cells was σ^B -dependent given the lack of increase in the level of fluorescence when the Δ*sigB* P*lmo2230*::egfp strain was exposed

the same antimicrobial stresses. Lastly, the data obtained by flow cytometry depicted a marked heterogeneity in fluorescence intensity within the population of wild-type positive-eGFP cells, which was noted by a broader bell-shaped peak (Figure 28).

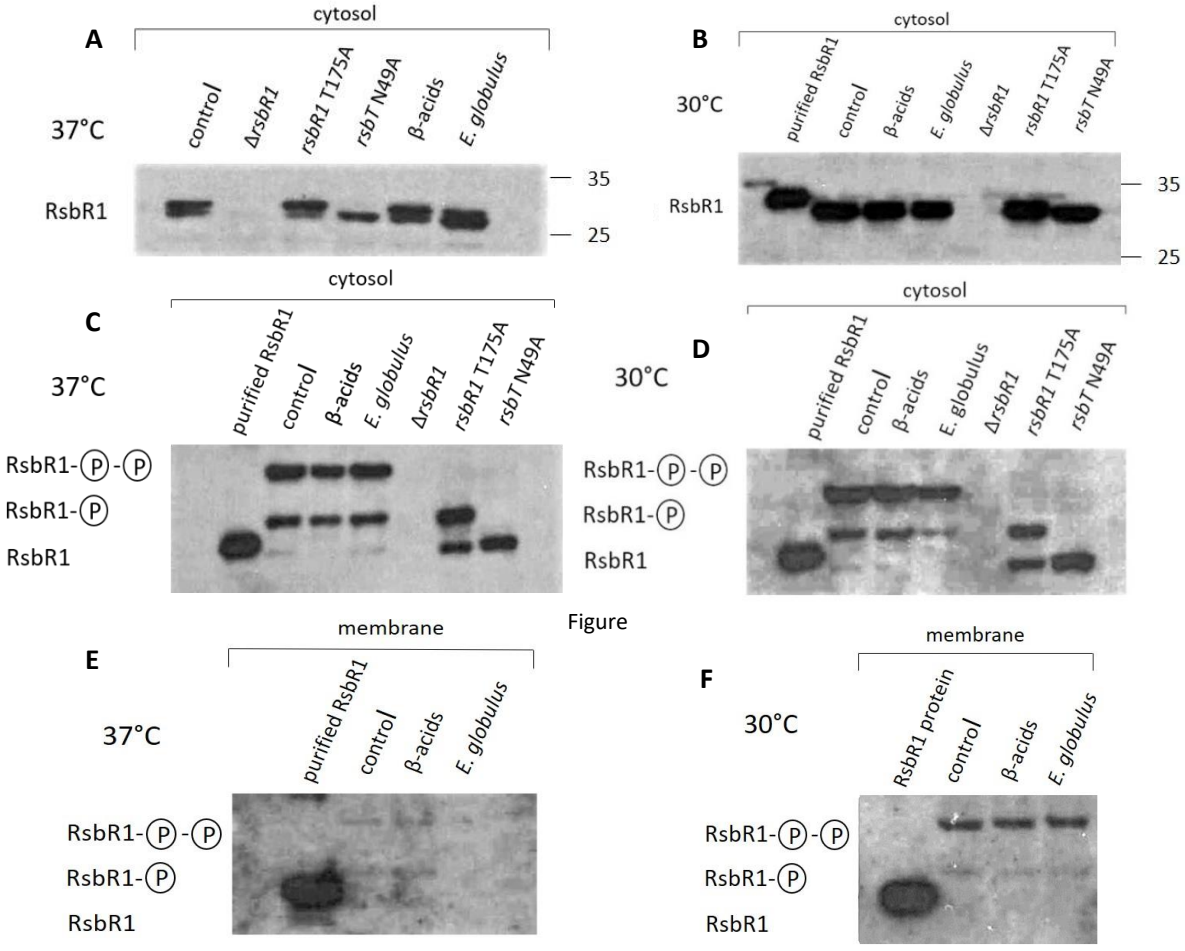
4.2.4. Subcellular localization and phosphorylation dynamics of RsbR1 in L. monocytogenes: the effect of antimicrobial stress

The current model of σ^B activation in *B. subtilis* predicts that RsbRA, whose ortholog in *L. monocytogenes* is known as RsbR1, and RsbS undergo phosphorylation/dephosphorylation modifications in order to regulate the stressosome activation and, consequently, the stimulation of the σ^B signaling cascade[86,87,88,89,90]. According to this model, in an unstressed state, RsbRA is mostly mono-phosphorylated and RsbS, non-phosphorylated. After imposing mild osmotic stress, RsbT exerts its kinase activity only towards RsbS, effectively producing its monophosphorylated isoform. Concerning RsbRA, this protein becomes doubly-phosphorylated only after imposing a strong stress, which in the particular study was 10% ethanol[111]. In *L. monocytogenes*, RsbR1 has two conserved threonine residues that can undergo phosphorylation – Thr175 and Thr209, while RsbS has only one, Ser56. Similar to *B. sublitis*, Misra *et al*. found that in the absence of stress only the amino-acid residue Thr175 is phosphorylated in *L. monocytogenes*, but not RsbR Thr209 or RsbS Ser59[69,79].

In this study, the impact of antimicrobial stress on the relative levels and subcellular localization of the SigB protein and the core stressosome component RsbR1 were investigated in *L. monocytogenes*. Additionally, the phosphorylation state of RsbR1 in the absence and presence of antimicrobial stress was determined. For this purpose, two plant antibacterial agents, hops β-acids and an *E. globulus* fraction, EU FR2, were used as a source of antimicrobial stress. The selection of these agents was based on previous observations that they are capable of inducing strong σ^B activation (Figure 28). To this aim, exponentially grown *L. monocytogenes* EGD-e wt cells at 30°C and 37°C were stressed with sublethal concentrations of the plant agents (Table 33). After 30 min, the cells were recovered and subcellular fractionation was performed. Immunoblot analyses using affinity-purified antibodies were carried out to determine the relative levels and subcellular location of RsbR1 and SigB. Non-phosphorylated histidine-tagged proteins produced exogenously in recombinant *E. coli*, a Gram-negative bacterium that lacks the *sigB* operon, were used as controls for identification of the proteins. Moreover, three *L. monocytogenes* EGD-e mutant strains were included as additional controls. The first strain, EGD-e *rsbT* N49A had a *rsbT* mutation created on the glutamine residue (N49), critical for the kinase activity of RsbT towards RsbR1 and RsbS. As a result,

this RsbT-N49A cannot phosphorylate these two stressosome proteins. The second control was EGD-e T175A, a strain with an RsbR1 protein that can only be monophosphorylated because of a mutation on Thr175. The third control strain, EGD-e Δ*rsbr1*, has the gene encoding for RsbR1 protein deleted from the genome. Coomassie blue staining was used as protein loading control in the Westerns blots.

Surprisingly, *L. monocytogenes* RsbR1 was found mostly cytosolic (Figure 29), which contrasted a previous study in which subcellular fractionation revealed that RsbR1 was 60% membrane-associated[93]. However, more recent studies are in agreement with our data since RsbR1 was visualized mostly in the cytosol of *L. monocytogenes* exposed to osmotic stress[128]. In our analyses, RsbR1 was difficult to visualize in the membrane fraction at the respective ratio of cytosol to membrane 1:1, so a 5-times more concentrated membrane fraction was prepared for the samples grown at 30°C to improve RsbR1 visualization. Phos-Tag SDS-PAGE technology was used to discriminate between the isoforms of RsbR1 of different phosphorylation states. This technique uses a functional molecule with a Zn^{+} ion that can specifically capture and separate proteins phosphorylated on Ser, Thr, and/or Tyr residue(s).



Figure

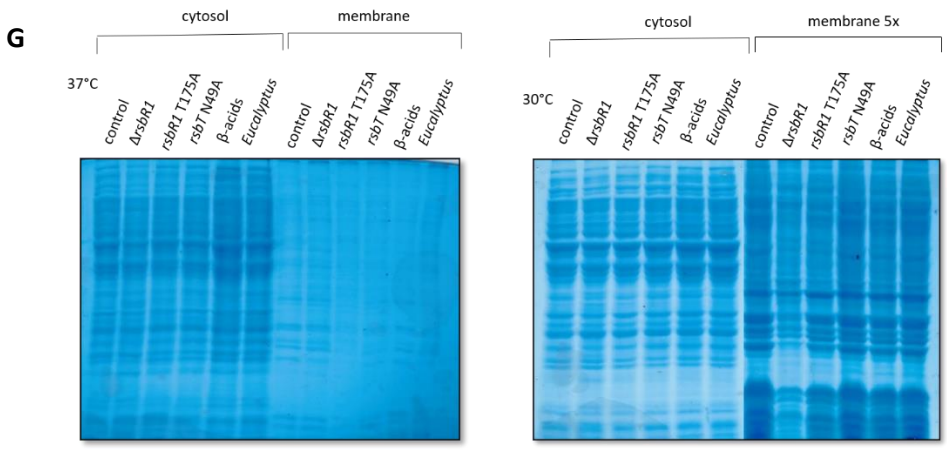


Figure 29. Exposure of *L. monocytogenes* to antimicrobial stresses does not alter the expression, subcellular localization and phosphorylation status of the core stressosome protein RsbR1 upon exposure antimicrobial extracts. *L. monocytogenes* EGD-e wild-type (control), Δ*rsbr1, rsbR* T175A and *rsbT* N49A were grown in BHI medium at 37°C and at 30°C to OD_{600nm}=0.3. Cytosol and membrane extracts were prepared from unstressed and stressed bacteria (exposed 30 min to subinhibitory concentration of hops β-acids and the extract of *E. globulus*) bacteria. A-F – immuno-blots against RsbR1. **A)** Anti-RsbR1 immuno-blot of a Tris-Glycine SDS-PAGE gel for bacteria grown at 37°C. **B)** Anti-RsbR1 immuno-blot of a Tris-Glycine SDS-PAGE gel for bacteria grown at 30°C. Molecular weight markers are shown on the right side. **C)** Anti-RsbR1 immuno-blot of a Phos-Tag SDS-PAGE gel for bacteria grown at 37°C – cytosolic fraction. **D)** Anti-RsbR1 immuno-blot of a Phos-Tag SDS-PAGE gel for bacteria grown at 30°C – cytosolic fraction. **E)** Anti-RsbR1 immuno-blot of a Phos-Tag SDS-PAGE gel for bacteria grown at 37°C – membrane fraction. **F)** Anti-RsbR1 immuno-blot of a Phos-Tag SDS-PAGE gel for bacteria grown at 30°C – membrane fraction. The immune-blots are representative of two independent analyses. **G)** Coomassie staining showing the protein loading in the immunoblots.

As expected, no RsbR1 protein was detected in the Δ*rsbR1* mutant strain. The appearance of exclusively unphosphorylated protein in the kinase-inactive *rsbT* N49A strain confirmed that RsbR1 is effectively the substrate of RsbT and that the kinase-activity of RsbT is essential for phosphorylation of RsbR1 (Figure 4.2.4C-D). In the EGD-e T175A mutant producing the RsbR1-T175 variant, the protein was detected in both unphosphorylated and monophosphorylated form, with the monophosphorylated form being more abundant than the unphosphorylated form, 67% to 33% respectively at both temperatures (Figure 29 C-D). This result showed that phosphorylation on the first site of RsbR1 (T175) is not a prerequisite for the phosphorylation of the second site (T209). Considering the samples treated with plant antimicrobials, the results indicated that cytosolic RsbR1 is 62% doubly-phosphorylated and 38% mono-phosphorylated in actively growing *L. monocytogenes* EGD-e cells at 37°C. This ratio was similar for the samples grown at 30°C, 71% and 29% respectively. Surprisingly, the same phosphorylation pattern was observed between the untreated (control) sample and the ones exposed to plant antimicrobial stresses, with no switch of the mono-phosphorylated protein pool to the doubly-phosphorylated one following stress. At the same time, no significant difference of the relative levels of RsbR1 was observed in the bacteria exposed to the plant antibacterial agents. Altogether the results indicate that the relative levels

and the phosphorylation status of RsbR1 do not change significantly with exposure to the specific plant extracts used.

4.2.5. Subcellular localization of SigB in L. monocytogenes: the effect of antimicrobial stress

Apart from RsbR1, the subcellular location and relative levels of SigB protein in *L. monocytogenes* EGD-e wt were also investigated. Unlike RsbR1, SigB was detected in relevant levels in both the cytosolic and membrane fractions with a higher presence of SigB in the cytosolic fraction of 65%, compared to the 35% of SigB in the membrane fraction in exponentially grown bacteria at both temperatures, 30°C and 37°C (Figure 30).

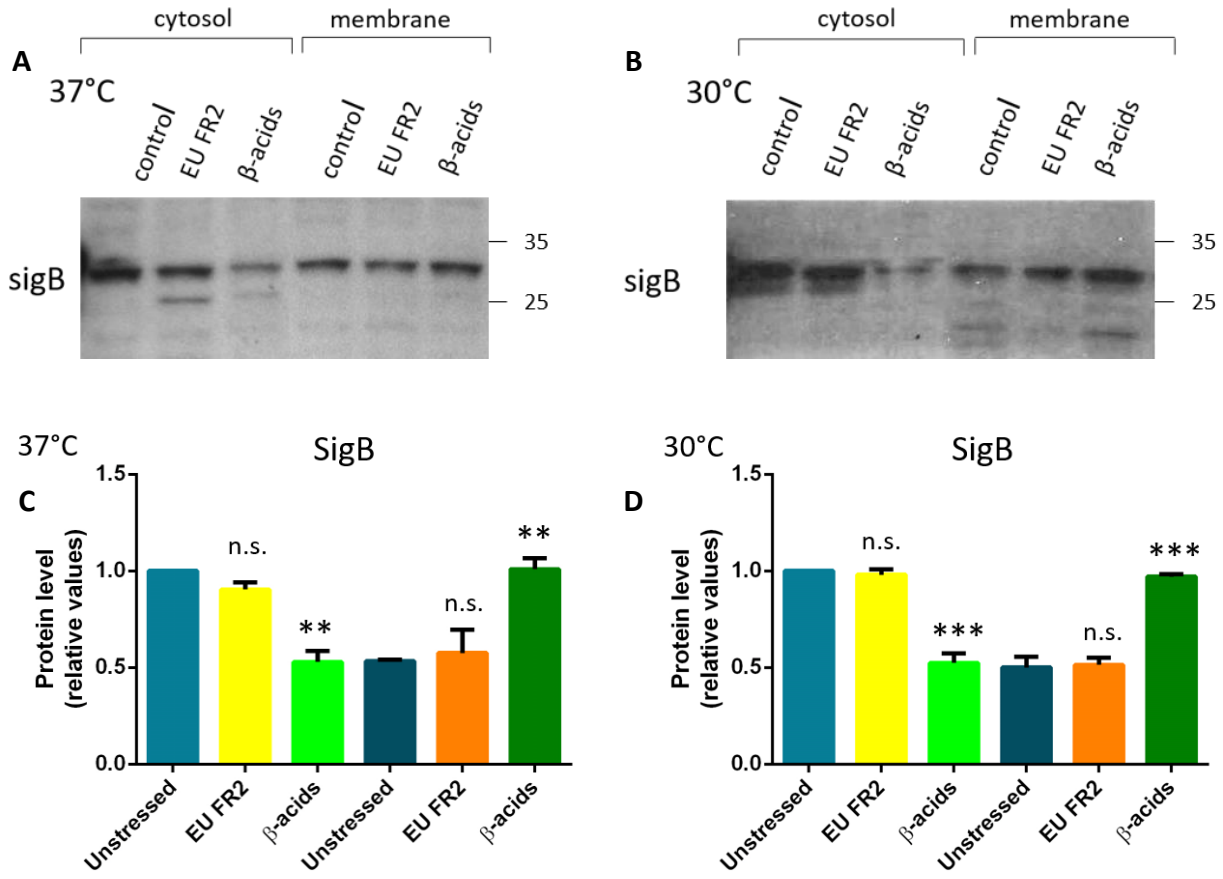


Figure 30. Exposure of *L. monocytogenes* to antimicrobial stresses alters the subcellular location of SigB protein.
L. monocytogenes EGD-e wild-type (control), was grown in BHI medium at 37°C and at 30°C to OD_{600nm}=0.3. Cytosol and membrane extracts were prepared from unstressed and stressed (exposed 30 min to subinhibitory concentration of hops β-acids or the fraction of *E. globulus* – EU FR2) bacteria. **A)** – Anti-SigB immuno-blot for bacteria grown at 37°C. **B)** – Anti-SigB immuno-blot for bacteria grown at 30°C. Molecular weight markers are shown on the right side. **C – D**) – Quantification of the signals obtained in the blots. Quantification of the signals was obtained from two independent biological replicates and the results are shown as mean values with standard deviation. Significance was determined by one-way ANOVA followed by Dunnett's multiple comparison test, and significance is shown relative to WT in each condition (***P < 0.001; **P < 0.01; n.s. not significant).

