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Natural compounds in attenuating virulence in *Listeria monocytogenes*

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Resumo Alargado

A segurança alimentar e a contaminação de alimentos são preocupações da saúde pública por todo o mundo. Segundo a Organização Mundial de Saúde, a contaminação de alimentos por organismos patogénicos está associada a mais de 200 doenças, e é estimado pela mesma organização que 1 em 10 pessoas acabam doentes devido ao consumo de alimentos contaminados. Estes organismos patogénicos afetam facilmente grupos vulneráveis, tais como imunocomprometidos, idosos, grávidas e crianças. Adicionalmente, ao impacto na saúde pública também possuem um impacto na economia nacional dos países, comércio alimentar, turismo e na indústria alimentar.

As doenças alimentares são normalmente causadas por diversos organismos, tais como bactérias, vírus, ou parasitas através da ingestão de alimentos contaminados. Entre os agentes patogénicos salienta-se a bactéria *Listeria monocytogenes*, que pode ser encontrada em diversos alimentos, tais como productos lácteos, vegetais, peixes e alimentos prontos a consumir.

L. monocytogenes é uma bactéria Gram-positiva, anaeróbia facultativa, não formadora de esporos com forma de bacilo e ubíqua, que quando ingerida pode causar listeriose. Esta doença afeta principalmente imnunocomprometidos, pessoas idosas e grávidas e possui uma taxa de mortalidade entre 20 e 30%. Esta doença pode apresentar sintomas ligeiras como dores musculares, náuseas, vómitos e diarreia ou evoluir para uma forma mais grave e sistémica, apresentando sintomas mais severos como febre, confusão perdas de equilíbrio e sépsis, cujo tratamento requer hospitalização e que em casos mais graves pode levar à morte. No caso de infeção de grávidas esta doença está associada a morbilidade fetal e neonatal em cerca de 25% dos casos. Esta situação resulta desta bactéria ter a capacidade de se disseminar através da corrente sanguínea e e de atravessar a barreira placentária e de infetar o feto.

O sucesso da bactéria *L. monocytogenes* está relacionado com a sua capacidade em promover a sua internalização em células hospedeiras e de possuir diversos fatores de virulência que lhe permitem sobreviver a agentes antimicrobianos e proliferar em condições adversas. Esta bactéria apesar da temperatura ideal de crescimento ser entre 30 e os 37 °C possui diversos mecanismos de proteção contra condições adversas que lhe permite proliferar a baixas temperaturas (0,4 a 4°C), altas temperaturas (55°C), altas concentrações sal e baixo pH. Como diversas destas condições podem ser encontradas na indústria alimentar, faz com que esta bactéria represente um risco para estas indústrias. Outros fatores importantes da virulência *L. monocytogenes* é a sua capacidade de motilidade mediada por flagelo e a formação de biofilmes. Quando a uma temperatura de 30 °C esta bactéria consegue movimentar-se devido ao flagelo, permitindo assim uma melhor procura

de nutrientes e colonização de locais para propagação do organismo. Esta motilidade mediada por flagelo também está relaciona com a formação de biofilmes tanto na adesão inicial à superfície como na formação do biofilme. Biofilmes são comunidades de células bacterianas ligadas entre si ou/e a uma superfície e envolvidas por uma matriz autoproduzida de substâncias poliméricas extracelulares. A formação de biofilmes permite uma melhor sobrevivência em superfícies como aço ou vidro, contra condições adversas e a agentes antimicrobianos usados na indústria alimentar. Apesar dos biofilmes protegerem a bactéria dos agentes antimicrobianos, esta bactéria também possui mecanismos como bombas de efluxo que lhe confere resistência a agentes antimicrobianos e a antibióticos.