The stress treatment with both plant antimicrobial agents, hops β-acids the *E. globulus* L. fraction EU FR2, resulted in increased SigB protein levels in the membrane fraction in the cells grown at both temperatures. Considering the EU FR2 treatment, the relative level of SigB in both the cytosolic and membrane fraction remained the same in the cells grown at 30°C, but changed slightly in the cells grown 37°C, with a moderate decrease in SigB levels in the cytosolic fraction and an increase of SigB levels in the membrane fraction. On the other hand, this effect was more pronounced when *L. monocytogenes* was exposed to the hops β-acids. The stress exposure at 37°C lowered the relative level of cytosolic SigB to 32% to the 65% of cytosolic SigB in the control, while the levels of SigB in the membrane fraction increased almost two-fold to 68% (1,8 times higher). The treatment at 30°C resulted in similar observations, changing the SigB levels in cytosol fraction to 36% and the levels of SigB in the membrane fraction to 64% (2,1 times higher) (Figure 30). These results agree with previous reports in *B. subtilis*, in which alterations in SigB levels were noticeable after the cells were exposed to ethanol stress[111].

4.2.6. Membrane integrity of L. monocytogenes after exposure to plant antimicrobials

Antibacterial agents are able to exert their effects on various structures of the bacterial cell, including the cell membrane. Membrane integrity is crucial for the survival of bacterial cells, as cells with damaged or compromised membranes cannot maintain or generate the electrochemical gradient, and, hence, the membrane potential. Consequently, the metabolic activity in these cells is impaired and they are considered as dead. Membrane targeting antibacterial agents can produce several types of injuries on the cell membrane, such as, membrane thinning and expansion, pore formation, increased membrane fluidity and permeability, disturbance of membrane-embedded proteins, inhibition of respiration and alteration of ion transport processes. Several methods can be used to evaluate membrane integrity, one of which is the stain exclusion method, like the one used by the LIVE/DEAD BacLight Bacterial Viability Kit.

In this study, the LIVE/DEAD BacLight Bacterial Viability Kit was used for assessing the membrane integrity of *L. monocytogenes* EGD-e wt after exposure of exponentially growing bacterial cells to the selected plant antibacterial agents at their previously determined MICs. The respective percentages of live and dead cells after exposure to the antimicrobial plant products were quantified using a fluorescence microplate reader. The membrane damage was assessed one hour after exposure to the products. The results are given in Figure 31. 10% DMSO was used as a positive control to compare the extent of membrane damage. DMSO is an aprotic solvent well-known for its deleterious effects on cell membranes. From lower to higher concentrations, DMSO starts inducing lateral expansion and progressive thinning of

the membrane, followed by formation of transient water pores, desorption of individual lipid molecules out of the membrane surface and completes by disintegration of the bilayer structure of the lipid membrane[115]. It should be noted that the concentrations of the plant compounds used in this experiment are the MICs for a culture containing 10^6 CFU/mL, hence, these concentrations are not sufficiently high to kill a culture of viable cells with $3x10^7$ CFU/mL during one hour, but are adequate to be able to draw conclusions of membrane integrity and changes in permeability.

The lowest percentage of membrane damaged cells was observed after the β-acid treatment – 94% of the bacterial cells were still viable after exposure, indicating that the membrane alterations caused by this plant agent were not substantially high to increase PI uptake, an indicator of large pore formation leading to cell death. The second least potent membrane damaging plant agent was the *O. stamineus* FR2, leaving 88% of live cells after one hour exposure. The treatment with hydroxytyrosol and α-acids resulted in a similar percentage of live cells after exposure – 75% and 75.8% respectively.

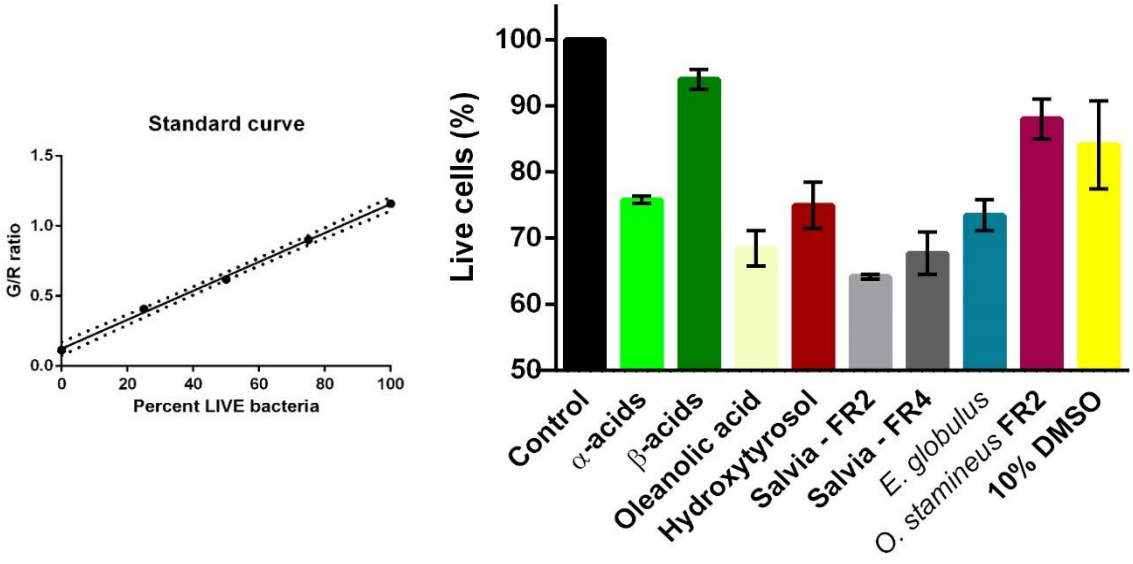


Figure 31. Evaluation of *L. monocytogenes* viability through membrane damage assessment using the LIVE/DEAD BacLight Bacterial Viability Kit. The standard curve is obtained by calculating the Green/Red ratio of 100%LIVE, 75%LIVE:25%DEAD, 50%LIVE:50%DEAD, 25%LIVE:75%DEAD and 100%DEAD cells. R^2=0.9982. The percentage for each plant agent shown in the bar graph was calculated as the mean value from three technical replicates. Error bars – mean with SD from three technical replicates. The results are representative of two biological replicates.

On the other hand, the most effective membrane damaging agents were the triterpenic (SAL FR2) and diterpenic fraction (SAL FR4) of *S. officinalis*, leaving 64.2% and 67.7% live cells after one hour exposure, followed closely by oleanolic acid – 68.4% and finishing with the *E. globulus* fraction – 73.4%. Surprisingly, exposure to 10% DMSO left a significantly lower percentage of membrane damaged cells compared to

some of the extracts. 84.1% of the bacterial cells were still viable after 1 hour exposure to this deleterious organic solvent. It has been postulated that the mechanism of antimicrobial activity of terpenes and terpenoids is through membrane disruption. Due to their hydrophobic nature, this class of compounds can partition into the membrane structure and disrupt the lipid bilayer producing deleterious effects on the membrane, such as membrane expansion, increased membrane fluidity and permeability, disturbance of membrane embedded proteins and ion transport channels or impairment of respiration[116]. The results of the LIVE/DEAD assay are clearly in alignment with these postulates, since the plant samples that contained a higher concentration of triterpenic acids or diterpenic compounds, thought to be the carriers of the antibacterial activities observed, resulted in a higher percentage of cells with disrupted membranes after exposure, even greater than the damage caused by a very high concentration of DMSO, namely 10% DMSO. This might suggest that the mechanism of activity of these samples is indeed bacterial cell membrane disruption. On the other hand, the results for the HBAs, hydroxytyrosol and the *O. stamineus* fraction suggest that these plant agents have a different cellular target and their mechanism of activity does not correspond with membrane disruption.

4.2.7. Determining the type of activity of the most active extracts – bacteriostatic or bactericidal

Ultimately, the type of antibacterial activity, bacteriostatic or bactericidal, exhibited by the most active plant antibacterial agents was investigated in more detail. A bacteriostatic agent is an agent that stops or inhibits microbial growth, while a bactericidal is responsible for killing the bacteria. To be able to distinguish whether the observed antibacterial activities of the selected plant agents were bacteriostatic or bactericidal, an experiment was designed where each agent was added at its MIC to an exponentially grown culture of 10^6 CFU/mL of *L. monocytogenes* wild-type at 37°C (t_0). A sample was drawn from the culture every hour during 5 h from the start point, and at a 24 h end point. The sample was plated on nutrient agar plates free from any antimicrobial agents and the approximate number of bacterial cells at each time point was compared to the cell culture where no growth inhibitors were added – control. During 24 h, as expected, all extracts were able to detain the bacterial growth, as no visual changes in the optical density at 600 nm could be observed. Moreover, if the number of viable cells was gradually decreasing with time, the plant antibacterial agent was capable of killing *L. monocytogenes*, implying bactericidal effects. The results of these assays are shown in Figure 32.

As expected, the cells in the control continued multiplying during the 24 hours as no inhibitory agent was present in the nutrient medium. Considering the cultures where plant antibacterial agents were

added, two different types of activity could be observed. On one hand, the HBAs purified from *H. lupulus*, α-acids and β- acids, as well as hydroxytyrosol, displayed only growth inhibition effects, given that as soon as the bacteria were removed from the presence of the antibacterial plant agent, they were able to restore their growth capabilities. Moreover, the number of viable bacteria at time points 1h, 4h and 24h is comparable to the number of viable bacteria at t_0. On the other hand, oleanolic acid, the *E. globulus* FR2 and FR2 and FR4 from *S. officinalis* indicate bactericidal effects, as the number of viable cells in the culture was brought to zero after 24 hours. *S. officinalis* FR2, the *E. globulus* fraction FR2 and oleanolic acid were especially active, since adding the antibacterial agent to the bacterial culture caused a reduction of 4 or 5 logs in the inoculum during the first hour. The bactericidal effects of *S. officinalis* L. FR4 were delayed, but nonetheless effective since no viable cells could be observed 24 hours after addition of the agent.

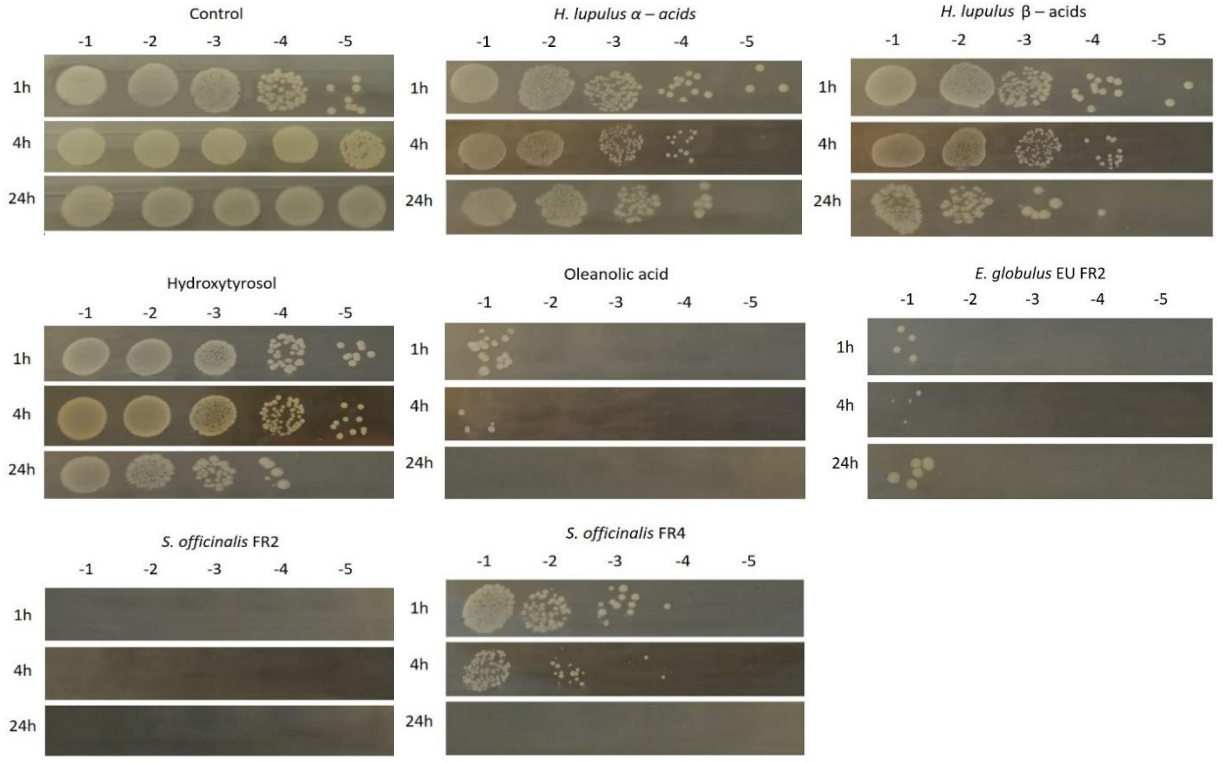


Figure 32. Cultivability test to distinguish between bacteriostatic or bactericidal activities of selected plant antibacterial agents. Each plant agent was added at its MIC (see Table 31) to an exponentially grown culture of *L. monocytogenes* EGD-e wild-type containing 10^6 CFU/mL bacterial population. Control – no antibacterial agent added.

4.3. Chapter III: Testing selected plant extracts and purified plant compounds in food matrices and on other food pathogens

Food products are perishable by nature and require protection from microbial contamination and pathogen and spoilage growth during their preparation, storage and distribution. Due to continuous expansion in the international food trade sector, a lot of food products are now sold in areas of the world far away from their production sites, implying the need for extended shelf-lives for these products. Improvements in the cold temperature distribution chain have allowed the economic manufacture of perishable foods, but refrigeration alone cannot ensure the quality and safety of all perishable foods. This is especially true when it comes to *L. monocytogenes*, a restrictive food pathogen for minimally processed products on account of its ability to reproduce at refrigeration temperatures. As a result, additional food preservation methods need to be taken into account, one of which is the use of antimicrobials able to inhibit microbial growth or kill food pathogens and spoilers.

Numerous studies have reviewed the antimicrobial potential of natural products[10,11,12,23,107]. However, most have been carried out on planktonic bacterial cells in laboratory media, and this doesn't represent the conditions of real food systems where bacteria generally live as part of a community with other native microorganisms[119,120]. Even more relevant is the potential reactivity of the antimicrobial with food components. Reactions with lipids, proteins, carbohydrates and other food additives could result in an overall decrease in the activity of the antimicrobial agent[122,123,124,139,150]. In addition, the intrinsic (pH, salt concentration, antioxidants) and extrinsic (temperature, vacuum, modified atmosphere packaging, innate microbial community) properties of the food also have a significant influence on the antimicrobial efficacy of the agent. Often, a greater concentration of the antimicrobial agent is needed to achieve the same effect in real food systems than the one observed in *in vitro* screenings in laboratory media. The reasons for the decreased activity of antimicrobials in food systems can either be dictated by the chemical nature of the antimicrobial agent, including partitioning of the agent in areas of the food away from where microbial growth occurs or transformation of the antimicrobial into less effective forms[119,120]; or by bacterial factors such as the induction of protective responses that offer cross protection due to overall increased stresses present in the food system, such as acidic pH, osmolytes etc.[38,42,63].

In the final part of the study, the antibacterial activities against *L. monocytogenes* of a selection of the most efficient antibacterial plant agents (detailed in Section 4.1.4), were determined in combination with other food-related stresses (Section 4.3.1), in simple food systems (Section 4.3.2 and 4.3.3) and on other bacterial food pathogens (Section 4.3.4). The antibacterial potential of these agents in food matrices was assessed by measuring the expression of luminescence of a luciferase-tagged *L. monocytogenes* strain called EGDe*lux*. As luminescence is expressed constitutively in this strain, the luminescence level was a measure of growth through metabolic activity and increase in number of cells[125].

4.3.1. Antibacterial activities of selected agents in combination with common food hurdles

In the food industry, various food preservation technologies known as hurdle technologies are applied to foods with the purpose of killing or inhibiting the growth of pathogenic and food spoilage microorganisms and creating microbiologically safe and stable food products[62]. The principal hurdle factors used by the industry are: applying temperature (high or low), reducing the water activity, modifying the pH, adding preservatives (nitrite, sorbate, sulphite) or utilizing the competitiveness of naturally occurring microorganisms in raw materials, ingredients and finished food products to supress the growth of food pathogens (for example, lactic acid bacteria)[132,133]. Nowadays, minimally processed foods are preferred by the consumer, since an increase in the severity of the food preservation process usually decreases the quality of the treated food. Therefore, it is important to secure maximum lethality or inhibition of microbial growth by using the lowest degree of a hurdle preservation factor, in favour of conserving the nutritional and sensory aspects of the food. The easiest approach to achieve this target is to combine several hurdle factors, for example, sub-lethal pH combined with the addition of an antimicrobial compound. The concept behind this principle is that the simultaneous application of two or more hurdle factors would metabolically exhaust the microorganisms, as they will employ all their energy to overcome the hostile environment and restore homeostasis[132]. This way, bacterial cells that are sub-lethally injured by one stressing condition, become sensitive to other physical or chemical agents to which healthy cells are normally resistant when only one stressing condition is applied[119,133].

Following this principle, a way of improving the efficacy of plant antimicrobial agents could as well be through their combined application with sublethal stresses, so that the microorganisms would have more difficulty in overcoming the multiple hurdles present in the system, resulting in a lower minimal concentration of the agent needed to supress the growth of the pathogen. To test this concept, the growth inhibition properties of selected plant extracts (Section 4.1.4) were tested in combination with commonly applied food-related stresses – low temperature stress, acidic stress and osmotic stress. To this end, a serial dilution of the antibacterial compounds starting from 1 mg/mL was carried out in BHI media previously adjusted to either pH 5.5 or to a NaCl concentration of 3.5% (sublethal stresses). Exponentially grown *L. monocytogenes* EGD-e was added to a final cell suspension value of 10^6 CFU/mL. For the cold stress combination, bacterial cells were left to grow for 21 days at 4°C in BHI media with the plant agents at various concentrations (serial dilution from 1 mg/mL). The broth dilution method that uses the coloured indicator resazurin was used in this method.

The results presented in Figure 33 suggest that exposing the bacteria to low temperature did not change the MICs of the plant antimicrobial agents at the 21st day of growth. At day 15, the resazurin metabolization rate indicated that the bacteria not exposed to plant antimicrobials (control) had already reached a suspension concentration at least 2-logs higher than the initial, noted by the colour change of the indicator resazurin. However, the inoculum concentration of the cells exposed to the plant agents at concentrations two or four times lower than the MICs observed at optimal temperatures was still not high enough to observe a change in colour at this time point. Nonetheless, at the 21st day after inoculation, the same MICs were observed for all plant antimicrobials as the ones previously established for 30°C.

The osmotic stress combination caused the most efficient reductions in minimal concentrations of the plant agents needed to inhibit the growth of *L. monocytogenes.* The MICs observed at a 3.5% NaCl concentration decreased from 2 to 10-fold. The only exception was oleanolic acid. The activity of OA was not influenced by the application of additional food hurdles, and its MIC remained equal at all conditions, 5 µg/mL. Considering the acid stress, most agents were able to exert their antibacterial properties at lower concentrations than the ones at pH 7. This was especially evident for *E. globulus* L. EU FR2 whose active principle exhibited a 25-times MIC reduction, from 25 µg/mL to 1 µg/mL. In contrast, hydroxytyrosol and *O. stamineus* Benth. OS FR2 completely lost measurable antimicrobial activities at acidic pH, suggesting that the protonation of the active principle generated an inactive form of the antimicrobial agent.

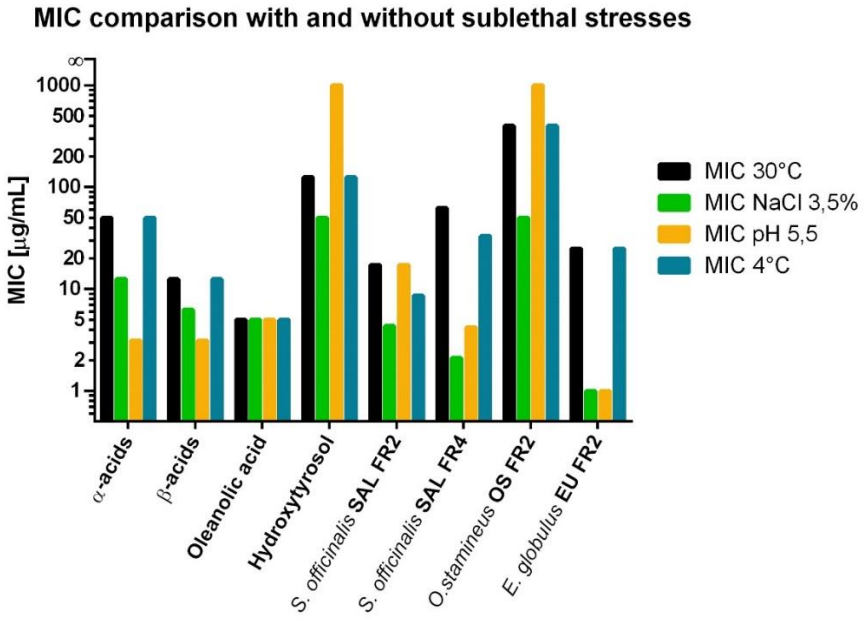
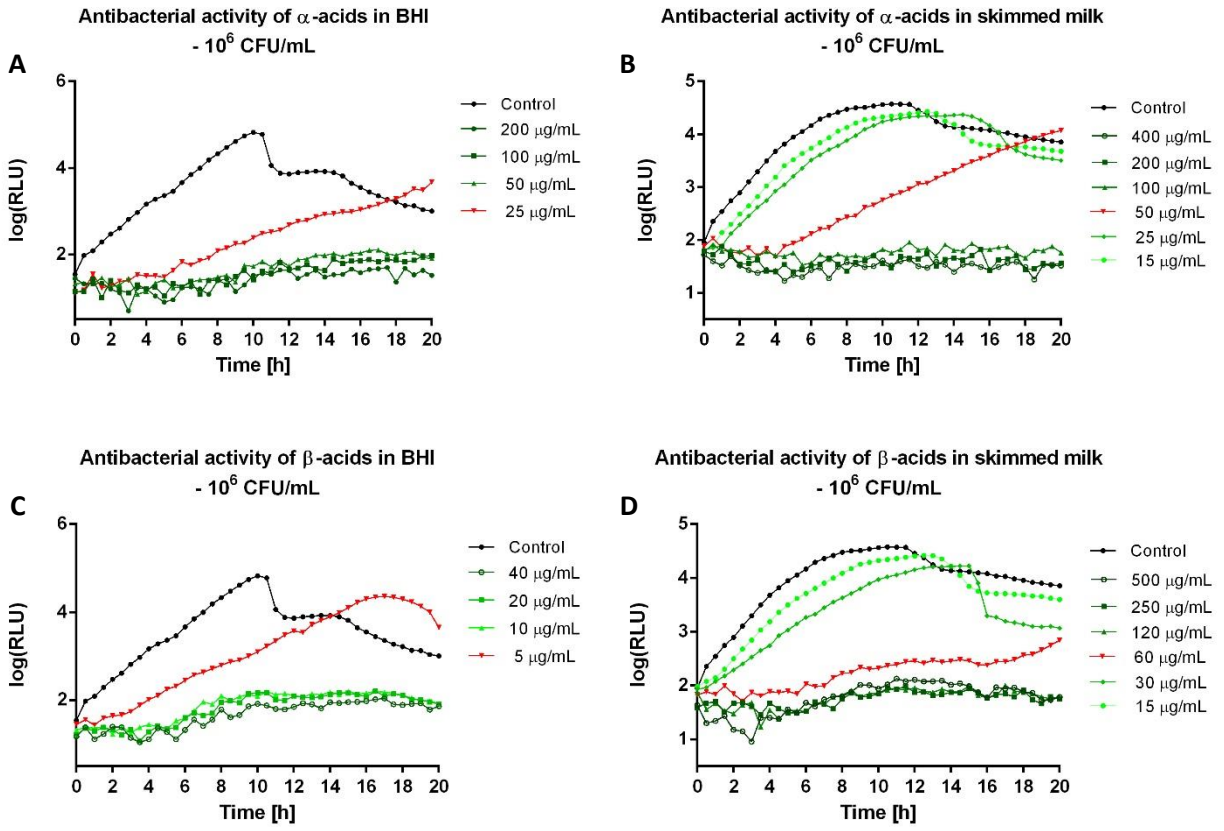


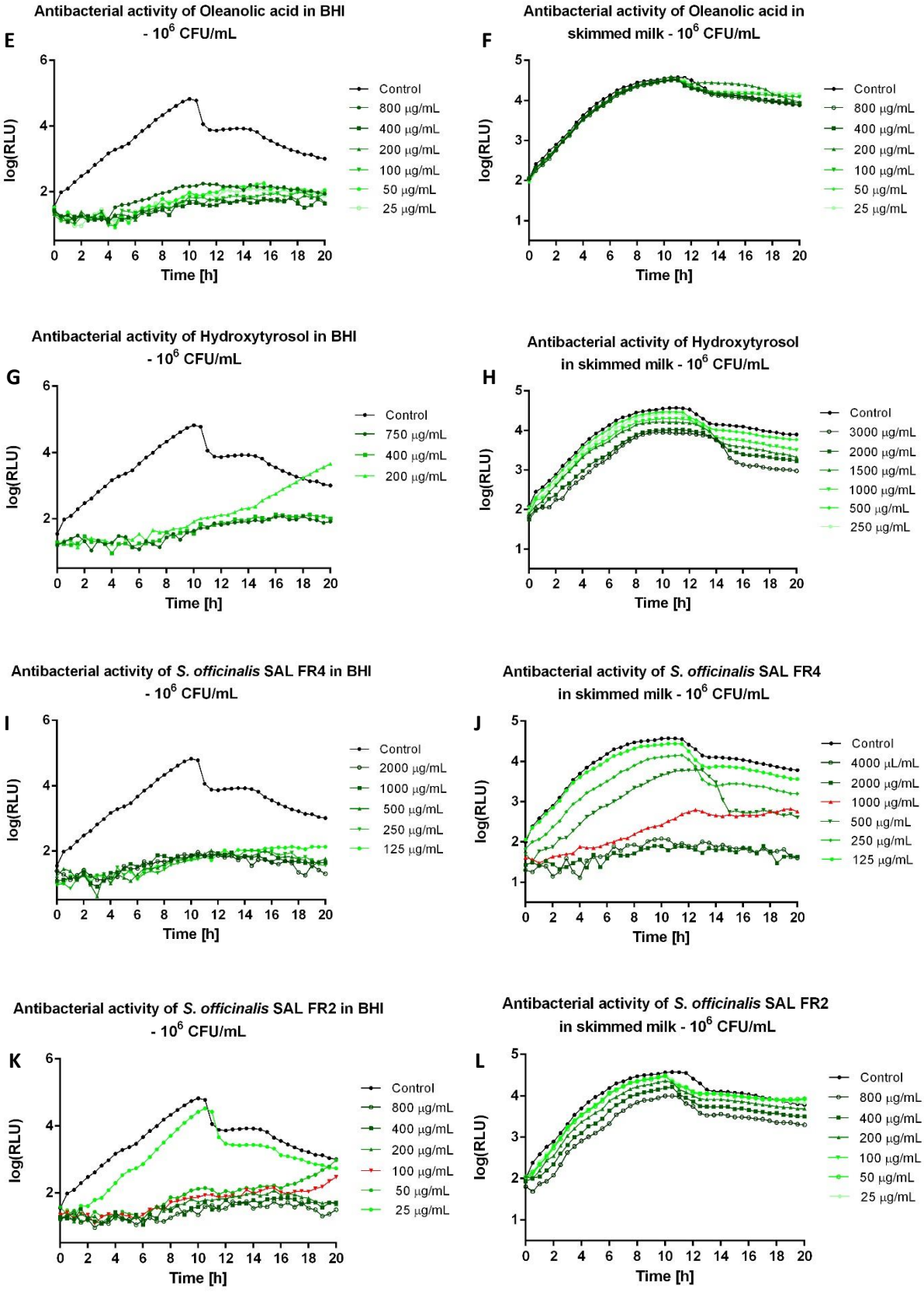
Figure 33. Antibacterial activities of selected plant antimicrobial agents in combination with osmotic stress (3.5% NaCl), acidic stress (pH 5.5) and refrigeration temperature (4°C). MIC – minimal inhibitory concentration. ∞ - no antibacterial activity at the tested concentration. The MICs for each condition are represented as the mean of the MICs of three independent replicates.

4.3.2. Testing of selected natural products in simple food matrices

The *L. monocytogenes* EGDe*lux* strain was used to assess the effects of the plant antimicrobial agents in reconstituted skimmed milk powder (MSK), 20% fat cooking cream and a popular cooking sauce, Béarnaise sauce. Just like *L. monocytogenes* $P_{lmo2230}$*::egfp*, the EGDe*lux* strain did not show any significant differences in growth rate or sensitivity to the plant antimicrobial agents compared to the wild-type strain, suggesting that results can be extrapolated from one strain to another (Table 32).

All selected plant antimicrobial agents showed diminished or even absent antibacterial efficacies when they were analysed in food matrices. Since a decreased activity was expected, all samples were tested at concentrations equal to and higher than their respective MICs in rich media (BHI). Starting with MSK, only SAL FR4 from *S. officinalis* L. and the hops bitter acids (HBAs) from *H. lupulus* L. retained their antibacterial activities. On the contrary, the antibacterial activities of hydroxytyrosol, oleanolic acid, SAL FR2, EU FR2 the *E. globulus* L. and OS FR2 from *O. stamineus* Benth. were almost completely attenuated in MSK (Figure 34). The conclusions of growth inhibition were derived by lack of increase in the log RLU signal readout in comparison to the controls, indicating no cellular replication.




E
Antibacterial activity of Oleanolic acid in BHI - 10^6 CFU/mL
log(RLU)
Time [h]
Control
800 µg/mL
400 µg/mL
200 µg/mL
100 µg/mL
50 µg/mL
25 µg/mL
F
Antibacterial activity of Oleanolic acid in skimmed milk - 10^6 CFU/mL
log(RLU)
Time [h]
Control
800 µg/mL
400 µg/mL
200 µg/mL
100 µg/mL
50 µg/mL
25 µg/mL
G
Antibacterial activity of Hydroxytyrosol in BHI - 10^6 CFU/mL
log(RLU)
Time [h]
Control
750 µg/mL
400 µg/mL
200 µg/mL
H
Antibacterial activity of Hydroxytyrosol in skimmed milk - 10^6 CFU/mL
log(RLU)
Time [h]
Control
3000 µg/mL
2000 µg/mL
1500 µg/mL
1000 µg/mL
500 µg/mL
250 µg/mL
I
Antibacterial activity of S. officinalis SAL FR4 in BHI - 10^6 CFU/mL
log(RLU)
Time [h]
Control
2000 µg/mL
1000 µg/mL
500 µg/mL
250 µg/mL
125 µg/mL
J
Antibacterial activity of S. officinalis SAL FR4 in skimmed milk - 10^6 CFU/mL
log(RLU)
Time [h]
Control
4000 µL/mL
2000 µg/mL
1000 µg/mL
500 µg/mL
250 µg/mL
125 µg/mL
K
Antibacterial activity of S. officinalis SAL FR2 in BHI - 10^6 CFU/mL
log(RLU)
Time [h]
Control
800 µg/mL
400 µg/mL
200 µg/mL
100 µg/mL
50 µg/mL
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L
Antibacterial activity of S. officinalis SAL FR2 in skimmed milk - 10^6 CFU/mL
log(RLU)
Time [h]
Control
800 µg/mL
400 µg/mL
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100 µg/mL
50 µg/mL
25 µg/mL

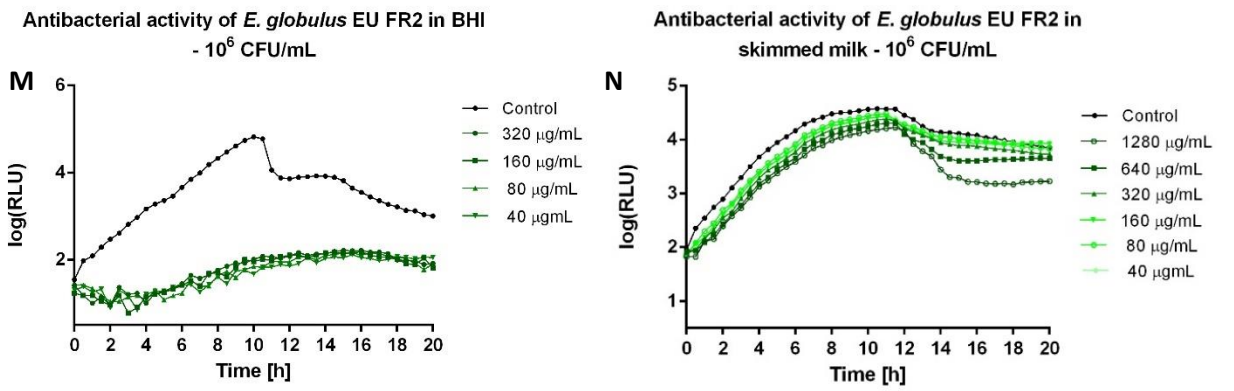


Figure 34. Growth curve of *L. monocytogenes* at different concentrations of plant antimicrobial agents in BHI and skimmed milk (MSK). Hops α-acids in **A)** BHI and **B)** MSK; Hops β-acids in **C)** BHI and **D)** MSK; Oleanolic acid in **E)** BHI and **F)** MSK; Hydroxytyrosol in **G)** BHI and **H)** MSK; SAL FR4 from *S. officinalis* in **I)** BHI and **J)** MSK; SAL FR2 from *S. officinalis* in **K)** BHI and **L)** MSK; and EU FR from *E. globulus* in **M)** BHI and **N)** MSK. An inoculum of 10^6 CFU/mL *L. monocytogenes* EGDe*lux* was introduced in BHI or MSK supplemented with various concentrations of plant antimicrobial agents and luminescence intensity was continuously measured for 20h. Control – *L. monocytogenes* EGDe*lux* in BHI or in MSK. The growth curve of *L. monocytogenes* represented in red indicates the highest concentration of the agent where growth could be be observed during the measurement.

Plotting the log RLU signal against time revealed that at least two-fold and 10-fold higher concentrations of the α-acids and the β-acids respectively, as well as at least 5-fold higher concentrations of SAL FR4 were needed to achieve the growth inhibition properties in MSK previously observed in BHI.

Going forth, the growth dynamics of *L. monocytogenes* in MSK in the presence of the plant antimicrobial agents were established by fitting the experimental data in a primary model (logistic growth model[141]). The following parameters were fitted using Excel Solver and least squares of the error; initial (N_0) and final cell population (N_{max}), the maximum growth rate (μ_{max}) and the duration of the lag phase (λ). A plot of the log RLU signal against time was used for calculating the growth kinetics parameters of the bacteria. Both a growth curve fitting and a time-to-detection approach[142] (fixed at log RLU = 2,4 as the time point threshold) were used to estimate the growth parameters. Since the lag phase duration could not be accurately predicted by these measurements, the maximum growth rate was used as the principal growth parameter to demonstrate a change in behaviour or response to the antibacterial agent. With that objective, the growth rate values derived from the primary model fittings were used to construct a dose-response curve and to establish a relationship between the growth rate changes with respect to the concentration of the plant antibacterial agent.