Devido à bactéria *L. monocytogenes* conseguir sobreviver a agentes antimicrobianos e proliferar em diversas condições adversas, novas alternativas têm vindo a ser estudadas com o objetivo de reduzir a presença deste organismo patogénico, tais como a utilização de bacteriófagos, bacteriocinas e compostos naturais, como os óleos essenciais. Focando nos compostos naturais, os óleos essenciais extraídos de plantas já revelaram ter capacidade bactericida contra organismos patogénicos e apresentam a vantagem de ser também uma alternativa a agentes sintéticos ou químicos. Porém estes óleos essenciais são compostos por uma mistura de 20 a 60 diferentes compostos naturais que variam facilmente em termos de constituição. Esta variação na constituição dos óleos essenciais não permite um efeito consistente, e com base neste problema surgiu o estudo de compostos naturais isolados. Os compostos naturais isolados ao contrário dos óleos essenciais não possuem o problema de variação na sua composição. Outra vantagem é que podem ser combinados com outras substâncias como antibióticos para aumentar ou restaurar o seu efeito. Diversos compostos naturais isolados já têm vindo a ser estudados e vários apresentam propriedades bactericidas e anti virulência contra diversos organismos patogénicos. Apesar de, em *L. monocytogenes,* já existirem alguns estudos com compostos naturais isolados, existem, no entanto, diversos compostos cuja ação é desconhecida nesta bactéria ou que requerem mais estudos na sua virulência.

Com base nisto foram selecionados quatro compostos (resveratrol, ácido *p* -cumárico, cânfora e linalool) para analisar o seu impacto na virulência desta bactéria.

Para entender o efeito destes compostos selecionados na virulência de *L. monocytogenes* foi determinada a concentração mínima inibitória de cada composto. Posteriormente concentrações sub-inibitórias foram também determinadas de forma a ter certeza que o efeito inibitório dos compostos nos ensaios seguintes era devido a um efeito do composto e não devido a morte celular. De seguida procedeu-se ao estudo do efeito dos compostos em diversos fatores de virulência da bactéria tais como "*quorum sensing*", biofilmes, motilidade, capacidade hemolítica e tolerância a condições adversas.

Primeiramente foi estudado o efeito dos compostos selecionados no "*quorum sensing"* devido a associação deste com a comunicação celular e outros fatores de virulência como motilidade e formação de biofilmes. De forma a avaliar este efeito foi usado *Chromobacterium violaceum* como biossensor. Esta bactéria produz violaceína, no entanto, quando o "*quorum sensing*" é inibida a produção de violaceína também é afetada. Assim dos compostos testados só o resveratrol e o linalool inibiram de forma significativa a produção de violaceína.

De seguida, devido a ligação existente entre o "*quorum sensing"* e a formação de biofilmes e motilidade foram analisados os efeitos dos compostos em estudo nestes fatores de virulência. A motilidade mediada por flagelo da *L. monocytogenes* é um importante fator de virulência, que lhe permite procurar por nutrientes e melhores locais de proliferação. De forma a avaliar o efeito dos compostos em estudo na motilidade foram usadas placas de TSA com 0,3% (m/v) de agar e compostos naturais. No final do ensaio todos os compostos, expeto resveratrol, mostraram alguma inibição na motilidade deste organismo ao longo das 72 horas de incubação a 30°C. A etapa seguinte deste trabalho consistiu no estudo do efeito destes compostos naturais na formação de biofilmes. A formação de biofilmes permite à bactéria sobreviver a diversas condições e agentes antimicrobianos e proliferar em superfícies como aço ou vidro. Para estudar a capacidade de formação de biofilmes foi usada a metodologia de coloração com violeta de cristal usando placas de 48 poços. Entre os compostos estudados, o resveratrol e o linalool mostraram uma inibição significativa na formação de biofilmes, sendo o efeito do linalool mais acentuado.