Fitting the experimental data in a primary model confirmed that only the α-acids, the β-acids and SAL FR4 retained their growth inhibition properties in MSK, since no growth rate changes were observed for the rest of the plant agents when compared to a control. As for the active compounds, an excellent dose-response relationship with a decrease in the maximum specific growth rate with increasing plant

agent concentration was observed (Table 34 and Figure 35). The specific growth rate values at each plant compound concentration generated by the both log RLU growth curve fitting and the TTD fitting showed excellent goodness of fit (as shown by R^2 and RMSE of model fittings), and both were used to plot the dose-response curves shown in Figure 35 (error bars). The growth rates in the dose-response figures were standardized to 1 (where 1 equalled the optimal growth rate in food matrix without the plant agent). This way, different food matrices and TTD to logistic growth rate values could be directly compared.

Table 34. Growth kinetics of *L. monocytogenes* in skimmed milk (MKS) in various concentrations of hops bitter acids (HBAs) and SAL FR4. Maximum growth rate (μ_{max}) and the goodness of fit expressed through the R^2 coefficient and the root mean square error (RMSE) were calculated with non-linear regression fitting. Data showing $\mu_{max} \leq 0,05$ were considered as no growth.

Plant agent	Concentration	μ_{max} (log RLU/h)	R^2	RMSE
Hops α-acids	0 ppm	0.862	0.99	0.0380
	15 ppm	0.744	0.99	0.0360
	25 ppm	0.561	0.99	0.0340
	50 ppm	0.166	0.99	0.0480
	100 ppm	0.026	-	-
	200 ppm	0.001	-	-
	400 ppm	0	-	-
Hops β-acids	0 ppm	0.869	0.99	0.0470
	15 ppm	0.744	0.99	0.0350
	30 ppm	0.561	0.99	0.0170
	60 ppm	0.166	0.87	0.1730
	120 ppm	0.026	-	-
	250 ppm	0.016	-	-
	500 ppm	0	-	-
S. officinalis SAL FR4	0 ppm	0.859	0.99	0.0370
	250 ppm	0.803	0.99	0.0270
	500 ppm	0.710	0.99	0.0230
	1000 ppm	0.677	0.99	0.0300
	2000 ppm	0.564	0.99	0.0390
	4000 ppm	0.217	0.93	0.0800
	8000 ppm	0.813	-	-

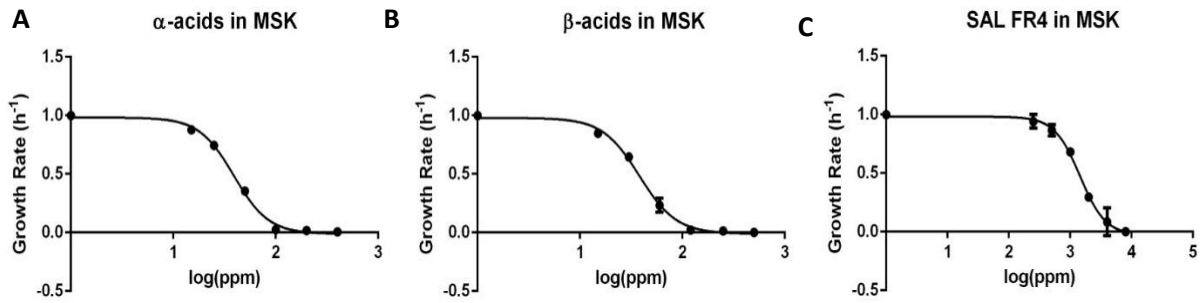


Figure 35. Hill Plot curves showing the dose-response relationship of α-acids, β-acids and SAL FR4 on the growth of *L. monocytogenes* in skimmed milk (MSK). Concentrations of the plant antibacterial compounds were included at six two-fold serial

dilutions, starting at 50 – 100 × MIC measured in BHI. Luminescence intensity was measured for each antibacterial concentration, in both the presence and the absence of the plant antimicrobial agents and the growth rate of the bacteria was calculated in each condition. Triplicate values for each concentration were averaged and then standardized to 1, where 1 equalled the growth levels without the added antimicrobial. The resulting standardized data were analysed in GraphPad using the log(inhibitor) vs. response -- Variable slope (four parameters) non-linear fitting to establish the Hill Plot dose-response of growth in the presence or absence of the antimicrobial agent. Dose-response relationships of *L. monocytogenes* growth in skimmed milk (MSK) were established for MSK supplemented with **A)** Hops α-acids; **B)** Hops β-acids and **C)** SAL FR4 from *S. officinalis* L.

The goodness of fit was excellent in all three secondary models as indicated by the R^2 coefficient and the small sum of squares values (Table 35). The predicted MICs and IC50 values were calculated from the linear regression and Hill Plot dose-response curves (Table 35). Complete growth inhibition was only seen at concentrations 8-fold higher for the α-acids, 50-fold higher for the β-acids and 60-fold higher for SAL FR4 than the MICs of the agents in laboratory media. Nonetheless, the α- and β-acids were needed in much lower concentrations to inhibit the growth of *L. monocytogenes* in MSK in comparison to SAL FR2.

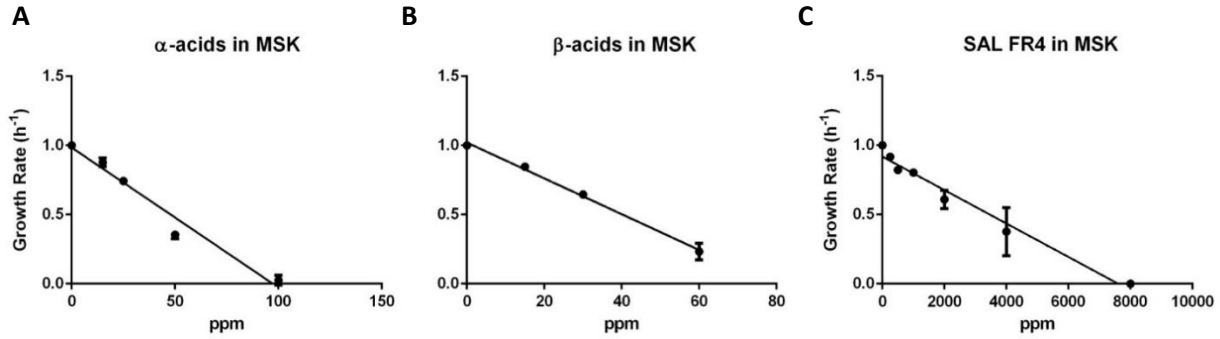


Figure 36. Linear regression curves showing the dose-response relationship of α-acids, β-acids and SAL FR4 on the growth of *L. monocytogenes*. Triplicate values for each concentration using the log RLU growth rate and the time-to-detection growth rate were averaged and then standardized to 1, where 1 equalled the growth levels without the added antimicrobial. The resulting standardized data were analysed in GraphPad using the linear regression fitting to establish the dose-response of growth in the presence or absence of the plant antimicrobial agent. Dose-response relationships of *L. monocytogenes* growth in skimmed milk (MSK) were established for MSK supplemented with **A)** Hops α-acids; **B)** Hops β-acids and **C)** SAL FR4 from *S. officinalis* L.

Table 35. Predicted values for the MIC and IC50 for the growth of *L. monocytogenes* in the presence of α-acids, β-acids and SAL FR4 in skimmed milk (MSK). The IC50 was predicted from the Hill Plot curves, while the MIC value from linear regression fitting. The goodness of fit of both models is presented by the R^2 coefficient, the absolute sum of squares (SS) and the standard deviation of the residuals (Sy.x).

Parameter	α-acids (ppm)	β-acids (ppm)	SAL FR4 (ppm)
		Hill Plot	
IC50	39.1	38.07	1441
log IC50	1.59	1.58	3.16
R^2	0.99	0.99	0.99
Absolute SS	0.0117	0.0105	0.0235

Parameter	α-acids (ppm)	β-acids (ppm)	SAL FR4 (ppm)
		Linear regression	
MIC	97.3	78.9	7609
R^2	0.97	0.99	0.95
Sy.x	0.075	0.032	0.079

Next, the antilisterial abilities of the selected plant antimicrobial agents (Table 31) were evaluated in cooking cream. Hydroxytyrosol, oleanolic acid, SAL FR2, EU FR2 the *E. globulus* L. and OS FR2 from *O. stamineus* Benth. were once again not active at the tested concentrations (Figure 34) in the food matrix. Additionally, both the β-acids and SAL FR4 lost their antibacterial effects in cooking cream at the concentrations tested, up to 100 ppm for the β-acids and up to 8 mg/mL for SAL FR4. On the other hand, the α-acids maintained their growth inhibition properties in 20% fat cooking cream. Unfortunately, fewer concentration points were used in measuring the response of the bacteria in this food matrix, so the goodness of fit of the model was of less confidence. A linear regression fitting predicted the MIC of the α-acids in cooking cream at 267 ppm with a coefficient R^2=0.99, and the IC50 value could be predicted by fitting to a Hill plot and was estimated at 63.4 ppm, with a R^2=0.99.

Ultimately, several plant agents that showed marked differences in antibacterial activity in BHI at pH 5.5 compared to pH 7, such as the α- and β-acids, and EU FR2 were tested in Béarnaise sauce. EU FR2 did not demonstrate any growth inhibition properties in this food matrix, while the α- and β-acids showed a very good dose-response relationship, both with an increase in the lag phase duration and a decrease in the maximum specific growth rate. The responses of the bacteria due to increase in concentration of α-acids was different than the one observed in MSK, and the increase in concentration of the plant agent did not show a linear increase in effectiveness, possibly due to interactions with the matrix.

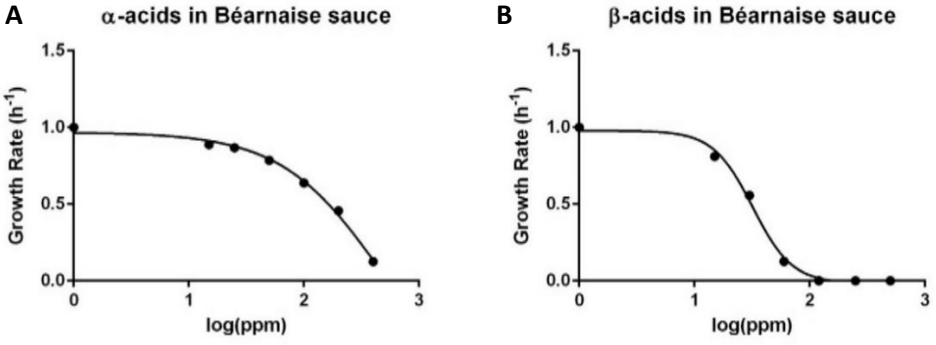
Table 36. Growth kinetics of *L. monocytogenes* in Béarnaise sauce in various concentrations of hops bitter acids (HBAs). Lag phase duration, initial (N_0) and final cell count (N_{max}), maximum growth rate and standard errors of fit were calculated with non-linear regression fitting. initial (N_0) and final cell population (N_{max}); maximum growth rate (μ_{max}); lag phase duration (λ).

Plant agent	Concentration	N_0 (log RLU/mL)	N_{max} (log RLU/mL)	λ (h)	μ_{max} (log RLU/h)	R^2	RMSE
Hops α-acids	0 ppm	1.705	3.530	1.3	0.447	0.99	0.0660
	15 ppm	1.702	3.466	1.4	0.397	0.99	0.0610
	25 ppm	1.736	3.378	2.2	0.388	0.99	0.0540
	50 ppm	1.666	3.326	2.1	0.351	0.99	0.0380
	100 ppm	1.775	3.217	2.5	0.285	0.99	0.0290
	200 ppm	1.666	3.033	3.2	0.204	0.99	0.0340
	400 ppm	1.589	2.285	4.0	0.056	0.78	0.0540

Plant agent	Concentration	N_0 (log RLU/mL)	N_{max} (log RLU/mL)	λ (h)	μ_{max} (log RLU/h)	R^2	RMSE
Hops β-acids	0 ppm	1.676	3.623	0	0.411	0.99	0.0530
	15 ppm	1.673	3.332	1.4	0.333	0.99	0.0370
	30 ppm	1.587	2.586	2.1	0.229	0.99	0.0290
	60 ppm	1.660	-	2.1	0.052	0.87	0.0710
	120 ppm	1.640	-	2.5	0	-	-
	250 ppm	1.377	-	3.2	0	-	-
	500 ppm	1.305	-	4.0	0	-	-

The MIC for the β-acids was predicted at 68.6 ppm, and for the α-acid at 440 ppm, while the IC50 for the β-acids was estimated at 32 ppm. Because of the unusual response of the bacteria to the increasing concentration of α-acids, the IC50 value was difficult to predict, and the 95% confidence interval placed it between 203 – 1078 ppm. However, the goodness of fit of the models was excellent, with R^2=0.99 in both cases (Figure 37). The Hill equation (Hill Plot) was used to fit the responses of the bacteria not only so the IC50 could be determined, but because it provides a potential to extrapolate the degree of lethality at higher concentrations, i.e., to predict inactivation, as well as growth (shelf-life) to control food safety.

Overall, it could be noticed that the most significant loss in activity of the plant agents happened in 20% fat cooking cream, possibly due to partitioning of the non-polar molecules in the fat of the matrix where it loses contact with the bacterial cells that usually grow in the aqueous phase. The results of the analyses conducted in Béarnaise sauce were similar to the results in MSK, and showed that both the α- and β-acids retained their growth inhibition properties in a more complex food matrix. While the α-acids were needed in a higher concentration to inhibit the growth of the bacteria in comparison to MSK, the β-acids had stronger antibacterial activities in Béarnaise sauce. An explanation for this, could be due to the moderately acidic pH of the sauce that acted as an additional hurdle in inhibiting the growth of *L. monocytogenes*.



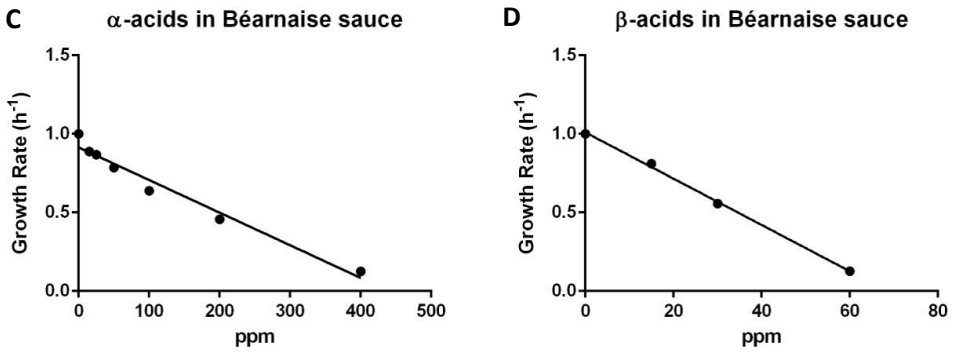


Figure 37. Hill Plot and Linear regression curves showing the dose-response relationship of α-acids and β-acids on the growth of *L. monocytogenes* in Béarnaise sauce. Concentrations of the plant antimicrobial compounds were included at seven twofold serial dilutions, starting at 50 – 100 × the MIC measured in BHI. Luminescence intensity was measured for each concentration, in both the presence and the absence of the plant antimicrobial agents and the growth rate of the bacteria was calculated in each condition. Triplicate values for each concentration were averaged and then standardized to 1, where 1 equalled the growth levels without the added antimicrobial. The resulting normalized data were analysed in GraphPad using the log(inhibitor) vs. response -- Variable slope (four parameters) non-linear fitting to plot the Hill Plot and the linear regression curves. Hil Plot curve for the dose-response relationship of *L. monocytogenes* growth in Béarnaise sauce supplemented with **A)** Hops α-acids; **B)** Hops β-acids; and linear regression cruve of *L. monocytogenes* growth in Béarnaise sauce supplemented with **C)** Hops α-acids; **D)** Hops β-acids.

In a final exercise, predictions on the antibacterial effects of the SAL FR4 were modelled using the parameters and equations based on the Gamma concept found in the Food Spoilage and Safety Predictor (FSSP) software[143], to estimate the effect of this plant antimicrobial agent on the shelf-life and the growth of *L. monocytogenes* in seafood under constant or fluctuating temperature conditions. The model predicted that at a SAL FR4 concentration of 3000 ppm, storage temperature of 10°C, pH 7 and 1% NaCl concentration, the plant antimicrobial agent would be able to preserve the seafood product for 12 days before hazardous levels of *L. monocytogenes* are reached[144], compared to 6 days of storage in the same conditions without the agent. In other words, if there is 1 CFU/g of *L. monocytogenes* initially in the product, the hazard limit of 100 CFU/g will be reached in 6 days without the plant antimicrobial, and the addition of SAL FR4 at 3000 ppm would slow the growth of *L. monocytogenes* and the hazardous levels of 100 CFU/g will be reached in 12 days. The model predicted the MIC for SAL FR4 in seafood at 6000 ppm. This example clearly indicated how by using predictive modelling, a realistic antimicrobial concentration as one hurdle in a simulated food product, could double the safe and useable shelf-life.

μ_{max} (1/h)	**0,0152**
t_{lag} (days)	4,63
time to hazardous increase (TTH) (days) **without** lag	12,59
TTH (days), based on with lag	17,22

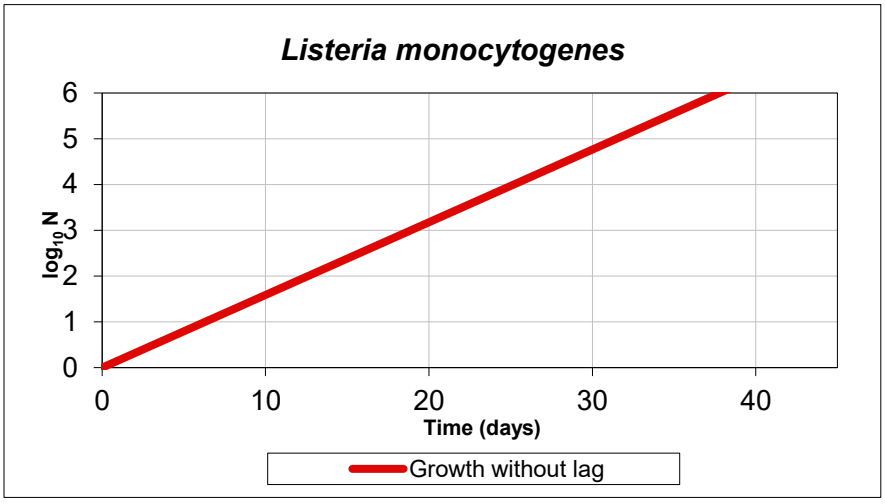
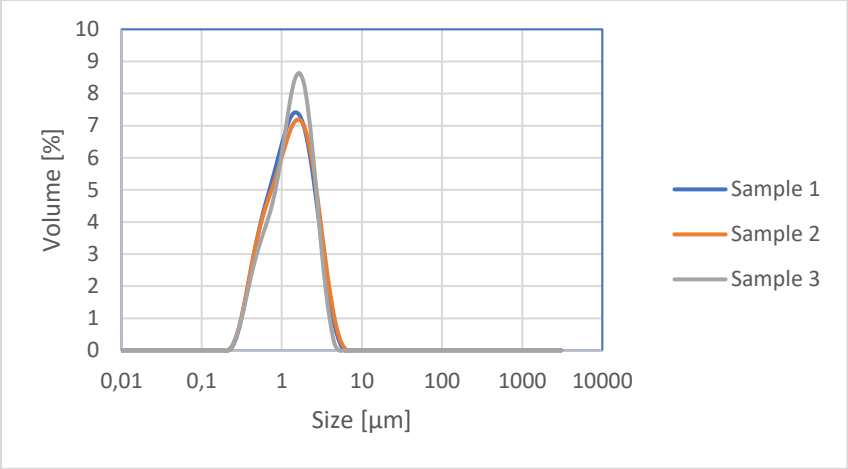


Figure 38. Estimation of growth inhibition effects of SAL FR4 in seafood. The growth rate of *L. monocytogenes* in the presence of SAL FR4 was used to make predictions on the growth of this pathogen in seafood is applied at a concentration of 3000 ppm. The impact of the plant antimicrobial on shelf-life of the seafood products was calculated using the Food Spoilage and Safety Predictor (FSSP) based on the gamma model concept. Time to hazardous increase was calculated with lag.

4.3.3. Testing of selected natural products in a laboratory produced basic food matrix

The objective of this part of the study was to assess the antimicrobial activities of three plant antibacterial agents in a basic food recipe named KonoMatrix (KM) (Section 3.16.2 – Materials & Methods) containing only essential food ingredients – water, oil, salt, glucose and whey protein (source of essential amino acids and vitamins for the fastidious microorganism *L. monocytogenes*). The chosen plant agents were: a *S. officinalis* L. extract – PS-024, oleanolic acid and β-hops bitter acids isolated from *H. lupulus* L that were added to the KM at three concentrations each: 50 µg/mL, 100 µg/mL and 500 µg/mL for the *S. officinalis* extract; 10 µg/mL, 100 µg/mL and 500 µg/mL for oleanolic acid and 10 µg/mL, 50 µg/mL and 100 µg/mL for β-hops bitter acids. A sample was taken from several random KM samples for determining the particle size, water activity and total solids in the food matrices. The results are presented in Figure 39. The pH of the samples was in the range between 6.30 – 6.35.



Analysis	Sample 1	Sample 2	Sample 3	**Average**
Particle size Dv(50)	1,37 µm	1,41 µm	1,45 µm	1,41 µm
Water activity	0,989	0,984	0,988	0,987
Total solids (dry matter)	12,6 g/100g	16,7 g/100g	16,6 g/100g	15,3 g/100g

Figure 39. Particle size distribution, water activity and total solids composition of KonoMatrix (KM).

The antibacterial properties of the extracts as a part of KM were assessed by two methods – measuring the relative luminescence units (RLU) produced by *L. monocytogenes* EGDe*lux* in a multimode plate reader and by counting the colony forming units (CFU)/mL in plate count growth curves. The study was divided in two stages. In stage one, the antibacterial properties of all three natural products were assessed at the natural pH of KM – pH 6.3. The general results of stage one indicated that extract PS-024 was the most active plant agent for inhibiting the growth of *L. monocytogenes* EGD-e amongst the three, so the matrices containing this extract were selected for further antibacterial food hurdle studies in combination with a sublethal stress - acidic pH. To this end, a series of PS-024 containing solutions with a pH range from pH 4.0 to pH 7.0 with 0.25 increments were produced. The antibacterial properties of all samples were first assessed by luminescence measurements, and only the pH solutions of the highest concentration of the *S. officinalis* extract PS-024 – 500µg/mL were tested by the plate count method.

Results Stage 1– All extracts at pH natural

The luminescence-based assays of all the extracts at the natural pH of KM clearly indicate that in the KM 5% food matrices containing the *S. officinalis* extract at 500 µg/mL and β-hops bitter acids at 100 µg/mL the growth of *L. monocytogenes* EGDe*lux* is completely inhibited as there is no luminescence signal detected above the basal level of 50-100 RLUs. In the food matrix containing β-hops bitter acids at 50 µg/mL the growth of *L. monocytogenes* is partially inhibited, as the intensity of the signal and area under

the curve are much lower than in Control KM 5%. All food matrices containing oleanolic acid did not show antibacterial activities against EGDe*lux* at the tested concentrations. The differences in oil concentration did not cause any observable differences in the antibacterial activities of the natural products (results not presented). Unfortunately, even though all KMs were pasteurized often by the low temperature long-time method, the KM was not sterile and present native microflora was detected from the beginning of the study. We believe that the double-peak shape of the luminescence curve, that was not observed in any other food matrix, was a consequence of *L. monocytogenes* growing in competition with the native microflora of KM.

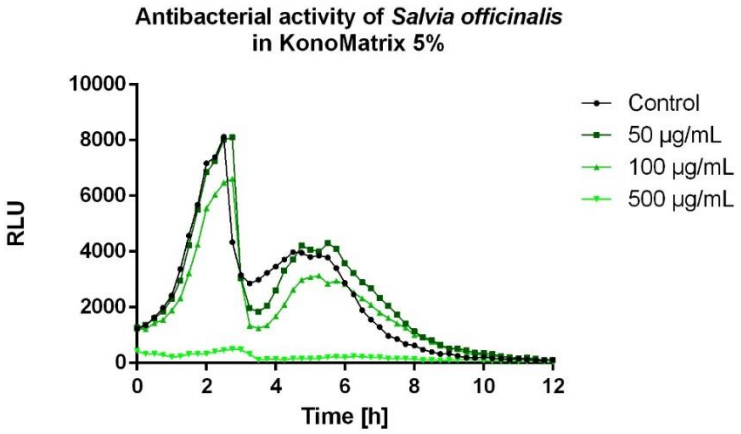


Figure 40. Growth curve of *L. monocytogenes* in KonoMatrix 5% with three concentrations of *S. officinalis* extract, 50 µg/mL, 100 µg/mL and 500 µg/mL.

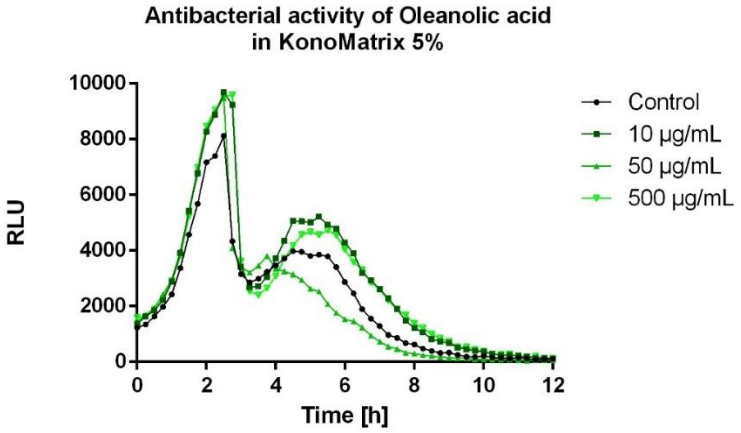


Figure 41. Growth curve of *L. monocytogenes* in KonoMatrix 5% with three concentrations of oleanolic acid, 10 µg/mL, 100 µg/mL and 500 µg/mL.

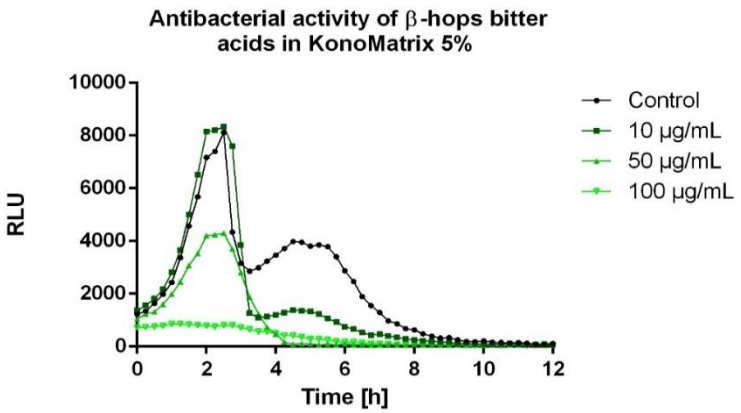


Figure 42. Gowth curve of *L. monocytogenes* in KonoMatrix 5% with three concentrations of β-hops bitter acids, 10 µg/mL, 50 µg/mL and 100 µg/mL.

In plate count experiments only the food matrices with the highest concentrations of the natural products were tested. Matrices of both 5% and 8.3% oil concentrations were assayed. The DMFit software, based on the Baranyi and Roberts Full Growth Model[126] was used to fit the data (Table 37). The initial bacterial population densities were $2.65x10^6$ CFU/mL and $1.35x10^5$ CFU/mL. Overall, the results showed reduced maximum growth rate in all the matrices that contained the plant agents, with reduction values from 0.009 log CFU/h to 0.094 log CFU/h at the higher initial inoculum concentrations, and 0.042 log CFU/h to 0.074 log CFU/h for lower initial population densities. The average lag-phase duration was influenced only by the *S. officinalis* extract, which extended to lag phase to 1.8 and 1.6 h from 0.9 and 0.7 h, respectfully. In some cases, the lag phase duration could not be estimated by the model. The standard error of the fits (SE) was calculated between 0.069 – 0.109. These findings indicate that the selected plant antibacterial agents, particularly the *S. officinalis* extract and the hops β acids, have promising bacteriostatic effects against *L. monocytogenes* in food systems. These effects are more evident when the initial cell population is lower, reflecting the general observations, that greater effects are seen with smaller inoculum size[145].

Table 37. Growth kinetics of *L. monocytogenes* in KonoMatrix with various plant antimicrobial agents. Lag phase duration, initial (N_0) and final cell count (N_{max}), maximum growth rate and standars errors of fit were calculated with the Baranyi and Roberts Growth Fit model (DMFit). (-) Lag phase duration could not be predicted.

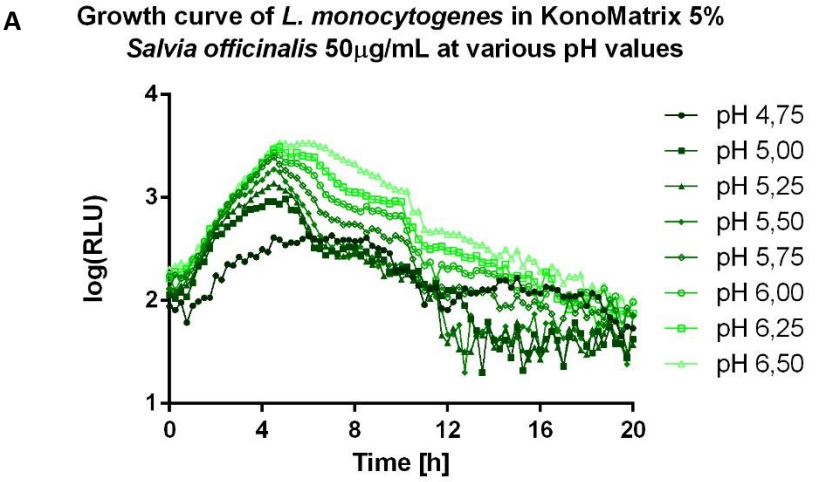
Plant agent	Concentration	N_0 (log CFU/mL)	N_{max} (log CFU/mL)	Lag phase	μ_{max} (log CFU/h)	R^2
Control	0 µg/mL	6.43	7.69	0.952	0.342	0.98
S. officinalis	100 µg/mL	6.23	7.53	-	0.248	0.98
S. officinalis	500 µg/mL	6.44	7.22	1.805	0.333	0.96
Oleanolic acid	500 µg/mL	6.40	7.84	0.833	0.332	0.96

Plant agent	Concentration	N_0 (log CFU/mL)	N_{max} (log CFU/mL)	Lag phase	μ_{max} (log CFU/h)	R^2
Hops β-acids	100 µg/mL	6.43	7.72	0.925	0.311	0.98
Control	0 µg/mL	5.10	8.67	0.696	0.301	0.99
S. officinalis	500 µg/mL	5.15	7.78	1.572	0.238	0.99
Oleanolic acid	500 µg/mL	5.13	8.47	-	0.259	0.99
Hops β-acids	100 µg/mL	5.12	7.73	0.800	0.227	0.99

Results Stage 2 – pH modified food matrices containing S. officinalis

After preparing the pH modified samples, the growth of *L. monocytogenes* in the control food matrix without antimicrobials was first examined. The initial bacterial inoculum concentration was approximately 10^7 CFU/mL. *L. monocytogenes* has the ability to survive and reproduce in acidic pH, with a minimum pH tolerance value of around pH 4.5 – 4.6 if not previously exposed to milder pH stresses in order to build tolerance to severe acidic conditions. This was confirmed in the study, as no growth was detected in the food matrix with a pH 4.5. Therefore, this value was excluded from further tests.

In the luminescence studies, it was observed that there was a gradual loss of intensity in the luminescence signal as the pH value decreased. This is believed to be a consequence of a slower growth rate of the bacteria as the pH value drops from the optimal and is consistent with OD_{600}/RLU growth curves in BHI. Total growth inhibition, interpreted by no increase in luminescence intensity, could be observed only for the *S. officinalis* extract at a concentration of 500 µg/mL at all pH values, while this complete lack of increase in signal was not observed for the other concentrations tested, even though the signal intensity peak decreased as the pH lowered. Decreased growth rate was also observed in the food matrix with 100 µg/mL of *S. officinalis* extract with an initial bacterial inoculum concentration of 10^6 CFU/mL.



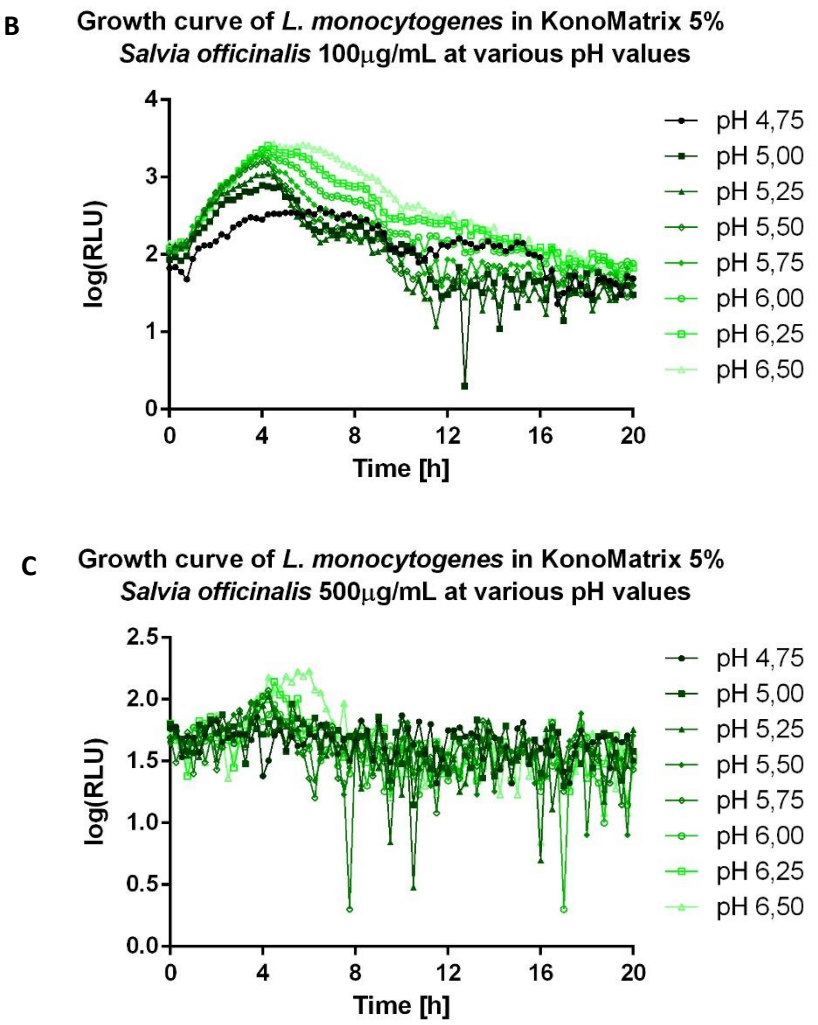


Figure 43. Growth curve of *L. monocytogenes* in KonoMatrix 5% with various concentration of *S. officinalis*. A) 50 µg/mL; **B)** 100 µg/mL; and **C)** 500 µg/mL at a pH range between 4.75 and 6.50.