Outro importante fator de virulência de *L. monocytogenes* está relacionado com a sua capacidade de infeção de células hospedeiras. Durante este processo esta bactéria precisa de escapar de um vacúolo formado durante o processo de internalização. Para isso a bactéria secreta toxinas capazes de formar poros na membrana do vacúolo. Umas das toxinas é a Listeriolisina O, que é uma citolisina fundamental para o processo de infeção deste organismo. Uma forma de estudar a presença desta toxina é através de um ensaio de hemólise, que quando estudado em *in vitro* esta citolisina é a capacidade de provocar a hemólise de eritrócitos. Analisando assim a hemólise que ocorre em eritrócitos quando em conjunto com *L. monocytogenes* após uma exposição aos compostos naturais é possível determinar se os compostos têm algum efeito nestas propriedades desta bactéria. Em primeiro lugar foi realizado um estudo de citotoxicidade dos compostos em relação aos eritrócitos humanos utilizados. Através deste estudo foi possível concluir que as concentrações sub-inibitórias dos compostos não apresentam efeito citotóxico para com os eritrócitos humanos, porém em algumas das concentrações superiores já apresentam efeito citotóxico. Relativamente ao ensaio que permite analisar o efeito na capacidade hemolítica deste organismo após a pré-exposição aos compostos naturais foi possível que certos

compostos como o ácido *p*-cumárico e o linalool foram capazes de inibir a hemólise, sendo que no caso do linalool foi possível inibir totalmente a hemólise provocada por esta bactéria.

Após o estudo da influência dos compostos naturais na virulência de diversos fatores de virulência da *L. monocytogenes* foi analisado se haveria algum efeito por parte dos compostos naturais na tolerância a condições adversas de *L. monocytogenes*. A capacidade desta bactéria de sobreviver a diversas condições tais como baixas e altas temperaturas, altas concentrações sal e pH ácido faz com que este organismo seja de difícil erradicação. De forma a estudar se os compostos naturais poderiam aumentar a suscetibilidade deste organismo às condições adversas mencionadas, foram misturados compostos naturais juntamente com a bactéria em TSB com cada condição adversa. No caso das concentrações de sal foram incubadas em TSB com 12% (m/v) de NaCl, para o de pH baixo o TSB foi acidificado até um pH de 2.4, no caso de temperaturas elevadas foi utilizado uma temperatura de 55°C e no caso temperaturas baixas uma temperatura de 4°C. Relativamente, ao estudo do stress osmótico provocado pelas concentrações salinas, ao pH baixo e ao ensaio com temperaturas elevados somente o linalool foi capaz de aumentar a suscetibilidade de *L. monocytogenes* a estas concentrações adversas, sendo que os restantes compostos não demonstraram nenhum efeito significativo. No que respeita ao estudo de temperaturas de refrigeração, este organismo foi incubado por 196 dias a uma temperatura de 4°C com compostos naturais. Os compostos e o organismo patogénico foram também incubados com e sem 12%(m/v) de NaCl. Neste estudo foi possível observar que o linalool, uma vez mais foi capaz de aumentar a suscetibilidade da bactéria à temperatura de 4°C com e sem presença de NaCl. Em relação aos restantes compostos, o resveratrol mostrou diminuir a tolerância deste organismo, porém só na presença de NaCl. O ácido *p*-cumárico por sua vez também conseguiu alterar a tolerância a temperaturas baixas, porém na presença de NaCl este efeito não ocorre. A canfora não mostrou ter efeito algum na tolerância de *L. monocytogenes*.

Por fim, devido às capacidades deste organismo para resistir a antibióticos e ao crescente aumento do aparecimento de resistências a antibióticos foi realizado um estudo para analisar se estes compostos naturais poderiam ter um efeito modulatório da concentração mínima inibitória dos antibióticos, potenciando assim o seu efeito. Para isso, os compostos naturais foram incubados com o organismo patogénico e 6 antibióticos separadamente (ampicilina, cefotaxima, gentamicina, eritromicina, tetraciclina e rifampicina). Entre os antibióticos testados só para a gentamicina ocorreram alterações da concentração mínima inibitória quando em contacto com resveratrol e linalool.