It is important to stress that at this stage, the luminescence signal was severely masked by the KM and probably the overgrown native microflora that competed for the nutrients with *L. monocytogenes*. Furthermore, the initial bacterial population density was quite high in the assays, 10^7 and 10^6 CFU/mL, limiting the replication to the first 5 hours after which the cells entered in stationary phase. The replication of *L. monocytogenes* is self-limiting at CFU/mL values higher than 10^9-10^{10} CFU/mL.

The effects of pH were further investigated by plate counts at three key pH values, pH 5.0, pH 5.5 and pH 6.0. The results agreed with the previous observations that the *S. officinalis* extract has a negative effect on the growth of *L. monocytogenes* at concentrations of 500 µg/mL (Table 38). Furthermore, at pH values below 6, the extract's exceptional antibacterial activities kill *L. monocytogenes* bacterial cells and not only inhibit their growth. This effect is especially evident at pH 5.0, since the final bacterial

concentration at the 24-hour end point was reduced almost one log compared to the initial bacterial population inoculum of $1.1x10^6$ CFU/mL. Because of the final cell count being lower than the initial cell count, indicating bactericidal activity of the extract, the Baranyi and Roberts model could not correctly fit the data. Hence, data showing less than 0.25 log CFU increase were considered to show no growth.

Table 38. Growth kinetics of *L. monocytogenes* in KonoMatrix with 500 µg/mL of *S. officinalis* extract at various pH values. Lag phase duration, initial (N_0) and final cell count (N_{max}), maximum growth rate and standars errors of fit were calculated with the Baranyi and Roberts Growth Fit model (DMFit). (-) Lag phase duration was not detected.

pH	Plant agent	N_0 (log CFU/mL)	N_{max} (log CFU/mL)	Lag phase	μ_{max} (log CFU/h)	R^2
5,0	Control	6.07	7.21	-	0.058	0.99
	S. officinalis	6.14	-	-	no growth	-
5,5	Control	6.03	8.48	1.059	0.259	0.99
	S. officinalis	6.01	6.46	-	0.162	0.88
6,0	Control	6.00	8.70	0.354	0.284	0.99
	S. officinalis	6.05	7.02	0.952	0.234	0.99

At pH 6.0 the lag phase duration could be predicted by the model, pointing out an increase in the lag phase duration by the effects of the extract, in addition to the growth rate decrease. Altogether, the results of the experiments conducted in the KM food system indicated that the *S. officinalis* extract at a concentration of 500 µg/mL could significantly inhibit the growth of *L. monocytogenes* in this food recipe KM, while in combination with acidic pH the antibacterial effects of the extract were not only inhibitory, but bactericidal as well. The hops β-acids at 100 µg/mL indicated growth inhibition properties as well, that were more obvious when the initial cell concentration was lower.

4.3.4. Antibacterial activities of selected plant compounds on other food pathogens

Due to greater consumer awareness and concerns regarding synthetic chemical additives, foods preserved with natural products have gained the interest of the general public. This has led researchers and food processors to embark on a quest for natural food additives with antimicrobial properties. While *L. monocytogenes* is one of the most resilient food pathogens with paramount importance to food safety issues, an ideal natural food preservative should have a broad spectrum of antimicrobial activity against a variety of the most common food pathogens and food spoilage bacteria. According to the report of WHO in 2015, almost 1 in 10 people fall ill worldwide after eating contaminated food, which accounts for approximately 600 million cases of foodborne diseases[4]. Preventing foodborne pathogens *(Clostridium*

botulinum, *Staphylococcus aureus*, *Campylobacter jejuni*, *Bacillus cereus*, *Listeria monocytogenes*, *Cryptosporidium*, *Escherichia coli* O157:H7, etc.) to cause intoxication and food-borne illnesses through the consumption of contaminated foods are one of the most challenging issues of the food industry and the food regulatory agencies.

In this regard, the antibacterial activities of ten of the most effective plant extracts or purified compounds against *L. monocytogenes* were tested using several other significant food pathogens, pathogen indicators or spoilage microbes, detailed in Table 1 in Material & Methods, as well as Table 38 below. Since the antibacterial efficacies of the selected plant agents were unknown against other bacterial strains, the screening was conducted at two stages. First, an initial assessment was performed using higher concentrations of the plant samples, up to 2 mg/mL, then a secondary step for all active samples included serial dilution tests. MIC determination was concluded by monitoring the changes in optical density after 24 hours. The results of all assays are grouped in Table 38.

The Gram-negative bacteria, *E. coli* and S. Typhimurim, were more resistant to the antibacterial effects of the investigated plant agents, and only two were able to retain their growth inhibiting potential: the phenolic compound hydroxytyrosol isolated from *O. europaea* L., and the punicalagins rich extract from *P. granatum* L, PS-011. Considering hydroxytyrosol, the MICs were even lower than the MICs observed for *L. monocytogenes*, 125 µg/mL for both Gram-negative bacteria, while PS-011 had similar antibacterial activities as the ones recorded for *L. monocytogenes,* 0,5 – 1 mg/mL. These results agree with numerous studies that have observed that Gram-negative bacteria are more resilient to the effects of various antimicrobials, including both antibiotics and natural antimicrobial agents such as essential oils, phenols, flavonoids, tannins etc[1,2,3].

On the other hand, it was interesting to discover that most of the plant agents used in this part of the study maintained their antibacterial activities against the other Gram-positive pathogens, with only a few agents not active against certain Gram-positive strains. *B. subtilis* and *B. cereus* had similar MICs to the ones observed for *L. monocytogenes*, while *S. aureus* was even more sensitive to the effects of all plant agents with the exception of oleanolic acid. Unfortunately, oleanolic acid also became ineffective when it was applied to a three-strain cocktail of *L. monocytogenes*, along with *S. officinalis* FR2. The *V. vinifera* L. extract was revealed as the least effective agent, showing activity only towards *B. cereus*. Similar observations were noted for *O. stamineus* Benth. OS FR2 that remained active only against *S. aureus*.

Table 39. Antibacterial properties of selected plant antimicrobial agents against various food pathogens. The antibacterial properties of the best plant antimicrobial agents were evaluated against several Gram-positive and Gram-negative food pathogens. The MICs of the plant agents were determined by broth serial dilution against exponential growth-phase cells at 10^6 CFU/mL inoculum concentration. 1 mg/mL was used as a staring concentration. (-) no activity at the tested concentration. MICs below 50 µg/mL are highlighted in green; between 50-100 µg/mL in yellow; between 100-250 µg/mL in orange; and above 250 µg/mL in red. *L. mono – L. monocytogenes*.

PLANT ORIGIN	FRACTION / ISOLATE	*E. coli* MG1655	*E. coli* 3 strain cocktail	*S. enterica* 14028s	*S. entérica* 3 strain cocktail	*B. subtilis* BG214	*B. subtilis* 168 wt	*S. aureus*	*S. aureus* 3 strain cocktail	*B. cereus*	*L. mono* 3 strain cocktail	*L. mono EGD-e*
Humulus lupulus	α - acids	-	-	-	-	12,5 µg/mL	15,6 µg/mL	25 µg/mL	31,3 µg/mL	7,8 µg/mL	31,3 µg/mL	35 µg/mL
	β - acids	-	-	-	-	6,25 µg/mL	15,6 µg/mL	6,25 µg/mL	15,6 µg/mL	62,5 µg/mL	15,6 µg/mL	5 µg/mL
Olea europaea	Olenolic acid	-	-	-	-	50 µg/mL	-	-	-	-	-	5-10 µg/mL
	Hydroxytyrosol	125 µg/mL	125 µg/mL	125 µg/mL	125 µg/mL	1000 µg/mL	1000 µg/mL	125 µg/mL	62,5 µg/mL	500 µg/mL	250 µg/mL	125 µg/mL
Salvia officinalis	Extract PS-024	-	-	-	-	100 µg/mL	500 µg/mL	250 µg/mL	250 µg/mL	250 µg/mL	1000 µg/mL	250 µg/mL
	SAL FR2	-	-	-	-	110,6 µg/mL	/	553 µg/mL	553 µg/mL	-	-	30 µg/mL
	SAL FR4	-	-	-	-	33 µg/mL	125-250 µg/mL	33 µg/mL	125-250 µg/mL	62-125 µg/mL	125-250 µg/mL	125 µg/mL
Orthosiphon stamineus	OS FR2	-	-	-	-	-	-	200 µg/mL	200 µg/mL	-	-	400 µg/mL
Eucalyptus globulus	EU FR2	-	-	-	-	10 µg/mL	10 µg/mL	10 µg/mL	200 µg/mL	50-100 µg/mL	13 µg/mL	25 µg/mL
Vitis Vinifera	Extract PS-030	-	-	-	-	-	-	-	-	62-75 µg/mL	-	250 µg/mL
Punica granatum	Extract PS-011	1000 µg/mL	500-1000 mg/mL	1000 µg/mL	1000 µg/mL	1000 µg/mL	500 µg/mL	125 µg/mL	125 µg/mL	1000 µg/mL	500 µg/mL	1000 µg/mL

DISCUSSION

Foodborne disease is a global issue with significant impact on human health. In response to the dynamic changes in current consumer demands for more high-quality foods with fresh-like attributes[119], improvements in conventionally used preservation techniques must be implemented. At the same time, it remains crucially important that the food product is microbiologically safe. Microbial growth in foods poses a potential health hazard as a result of the presence of foodborne pathogens or microbial toxins, but is also a source of economic loss as a result of spoilage. Several foodborne bacterial pathogens have emerged as a source of concern regarding the safety of foods, one of which, *L. monocytogenes*, a resilient bacterium, able to adapt to and grow in foods with high acidity and high salinity, to growth and proliferate under refrigeration temperatures and to resist technological factors by forming biofilms.

Traditional medicinal plants have a long history of use in the treatment of various human illnesses and infectious diseases. Plants synthesize a variety of secondary metabolites with diverse bioactive properties, some known to offer protection against a wide range of pathogenic microorganisms, either by inhibiting virulence factors or by targeting microbial cells. Various research studies have demonstrated the antimicrobial effects of plant compounds against pathogenic and spoilage bacteria and fungi, opening the possibility of introducing plant extracts and plant-derived phytochemicals as food antimicrobials. The revision of traditional practices paired with scientific evidence may provide a solid background on the use of plant antimicrobials and a basis for developing innovative food applications. Naturally occurring antimicrobials are, for the most part, only proposed for use in foods, but not yet exploited commercially. In Europe, there are relatively few compounds that are allowed as food preservatives, and these are primarily organic acids[139].The use of antimicrobial agents in foods is controlled by the guidelines and regulations of the authority agencies in the country of their intended use, like EFSA in Europe, and strict requirements are in place for the toxicological evaluation of novel direct food antimicrobials. However, many plants and plant extracts have a long history of use, a “generally recognized as safe” (GRAS) status and are already authorized for use in food. For example, AECOSAN (Spanish Agency for Consumer Affairs, Food Safety and Nutrition) has accepted the use of a HT extract as a functional ingredient in 2015[200], while in 2010, rosemary extracts obtained EU approval for food preservation[201]. In 2017, the U.S. Department of Agriculture Food Safety and Inspection Service (FSIS) approved HBAs for use as antimicrobial agents in the amount of 4.4 mg/kg of cooked meat and 5.5 mg/kg in casings for meat products[202].

5.1. Screening of plant extracts for antibacterial activities against L. monocytogenes

Initially in this study, a screening of the antibacterial properties of 120 plant extracts, used as food supplements, herbal medicinal products, in feed or cosmetics was completed. Plant extracts are incredibly

complex, and they are usually composed of hundreds to thousands of metabolites, many of which are represented in very small quantities. In addition, the bioactive effects of extracts can be caused by synergistic or additive effects between several compounds that once separated lose their potency. Therefore, a challenging aspect of this research was not only to quantitatively measure the bioactivity of plant extracts, but also to connect a particular chemical structure(s) with the observed effects. In developing plant antimicrobials designated for use in food preservation, it is important that the process for obtaining the active product is not overly complicated. In this sense, it is desirable that the plant product either contains the bioactive principal as one of its major components, or that the bioactive principle is effective at low concentrations. It is important to note that it is not always advantageous to isolate a pure compound from the plant extract. Many extracts have a long history of use and have already been given a GRAS status. On the other hand, purifying compounds from a mixture of compounds with similar physico-chemical properties is laborious, and usually requires various purification steps, which in turn can significantly impact the cost of the final product, to a point it is no longer economically viable for its intended use as a food preservative. Moreover, once purified, a risk exists that the high-purity phytocompound will be considered a "fine chemical" rather than an agent derived from nature[28].

The results of the initial screening revealed several plant species that contained phytocompounds with strong antilisterial activities, including *O. europaea* L., *S. officinalis* L., *E. globulus* L., *H. lupulus* L., *O. stamineus* Benth, *P. granatum* L and *R. officinalis* L.

Products from *O. europaea* L. have been previously studied for their antimicrobial potential. Markin *et al*.[173] studied the effects of olive leaf extracts on several bacteria, and found that the olive leaf 0.6% extract killed within 3 h almost all cells from cultures of *E. coli*, *B. subtilis*, *Klebsiella pneumoniae*, *P. aeruginosa*, and *S. aureus*. Our study found that both olive leaf and olive fruit extracts had good antilisterial properties, and identified oleanolic acid (OA) and hydroxytyrosol (HT) as the most active agents. OA (3β-hydroxyolean-12-en-28-oic acid) is a pentacyclic triterpenoid with widespread occurrence throughout the plant kingdom[176]. OA had one of the lowest recorded MICs in this study, 10 µg/mL. Szakiel *et al*.[174] placed the MIC of OA against *L. monocytogenes* at 15 µg/mL, while Kim *et al*.[175] at 16-32 µg/mL, and 32-64 µg/mL for *Enterococcus faecium* and *E. faecalis*. Fontanay *et al*.[171] also recorded excellent antibacterial activity for OA and ursolic acid (UA) against *S. aureus* and *E. faecalis*, but the activity was limited to Gram-positive bacteria. Kurek *et al*.[172] reported that OA and UA are potent inhibitors of various pathogenic bacteria like *Streptococcus pneumoniae*, *Streptococcus mutans*, methicillin-resistant *S. aureus* (MRSA), vancomycin-resistant enterococci (VRE) and *Mycobacterium tuberculosis*. Interestingly enough, while our study agreed with the results of abovementioned studies, and found that OA and UA had great

antilisterial properties, we did not find any antilisterial effects for maslinic acid and glycyrrhizic acid, compounds that also belong to the triterpenic acids group and are structurally very similar to OA and UA.

Unlike the other antibacterial plant agents investigated in this study that were able to inflict strong growth inhibition at half the MIC, oleanolic acid (OA) either completely killed the bacterial cells or there was no inhibition. We believe that this was due to the inoculum size effect and a consequence of a reduced ratio of available antimicrobial molecules per target bacterial cells when the concentration was below the MIC. This finding agreed with an observation by Kurek *et al*.[171] that a concentration of OA at 0.7 x MIC did not impact cell growth or survival of *L. monocytogenes*. In the same study, they speculated that OA exerts its antibacterial properties by inhibiting peptidoglycan autolysis. Our results suggest that the molecular target of this compound is more likely to be the bacterial membrane. Treating the cells with OA resulted in a high percentage of cells with compromised membrane integrity, significantly higher than the number of membrane injured cells by 10% DMSO, a known membrane targeting solvent (Figure 31). Moreover, the rapid drop in viability in the time-kill assay suggests a quicker action than interfering with cell wall homeostasis, although this effect could also be present.

Many studies have suggested that the molecular target of terpenes is the cytoplasmic membrane, since they are readily soluble in lipophilic structures[10]. It is believed that terpenes disrupt the membrane organisation, alter its permeability and affect its ability to effectively carry out osmoregulation. This might result in a permeable membrane, as observed in our study where the plant samples that contained a higher concentration of triterpenes had a higher percentage of cells whose membrane integrity was compromised, higher than the damage done by 10% DMSO. Araya-Cloutier *et al*.[131] used ampicillin as control in their study of membrane permeabilization of *L. monocytogenes* by prenylated phenolics and discovered that when the cells were treated with ampicillin, there was no increased uptake of propidium iodide (PI), suggesting increased permeabilization. This observation agrees with the mechanism of activity of ampicillin which exerts its effect on the cell wall, providing additional evidence that this test is sensitive to membrane acting antimicrobials.