Em suma, os compostos naturais testados neste trabalho foram capazes de inibir ou afetar diversos fatores de virulência de *L. monocytogenes*. No entanto, o linalool, devido a

mostrar efeito em todos os ensaios e estudos realizados, foi, entre os compostos estudados, o composto mais promissor contra *L. monocytogenes*.

Palavras-chave

Listeria monocytogenes; Resveratrol; Ácido *p*-cumárico; Cânfora; Linalool; "*Quorum sensing*"; Biofilmes; Motilidade; Condições adversas; Modulação de suscetibilidade a antibióticos

Abstract

Food safety is a pressing global concern with widespread implications. Contamination of food products is linked to the transmission of over 200 diseases. The World Health Organization (WHO) estimates that 1 in 10 people worldwide falls ill each year from consuming contaminated food. Among the foodborne bacteria, *Listeria monocytogenes* is a significant pathogen known for its impact on public health and economies. *Listeria monocytogenes* is commonly found in unpasteurized dairy products and ready-to-eat foods, posing a global food safety concern and potentially causing severe health consequences. *Listeria monocytogenes* causes listeriosis in humans and animals, a disease with a high rate of hospitalization and mortality. *Listeria monocytogenes* is challenging to control due to its virulence traits and ability to adapt to antibiotics and adverse conditions encountered during food processing. Researchers are studying alternative methods to reduce the presence of pathogens, with bacteriophages, bacteriocins, and natural compounds, like essential oils being explored. Nonetheless, isolated natural compounds offer a consistent approach compared to essential oils, which can vary in composition. This study investigated the effects of four natural compounds (resveratrol, *p*-coumaric acid, camphor, and linalool) on inhibiting the virulence factors of *Listeria monocytogenes*. Sub-inhibitory concentrations of these compounds were examined for their impact on motility, quorum sensing, biofilm formation, toxin production, tolerance to adverse conditions, and antibiotic minimum inhibitory concentration (MIC). Camphor only inhibited motility, while *p*-coumaric acid reduced the bacterium' hemolytic activity and increased susceptibility to low temperatures without NaCl. Resveratrol inhibited quorum sensing and biofilm formation and enhanced the impact of low temperatures and high osmolarity. Linalool emerged as the most promising compound, inhibiting all tested virulence traits, and potentiating the impact of stress conditions on bacterial survival. These findings highlight the potential of natural compounds in inhibiting *Listeria monocytogenes* virulence factors and their applicability as preservatives in the food industry.

Keywords

Listeria monocytogenes; Resveratrol; *p*-Coumaric acid; Camphor; Linalool; Quorum sensing; Biofilms; Motility; Adverse conditions; Modulation of antibiotics susceptibility

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List of abbreviations

ActA	Actin polymerization protein
ADI	Arginine deiminase
AMP	Ampicillin
ATR	Acid tolerance response
CSP	Cold shock proteins
CTX	Cefotaxime
DMSO	Dimethyl sulfoxide
EPS	Extracellular polymeric substances
ERY	Erythromycin
GABA	γ-aminobutyrate
GAD	Glutamate decarboxylase
GEN	Gentamicin
HSPs	Heat shock proteins
LB	Luria-Bertani
LLO	Listeriolysin O
MIC	Minimal inhibitory concentration
PC-PLC or PLC-B	Non-specific phosphotidylcholine phospholipase C
PI-PLC or PLC-A	Phosphatidylinositol-specific phospholipase C
RIF	Rifampicin
TET	Tetracycline
WHO	World Health Organization

1. Introduction

1.1. Food safety

Food safety is a public health concern since it can affect people around the world. Food contamination is often related with food safety and thus it can cause more than 200 diseases transmitted through food products. World Health Organization (WHO) estimated that 1 in 10 people in the world get sick due to ingestion of contaminated food, this means almost 600 million people per year get sick and about 420 000 of those die every year [1].