Hydroxytyrosol (HT), or 3,4-dihydroxyphenylethanol, is one of most potent natural antioxidants. The antimicrobial activity of HT and its potential use as a natural preservative have been studied by several researchers, however the reported MICs vary greatly. The lowest MICs for this phytocompound were described by Bisignano *et al*.[203] who reported MICs for HT against several bacterial reference strains and clinical *S. aureus* isolates between 3,9 – 31,25 µg/mL depending on the strain. On the contrary, Medina-Martínez et al.[204] reported that 400 µg/mL strongly inhibited the growth of four different *E. coli* strains, while the MIC for *L. monocytogenes* CECT 940 was reported to be higher than 1000 µg/mL. Concerning

food applications, Martinez-Zamora *et al*.[205] evaluated how the addition of hydroxytyrosol (synthetic and organic) affected the shelf-life of lamb meat burger patties. In this work, they demonstrated that the addition of 200 ppm of hydroxytyrosol better preserved raw lamb meat, with half the microbiological growth as total vial count after 6 days of storage compared to a control where sulfites and synthetic antioxidants were present. Moreover, Martinez *et al*.[206] showed that natural extracts from pomegranate, rosemary and hydroxytyrosol delayed the lipid oxidation and the microbiological spoilage in fish patties and extended the shelf life of these fish products under retail display conditions.

Considering *H. lupulus* L., the hops bitter acids (HBAs) were identified the most efficient antilisterial agents. HBAs are composed of hops alpha (α-) and beta (β-) acids which are pale yellow oils, soluble in hexane and characterized as prenylated phloroglucinols. The β-acids are less acidic (pKa=6.1) than the α-acids (pKa=5.4) due to the replacement on C-6 with an extra prenyl side chain. The mechanism of activity of HBAs was first proposed by Teuber and Schmalreck[136] by treating *B. subtilis* cells with humulone (an α-acid), lupulone (β-acid), isohumulone or humulinic acid fractions of hop resins. They hypothesized that HBAs inhibit the growth of Gram-positive bacteria by causing leakage in the cytoplasmic membrane and inhibiting the active transport of sugars and amino acids. Further studies performed with *Clostridium sticklandii* revealed that the β-acids had a negative effect on the pH gradient and membrane polarization, which led to a comprised protonmotive force[137]. These observations together with studies done on other Gram-positive rumen bacteria led the authors to suggest that HBAs act like phyto-ionophores, since they increased the ion permeability of the cell membrane. Our findings partially agree with the proposed mechanisms and suggest that hops α-acids indeed could cause substantial membrane damage, but the treatment with the β-acids resulted in 94% of the cells still having uncompromised cytoplasmic membranes. On the other hand, a key feature of a proton-transporting ionophore's effect on cell membranes is that the ionophore becomes more potent as the pH decreases[138]. Flythe *at al*.[137] found that *Peptostreptococcus anaerobius*, *C. sticklandii* and *C. aminophilumply* were sensitive at neutral pH at β-acid concentrations between 3 and 30 ppm, and decreasing the pH to 5.6 decreased the viable numbers in all cases. These results agreed with our findings that at pH 5.5 the MICs of both the α- and β-acids improved, especially the MIC of α-acids which decreased from 30 ppm to 2.5 ppm. This result could support the hypothesis that undissociated forms of HBAs are able to transport H^+ ions through the membranes of Gram-positive bacteria and compromise their homeostasis.

In this investigation, a methanolic extract of *E. globulus* L., further fractionated to isolate the water insoluble compounds was highly antilisterial with a MIC of 25 µg/mL. This plant product was characterized by a high concentration of triterpenic acids, in agreement with other studies that have investigated the

phytochemical profile of *E. globulus* using volatile organic solvents[177]. The low MIC of this fraction, combined with its bactericidal effects and a high ratio of *L. monocytogenes* cells with compromised membrane integrity, similar to the results observed for pure OA, led us to believe that the triterpenic acids were the antilisterial entities in this plant agent. Although, we do not exclude the contribution of other compounds, such as quercetin, a flavonoid compound with demonstrated antibacterial effects against several food pathogens[178], and antilisterial effects of 0.35 mg/mL[179], to have contributed to the bioactivity. It is noteworthy mentioning that the highly effective antilisterial compound eucalyptol was not detected in this fraction, excluding potential activity by the volatile compounds of *E. globulus*.

S. officinalis L. contains many biologically active compounds, including monoterpenes, diterpenes, triterpenes, and phenolic components[102]. Although many studies have evaluated the antibacterial properties of *S. officinalis* most have focused on the essential oil components, that were not the focus of our study. We found two active fractions of the methanolic extract of *S. officinalis*, SAL FR2 and SAL FR4. SAL FR2 was rich in pentacyclic triterpenic acids like OA and UA, that we believe were responsible for the observed antibacterial activities. On the other hand, the bioactive properties of SAL FR4 were on the account of other phytocompounds, since these acids were not present in this fraction. As we detected a high concentration of diterpenoids in the fraction, we suspected that these might be the antibacterial phytocompounds. The most common diterpenes of *S. officinalis* include: carnosic acid, carnosol and methyl carnosate[183]. The antilisterial activity of carnosic acid was demonstrated by Rozman *et al*.[184] in rosemary extracts. This result was later confirmed by Campo *et al*.[185] who found that carnosic acid was the most active from the investigated eight phytocompounds and had an inhibitory effect against *L. monocytogenes* at 25 µg/mL. It was found that this antilisterial activity was further improved at low pH and high NaCl content, which agreed perfectly with our observations. Interestingly, even though the methanolic extracts of the leaves of *S. officinalis* and *R. officinalis* L. have a similar profile of diterpenoids, and a similar concentration of carnosic acid and carnosol, we observed weaker effects for the *R. officinalis* extract PS-022. This indicated that apart from the diterpenoids, other phytocompounds with antibacterial activities, could also be present in the SAL FR4 fraction.

We included *O. stamineus* Benth. in this study as a less investigated species for its antilisterial activities, and found that the aqueous extract, and more particularly the polyphenols rich fraction of this extract were the most active products. *O. stamineus* is abundant in polyphenolic compounds, including 26 phenolic acids and 11 flavonoids identified by Guo et *al*[180]. We found 275 ppm of rosmarinic acid in this fraction (Table 23), but the studies done with pure rosmarinic acid suggested that this was not the moiety with antilisterial activity. Ho *et al*.[181] reported antibacterial activities of the 50% methanol extract and the

aqueous extract of *O. stamineus* against *L. monocytogenes,* and Alshawsh *et al*.[182] found that the best antimicrobial activity was shown by the aqueous extract of *O. stamineus* against *S aureus*, while no activity was observed against *E. coli* and *K. pneumoniae*. These findings agreed well with our results.

It has been widely demonstrated that phytochemicals are less effective against Gram-negative bacteria. The resistance of Gram-negatives to antimicrobial compounds is attributed to the presence of the outer membrane and the interplay between reduced influx and more effective efflux of antimicrobials compared to Gram-positives[130,131]. This study agreed with literature, as only hydroxytyrosol and the *P. granatum* L. extract impaired the growth of the Gram-negative bacteria *E. coli* and *S.* Typhimurium. For future work, a combination of compounds that strongly inhibit Gram-positive bacteria with one that inhibits Gram-negatives could prove higher overall efficiency at lower concentrations than the recorded.

For efficient control of foodborne pathogens, extensive data on the inactivation kinetics and growth inhibition of bacteria is needed, along with specifics on bacterial metabolism and genomic regulation in the presence of antimicrobials. SigB regulates the expression of genes responsible for bacterial survival under challenging environmental conditions by activating the general stress response (GSR) in *L. monocytogenes*. According to literature, a *sigB* null mutant is more susceptible to environmental stresses and attenuated in virulence[76]. SigB could serve as an important biomarker to assess the effects of cellular damage from antimicrobials on cells not able to activate the GSR and their possible sensitization to further preservation and inactivation treatments. Palmer at al.[112], found evidence that fluoro-phenyl-styrene-sulfonamide specifically inhibits activity of SigB across Gram-positive bacterial genera, in both *L. monocytogenes* and *B. subtilis*. If this SigB inactivation could sensitize the tolerance of bacteria to external stresses, this could be a novel approach used by the food industry to use milder preservation techniques in food production processes. To achieve this, it is first necessary to demonstrate that a *sigB* null strain responds differently to antimicrobial agents and food preservation techniques.

Our findings did not conclude significant differences in the antibacterial activities of the tested plant agents between the wild-type (wt) and the Δ*sigB* strains of *L. monocytogenes*. This might be due to the fact that the focus was primarily on the effect of the antibacterials at a single time-point, while not examining the length of the lag phase or the possible differences in bacterial tolerance between the strains in time-kill assays. Tolerant and non-tolerant bacteria may have the same MIC value, but the minimum duration of killing that can be obtained from time-kill curves can be distinct, suggesting a different level of tolerance[118]. Our results are in accordance with several studies that have investigated the differences in growth and survival of *L. monocytogenes* wt and Δ*sigB* in growth media supplemented with different antibiotics or bacteriocins. Bagley *et al*.[95] observed that even though the Δ*sigB* mutant was killed much

faster than its parental strain at a lethal dose of the antimicrobial, the zone of inhibition and the MICs of the bacteriocins nisin and lacticin 3147, and the antibiotics ampicillin and penicillin did not differ between the strains. Shin J.-H. *et al*.[108] noticed that even though σ^B activity was significantly induced in the wild type strain with vancomycin treatment, no differences in the logarithmic growth levels could be observed in comparison to the Δ*sigB* mutant. In contrast, Zhou et al.[135] demonstrated that in *L. monocytogenes*, σ^B contributed to tolerance of antibiotics acting on protein synthesis, since the MICs of tetracycline HCl and gentamicin sulphate for the Δ*sigB* mutant were lower than those for the wild type strain. On the other hand, when Palmer *et al*.[109] exposed both strains to sublethal levels of nisin, a lower degree of reduction in bacterial numbers could be observed in the Δ*sigB* mutant, indicating increased nisin resistance of the Δ*sigB* mutant relative to the wt strain of *L. monocytogenes* 10403S. The discrepancies in these studies suggest a complex SigB-regulated network responding to different conditions of antimicrobial stress.

5.2. Effects of plant antimicrobial agents on the general stress response in L. monocytogenes

Bacteria adapt to changes in their environment using signal transduction systems. These systems contain at least two elements, a sensor, responsible for sensing external stimuli, and a response regulator, in command of altering the expression profile of bacterial genes for survival and adaptation. While bacteria usually respond to environmental triggers using two-component regulatory systems[127], the activity of the alternative sigma factor SigB is controlled by a multicomponent signal transduction pathway that involves a partner-switching mechanism resulting in SigB being released from the anti-sigma factor RsbW[129]. Since the first report on SigB in *L. monocytogenes* in 1998[74], modulation of stress response has been recognized as a key role of this alternative sigma factor, and at least 73 SigB regulon members have been identified involved in different aspects of stress response and survival, including osmotic, oxidative, acid, alkaline and bile stress, or in antibiotic resistance[134]. The effective disruption of these regulatory systems has been proposed as a potential target for the development of antibacterial agents.

In this study, for the first time to our knowledge, we demonstrated that antibacterial agents originating from plants are capable of inducing the activity of SigB and to activate the general stress response in *L. monocytogenes*. Each plant antimicrobial agent from the selected (Table 31) showed a different degree of SigB activation not related to the MIC or the type of antibacterial activity (bacteriostatic or bactericidal). The *H. lupulus* compounds, especially the β-acids, as well as the *E. globulus* fraction, EU FR2, exerted a strong and proportional increase in SigB activity with a significant difference in eGFP positive cells after 30 min, 1 h and 2 h of exposure to the plant antimicrobials. The *O. stamineus* fraction, OS FR2, and the hops α-acids induced a weaker response, while no significant differences were

observed after the treatments with the rest of the plant antimicrobial agents – oleanolic acid, hydroxytyrosol and the *S. officinalis* fractions SAL FR2 and SAL FR4 (Figure 28). Shin *et al*.[108] observed that SigB activity was significantly induced by the addition of vancomycin and identified 18 vancomycin-inducible SigB-dependent stress response proteins associated with cell wall biogenesis, cell metabolism, intracellular transport, GSR and virulence. Begley *et at*.[95] speculated that the differences in tolerance they observed between *L. monocytogenes* wt and Δ*sigB* upon exposure to nisin, ampicillin and penicillin G were on account of the positive regulation of the SigB-regulon in the wt strain that contains genes that encode putative efflux pumps, penicillin-binding proteins, autolysins or proteins involved in cell envelope modification. In addition, Gravesen *et al.*[163] and Vadyvaloo *et al*.[164] hypothesized that, in *L. monocytogenes*, SigB adds to antibiotic tolerance by controlling membrane charge or lipid composition.

In the signal transduction pathway that leads to SigB activation, a supramolecular multiprotein RsbR1-RsbS-RsbT complex known as the stressosome is presumed to act as the stress sensing element. Several environmental stresses including low pH, high osmolarity, and blue light are known to be sensed by the stressosome[70,84]. The current model of stressosome activation inferred from *B. subtilis* predicts that RsbR1 T175 is already phosphorylated in unstressed conditions and that environmental stress would trigger phosphorylation at a second site on RsbR1, T209, and RsbS S56 phosphorylation[111]. Structural analyses have revealed that in *L. monocytogenes*, T209 and S56 are located near a flexible loop that could adopt two configurations preventing or allowing access for phosphorylation by the kinase RsbT[92].

Once the hops-β acids and EU FR2 from *E. globulus* were established as strong SigB inducers, the effects of exposure of *L. monocytogenes* to antimicrobial stress by these agents on the distribution of the core stressosome protein RsbR1 and SigB in subcellular compartments were investigated. The majority of RsbR1 was detected in the cytosol and no changes in the subcellular location of this protein were seen upon exposure to stress from the plant agents. The occurrence of the molecular modifications and the subcellular location of the stressosome proteins during osmotic stress were assessed in *L. monocytogenes* by Dessaux et al.[128] who also found that these proteins were mainly cytosolic. The findings of their study supported a short half-life for the RsbR-RsbS-RsbT complex in live cells, in contrast to the stable complexes that are assembled *in vitro* with purified proteins[93]. The transient nature of the stressosome complex could explain why, in cells exposed to antimicrobial stress, the majority of RsbR1 is located in the cytosol. In addition, this observation agreed with previous studies who showed that the RsbR, RsbS, RsbT and RsbU proteins remain relatively constant during σ^B activation[166]. This is related to the genetic organization of the *sigB* operon where the upstream half of the operon (rsbR-rsbS-rsbT-rsbU) is transcribed from a SigA-dependent promoter[167]. Hence, the levels of the upstream members would be unchanged in response to

stress. However, this notion does not explain the unchanged levels of SigB protein upon exposure to the antimicrobial stress. Since the transcription of SigB is positively autoregulated, higher SigB levels were expected after stress exposure. A possible explanation would be that the sublethal concentration of the plant antimicrobial agents was not sufficient to make a difference in SigB concentration at the protein level. Future studies with higher concentrations of the plant stressors should address this dilemma.

This change in location of the SigB protein from the cytosol to the membrane fraction due to antimicrobial stresses has not been explained before. Our findings agreed with a recent study [128] who also detected SigB in both the cytosol and membrane fractions of *L. monocytogenes*, and observed higher SigB levels in response to osmotic stress in both fractions. These authors investigated whether the membrane localization of SigB was facilitated by transertion of SigB-regulated proteins, suggesting that some protein(s) upregulated by SigB upon stress could favor the membrane localization of this sigma factor.

In contrast to the predicted monophosphorylated state of RsbR1 in unstressed *L. monocytogenes*, we found that RsbR1 is both mono- and doubly-phosphorylated, with a higher ratio of the doubly-phosphorylated isoform. This phosphorylation state of the protein did not change upon exposure to antimicrobial stress, which was in agreement with a previous study by Dessaux *et al*.[128] where it was observed that RsbR1 was mostly doubly-phosphorylated and the phosphorylation state did not change upon exposure to osmotic stress. Another possible explanation would be that the two phosphorylation isoforms detected in the immuno-blots are both mono-phosphorylated, but on different phosphorylation sites. The Phos-Tag gel system separates proteins based on their phosphorylation state, not on their sizes, hence conformational changes in the protein due to phosphorylation events could change the migration patterns of the protein. A top-down mass spectrometry approach could provide a solution to this dilemma. However, in *B. subtilis*, RsbRA (RsbR1 ortholog in *B. subtilis*) was found mainly mono-phosphorylated, and the doubly-phosphorylated state of RsbRA has been hypothesized to occur only during extreme stress in order to limit SigB signalling[92,168]. Furthermore, the control mutant strains harbouring a mutation in the one of the phosphorylation sites of RsbR1 (T175) and the kinase-inactive RsbT variant, demonstrated that phosphorylation at T175 was not a pre-requisite for RsbR1 T209 phosphorylation and that this reaction is mediated by the kinase activity of RsbT (Figure 29). This result was consistent with the dependence of RsbRA phosphorylation on the RsbT kinase in *B. subtilis*.

The results of our study indicated that the development of an effective screening method for assessing the antibacterial properties of natural products through measuring the activation of the general stress response in *L. monocytogenes* could not be accomplished for several reasons. First and foremost, even though highly active plant agents were selected for measuring the activation of SigB induced by

exposure to antimicrobial stresses, not all agents were able to generate a SigB regulated response. In more detail, about half of the plant agents were able to induce higher SigB activity compared to the control, with only two, the hops β-acids and EU FR2 indicating a strong and proportional increase in SigB activity. This means that even though SigB controls the GSR in *L. monocytogenes*, not all genes included in antimicrobial defenses are controlled by SigB. This is in agreement with a recent study done by Ranganathan *et al*.[169] investigating the protective effect of SigB in *S. aureus* during exposure to six different antibiotics. They found that SigB had a clear protective role when *S. aureus* cells were exposed to gentamicin, ciprofloxacin, vancomycin and daptomycin, but no differences were observed for oxacillin and clindamycin. Next, the development of a good screening method should involve the use of highly available and not overly complex analytical instruments. Since the fluorescence signal of wild type $P_{lmo2230}$::egfp could not be detected by a microplate reader, flow cytometry had to be used to measure SigB activation. Flow cytometry is an excellent and rapid instrumental tool, but it is also expensive, difficult to use and not available in many labs, especially for use with BSL-2 pathogens. Lastly, even if successfully developed, this screening method would have had a limited use for testing against other pathogenic or non-pathogenic bacteria for two main reasons. First, even though SigB functions as a central regulator of the stress response in bacteria of the genera *Bacillus*, *Staphylococcus* and *Listeria*, not all Gram-positive bacteria contain SigB. In fact, other alternative sigma factors more similar to σ^F are used by *Mycobacterium* and *Streptomyces* when exposed to stressful environments. In Gram-negative bacteria, the situation is completely different with σ^S identified as the general stress response regulator. Finally, the number of alternative sigma factors in different bacterial genera varies greatly depending on the life cycle of the bacterium, with some that have only one sigma factor like the intracellular pathogen *Mycoplasma genitalium* to bacteria that have more than 60 sigma factors like the soil bacterium *Streptomyces coelicolor*[68]. Hence, utilizing bacterial stress responses to screen for antibacterial activity would imply having previous knowledge of the genes and their control systems included in protection against environmental stresses and successful construction of a reporter strain able to generate a measurable and quantifiable signal as a result of the increased gene expression. In all, even though it is a highly innovative idea to exploit the stress state of the bacterium to screen for antibacterial properties, it is too complex to execute and requires extensive prior technological developments to execute effectively.

5.3. Antibacterial activities of selected plant antimicrobial agents in food matrices

The microbial safety and stability of most foods is based on the application of multiple preservative factors called hurdles. Hurdles can be defined as sublethal stresses which bacteria must

overcome in order to survive or thrive in food systems[62]. *L. monocytogenes* is a significant challenge in food production, particularly for minimally processed and chilled ready-to-eat products. The combination of antimicrobials with other hurdle factors, such as pH or temperature, are attractive approaches to control *L. monocytogenes* and enhance the safety and quality of minimally processed foods.

Predictive microbiology is a key aspect of food safety and spoilage. The concept of this research field is that a detailed knowledge of the behavior of microorganisms in food products (growth, survival, inactivation), condensed into mathematical models, enables an evaluation of the microbiological safety and quality of foods[151]. Most of the modelling studies are focused on the effect of temperature, pH, water activity and commonly used organic acids, while the effects of plant antimicrobial agents on inhibiting the growth of microorganisms are primarily based on endpoint MIC determinations. Modelling the kinetics of bacterial growth in the presence of antibacterial agents better describes the dynamic behaviour of antimicrobial activity, allows for a more accurate MIC value to be determined and provides quantitative insight into the effects at sub-inhibitory concentrations.

To successfully apply antimicrobial agents in food systems, in addition to fundamental studies in laboratory media, studies in representative food systems should also be conducted to determine potential interactions between the antimicrobial agents and food components that could impact on their antimicrobial efficacy[139]. A quantitative biological response to the presence and concentration of an antimicrobial can be most easily determined by measuring growth kinetics (i.e., lag phase and growth rate). In this study, the growth kinetics of *L. monocytogenes* were estimated in several food matrices, including reconstituted skimmed milk (MSK), 20% fat cooking cream, Béarnaise sauce and a laboratory-made simple food matrix named KonoMatrix (KM, see Section 3.16.2). We used the logistic primary growth model[141] to calculate the growth parameters of *L. monocytogenes* in the presence of different concentrations of selected plant antimicrobial agents, and used the fitted values to establish a dose-response relationship. The selection of a primary growth model in predictive food microbiology is often subjective, and usually based on convenience[146]. To evaluate the performance of the model, usual measures of goodness-of-fit like the Pearson coefficient (R^2) and the root mean square or the errors (RMSE) are used. A study by Pal *et al*.[146] that compared several primary predictive models to study the growth of 19 *L. monocytogenes* isolates showed no significant difference between the predictions of the linear, Gompertz, logistic, and Baranyi and Roberts models, although the Baranyi model showed the best goodness-of-fit for the greatest number of curves studied. This study agreed with the results of a previous study by Lopez et al.[147] who showed that the Baranyi model best determined the growth parameters.

Primary models estimate the response of a microorganism over time to a single set of conditions. The response can be measured either by direct or indirect measurements of microbial population density or products of microbial metabolism[152]. Quantification of bacteria at selected time points is usually done by laborious standard colony count methods, but automated methods such as absorbance, conductance or luminescence measurements can facilitate the generation of data. The population density of *L. monocytogenes* was primarily measured by the intensity of the emitted luminescence signal (RLU) in our study, generated by the EGDe*lux* strain through active metabolism and replication, although plate count measurements were also performed. Early on, we established a linear correlation between the optical density at 600 nm (OD_{600}) and the number of colony forming units per mL (CFU/mL) with a R^2=0.99 coefficient. Since food matrices are opaque solutions, the OD_{600} could not be used as an indicator of growth. Additional simultaneous measurements of OD_{600} and RLU in BHI revealed that the RLU signal gradually increased during the lag phase, then rapidly increased during exponential phase, reaching a maximum at the end of exponential phase, and rapidly decreased throughout stationary phase until the basal level was reached again. These observations provided us an overview of how the RLU signal relates to the growth phase of the bacteria, and enabled us to use this indirect measurement of microbial population to fit the growth curves, expressed as log RLU against time.

Estimations of the growth rates in different concentrations of the plant antimicrobial agents were calculated by two approaches, by a growth curve fitting and a time-to-detection approach[142]. The high R^2 and the RMSE values indicated a good quality of the fits. Furthermore, the excellent agreement in the dose response curves obtained by both approaches further validated the accuracy and adequate performance of either method. Subsequently, a secondary fitting of the dose-response relationship was plotted to establish a relationship between the growth rate changes with respect to the concentration of the plant antibacterial agent. Again, two approaches were used to translate primary model growth rate estimations to a secondary dose-response model. The constructed Hill plots using log-transformed concentration values allowed for the prediction of the half maximal inhibitory concentration (IC50) of the plant antimicrobial agents, while a simple linear regression fitting allowed the prediction of the MICs.

A clear direct relationship between the lag phase duration and the growth rate was observed only in Béarnaise sauce, although the correlation between the parameters was relatively weak, with an R^2 coefficient of 0.75. Unfortunately, no other relationship was observed in the rest of the food matrices. In growth studies, the lag phase is believed to reflect microbial adaptation to new environments, and can be influenced by many factors, such as the characteristics of the previous environment, the inoculum size and the growth stage of the cells[140]. Even though the lag time has been extensively studied in predictive

microbiology, accurate prediction of the lag time in foods remains difficult to obtain and it is necessary to improve predictions[148]. In the study by Pal *et al*.[146], the lag times of *L. monocytogenes* strains in slurries of both sliced turkey and frankfurters formulated with or without antimicrobials potassium lactate and sodium diacetate remained similar. These observations were in agreement with the data from Schlyter *et al*.[149], that found that a combination of sodium diacetate and sodium lactate had no effect on the lag time of *L. monocytogenes* in turkey slurry, but a significant effect on growth rate. These results allowed the authors to speculate that diluted antimicrobials in meat slurry preparations affected the metabolism of *L. monocytogenes* involved in the exponential phase growth with no or little effect on the metabolic activity involved in the lag phase. Hence, the growth rate of the bacteria was considered the main growth parameter of behavioral response and to construct the models.

Overall, the hops bitter acids (HBAs) isolated from *H. lupulus* and two *S. officinalis* products (SAL FR4 and extract PS-024), exhibited the best antibacterial properties in food systems. The lowest MIC values in food were recorded in MSK for the HBAs, 39 ppm and 38 ppm for the α- and β-acids, respectively, while SAL FR4 had a significantly higher MIC, 1441 ppm. In cooking cream, from the eight plant agents tested, only the α-acids retained their antibacterial properties, and a linear regression fit placed the MIC value at 267 ppm. *L. monocytogenes* growth can be influenced by many factors in a food system. While the interaction of *L. monocytogenes* with antimicrobial agents is more direct in laboratory media, the situation is more complex in a food system. Three types of interactions occur in the food system; an interaction between the microorganism and the food system, an interaction between the antimicrobial and the food system, and an interaction between the antimicrobial and the microorganism. Therefore, decomposition to specific mechanisms from the various contributions these make to the final responses observed is extremely difficult in food matrix systems. However, what can be assumed is that the efficacy of antimicrobial compounds in food systems is largely dependent on the chemical and physical properties of the antimicrobial. The polarity of the compound is probably the most important physical property[119]. Water solubility or hydrophilic properties are necessary to assure that the antimicrobial is soluble in the water phase, where microbial growth occurs, although, antimicrobials acting on the cell membrane appear to require some lipophilic properties[153,154]. It seems that a specific hydrophilic–lipophilic balance of the antimicrobial's properties is required for optimal activity. However, the balance needed in a laboratory medium, may differ significantly from the one needed for a food product[119]. Other important factors leading to reduced effectiveness among antimicrobials in food systems are the food component interactions. Lipophilic or amphiphilic antimicrobials can solubilize in or be bound by lipids or hydrophobic proteins in foods and tend to partition into the lipid areas of the food. This makes them less available to

inhibit microorganisms in the food product. Interaction with lipids probably results in the greatest interference with antimicrobial activity[119].

In Béarnaise sauce, both α- and β-acids showed a very good dose-response relationship, with an increase in the lag phase duration and a decrease in the maximum specific growth rate. The MIC for the β-acids was still quite low and placed at 68,6 ppm, while the α-acids lost the linear increase in effectiveness at higher concentrations, resulting in a MIC prediction of 440 ppm. This effect was possibly a result of interactions with the matrix, like partitioning in the lipid phase of the sauce. Béarnaise sauce is an acidic sauce with a pH of 4.7, and a high fat content in the recipe coming from butter, oil and yolks. HBAs are very hydrophobic compounds, and the acidic pH of the sauce allows for their solubility in the water phase, however, it seems like the hydrophilic–lipophilic balance changes at higher concentrations for α-acids, and their addition in the sauce does not necessarily guarantee a higher concentration in the water phase, where contact with bacteria occurs. The pH of the food can result in ionization of an antimicrobial and a change in activity. It is known that organic acids function at low concentrations only in highly acidic foods (pH 4.5 to 4.6)[119]. This is due to the fact that the most effective antimicrobial form is the undissociated acid, which increases in concentration at pH values lower than the pKa of the acid. In the undissociated form, organic acids can penetrate the cell membrane lipid bilayer more easily[107]. Surprisingly, even though EU FR2 showed significant improvement in activity at acidic pH in laboratory media, with a change in MIC from 25 ppm to 1 ppm, this plant agent was not active in Béarnaise sauce, suggesting that the antimicrobial compound(s) is probably not acidic and is of highly hydrophobic nature, and possibly partitions in the lipid phase of the sauce.

The evaluation of the antilisterial properties of the *S. officinalis* L. extract PS-024, β-acids and oleanolic acid in KM indicated that the *S. officinalis* L. extract at the highest concentration, 500 ppm had the best antibacterial properties and inhibited *L. monocytogenes* growth up to 10^7 CFU/mL in the produced food matrices. The β-hops bitter acids had good antibacterial activities at 100 ppm, with gradual loss of activity as the concentration of the active compound decreased, while oleanolic acid had very limited activity, observed only by the reduced growth rate obtained by the Baranyi and Roberts[126] fitting of the plate counts. To the best of our knowledge, we herein report the first results of the antibacterial activity of oleanolic acid in food matrices. The unusual double-peak curves posed difficulties in modelling the luminescence data in the growth curve studies, so observations were limited to where a complete lack in ΔRLU existed. For the *S. officinalis* extract at 500 ppm, pH made the difference between a bacteriostatic effect, and a bactericidal effect, shown by the lower final cell population density compared to the initial cell population. Generally, it was difficult to observe strong growth rate reductions in KM

produced by the plant agents as high inoculum populations were used. This notion was nicely reflected in the results from the plate count data fitted with the Baranyi and Roberts model, which showed that when an initial inoculum size of 10^5 CHU/mL was used, higher differences in log CFU reduction were observed compared to when an initial inoculum size of 10^6 CFU/mL was used. The similar growth kinetics with the control at low plant agent concentrations could be attributed to less demand for cellular energy expenditure, needed to overcome the low stress conditions[170].

Our results on the antilisterial efficacies of HBAs in food systems agreed with several studies. Larson *et al*.[150] showed that a hop extracts with 41% beta acids was inhibitory against *L. monocytogenes* at 100 ppm in skimmed milk, while this concentration was 10-fold higher for 2% and whole fat milk. In cottage cheese the MICs were between 100-3000 ppm. Furthermore, a 65% β-acids extract did not cause *L. monocytogenes* inhibition in full-fat Camembert cheese. The authors speculated that the antimicrobial activities of hop extracts in food appeared to increase with acidity and lower fat content, which entirely agreed with the results of our study. Kramer *et al*.[155] observed an identical MIC for β-acids against *L. monocytogenes* at neutral pH in BHI as ours, and even 2 to 4-fold lower MICs at pH 5 than the ones we observed at pH 5.5 suggesting that acidic pH indeed produces the most active antibacterial forms of HBAs. Lastly, the antibacterial effects of HBAs against *L. monocytogenes* in food models were shown in several other studies that examined deli-style meat and pork tenderloins[155,156,157,158].

On the other hand, studies reporting on the antilisterial properties of *S. officinalis* extracts in food systems were difficult to find. Several studies have reported the preservation effects of essential oil components of sage[159,160], but these compounds were not the objective of investigation of this study. Šojić *et al*.[161] observed that the addition of a sage extract in fresh pork sausages caused a significant inhibition of microbial growth and a positive effect on sensory properties. Karpinska-Tymoszczyk *et al*.[162] reported a reduction of 1 log CFU/g in mesophile levels due to the addition of sage ethanol extracts (1000 ppm) to turkey meatballs at 4°C, but a lower sensory acceptance was recorded at this concentration of the extract.

Antimicrobials will undoubtedly continue to be needed to provide the food supply that will be demanded in the future. The results from this study provide important preliminary information, and further studies are required on the cost-effectiveness of plant antimicrobials, and their stability and efficacy under processing, transportation, and storage conditions. Moreover, the adequate use of plant antimicrobials in foods in which they add to the positive sensory characteristics of the product additional to improving food safety should also be further assessed. Natural antimicrobials will be increasingly sought in hurdle technology, and detailed information on their growth inhibition properties in various food systems provide a solid foundation for their successful application.

CONCLUSIONS

Based on the results presented in this thesis, and in agreement with the previously defined objectives, the following conclusions can be drawn:

1. Plant extracts from several species can affect the growth and survival of *L. monocytogenes.* The most effective plant antibacterial agents were derived from *O. europaea* L., *H. lupulus* L., *E. globulus* L. and *S. officinalis* L.
2. The hops bitter α- and β- acids, and the triterpenic acids oleanolic acid and ursolic acid are highly efficient in inhibiting the growth of *L. monocytogenes* in laboratory conditions, with minimum inhibitory concentrations in range between 5 – 35 μg/mL.
3. Antibacterial agents originating from plants can induce the activity of SigB and activate the general stress response in *L. monocytogenes*. However, not all plant antimicrobial agents from the ones evaluated were able to generate this effect and the ones that did, exerted a varied degree of SigB activation not related to the MIC or the type of antibacterial activity.
4. The cytosolic subcellular localization and phosphorylation pattern of RsbR1 do not change in the presence of plant antibacterial agents in laboratory conditions. However, a portion of cytosolic SigB protein mobilizes to the membrane after exposure to purified hops β-acids.
5. The antibacterial efficacy of novel plant antimicrobials cannot be assessed indirectly by measuring SigB activation in *L. monocytogenes*.
6. The antibacterial efficacy of plant agents *in vitro* does not match the same effects in real food systems. Interactions of the plant antimicrobial agent with food components either reduced their bioactivity or completely impair it. Nonetheless, the hops bitter acids and selected *S. officinalis* L. products show good potential for application as food preservatives.
7. The antibacterial properties of plant agents differ between Gram-positive and Gram-negative bacterial food pathogens. The Gram-negative bacteria are more resistant to the effects of the plant antibacterial agents and only hydroxytyrosol and a *P. granatum* L. extract can inhibit the growth of the selected Gram-negative pathogens, *E. coli* and *S.* Typhimurium. On the other hand, the Gram-positive pathogens show similar sensitivity.

As a general conclusion of this thesis, it can be said that this study on plant-based antimicrobials has shown the great untapped potential of these agents and their extensive biodiversity available worldwide. Given the consumer demand for more natural products and the growing need for alternative preservatives to ensure food safety, it is imperative that plant-derived antimicrobial compounds be fully assessed for their feasibility for food application.

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REFERENCES

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