These foodborne diseases can easily affect immunocompromised individuals, elderly people, pregnant women and children. In fact, children under 5 years old can carry up to 40% of the foodborne diseases, being responsible for almost 125.000 deaths every year [1,2]. Additionally, another negative influence of these diseases is the impact that they have in national economies, trade of foods, tourism and agriculture industry. The World Bank studied this impact and indicated that the loss associated with foodborne disease in low- and middle-income countries was around US$ 95.2 billion per year, and the annual cost of treating foodborne diseases is about US$ 15 billion [1,3].

These foodborne illnesses are known to be of infectious or toxic nature and are usually caused by bacteria, viruses, parasites, or chemical substances entering the body through contaminated food [4]. A systematic review on food safety and public health analysed 81 full-text articles and by comparing them with each other concluded that mislabelling (38%), microbial contaminations (22%), and chemical contamination (19%) are the most common food safety issues. Furthermore, this study also stated that 21 of 81 analysed reported the presence of pathogenic microorganisms, such as *Salmonella* spp.*, Campylobacter* spp.*, enterohaemorrhagic Escherichia coli, Vibrio and Listeria* spp. in foods, such as eggs, milk, meat, seafood or vegetables and even water. These microorganisms are also mentioned by WHO for being commonly found in the same food products mentioned in the study [2,4].

1.1.1. *Listeria monocytogenes*

The WHO has stated Listeria as one of several bacteria that can cause food contamination. This pathogen is often found in unpasteurized dairy products and ready-to-eat foods. In addition, it can lead to various health consequences and therefore be considered a food safety concern worldwide.[4]. Currently, the genus *Listeria* contains up to 27 different species, being one of them *Listeria monocytogenes* [5]. Additionally, *L. monocytogenes* can also be divided in 13 serotypes, which are separated in 4 lineages,

I to IV, from these the lineages I and II are the more prevalent and virulent [6,7]. These are also the lineages for which more studies about *Listeria* can be found [8].

L. monocytogenes is a Gram-positive, facultative anaerobe, non-sporulating and ubiquitous rod shaped bacterium [6,7], which can be isolated from soil, water, meat, vegetables, fish, processed foods, ready to-eat foods and dairy products. In addition, it can colonize plants and infect mammals and birds [9,10].

L. monocytogenes causes a disease denominated by listeriosis, which is associated with the ingestion of contaminated food and affects mainly immunocompromised individuals, such as those with cancer, leukaemia or acquired immune deficiency syndrome, elderly people, or pregnant women [10]. Listeriosis can appear as sporadic infections or disease outbreaks with a significant mortality rate between 20–30% [11]. The infection in humans can vary, depending on the age of the infected person, the immune system, the amount of ingested bacterial cells and the virulence of the bacteria [12]. Although this disease in most cases is expressed as a mild febrile illness with symptoms, such as fever, muscle aches, nausea, vomiting and diarrhoea that can last some days, it can also develop into a more severe and systemic form of listeriosis that affects primarily fragile and immunocompromised people. In the case of more severe listeriosis, several symptoms may occur, such as headache, cervical stiffness, confusion, loss of balance, convulsions and sepsis, which can last for weeks, and are usually associated with high hospitalization and even fatality [11,12].

Maternal-neonatal listeriosis is associated with increased fetal and neonatal morbidity, leading to fetal loss in up to 25% of cases of infection. This condition is associated with the ability of *L. monocytogenes*, after ingestion, to cross the intestinal barrier, spread through the bloodstream and potentially cross the placental barrier, infecting the fetus [13].

Furthermore, the success of *L. monocytogenes* in inducing infection and causing listeriosis is related to its ability to promote its own invasion through host cells. This ability derives from several virulence factors that contribute to its pathogenicity, but it is also influenced by the bacteria's ability to survive adverse conditions, such as low (0.4 to 4 °C) and high (55 °C) temperatures, osmotic stress, low pH and antimicrobial agents [14]. These stressful conditions may often be found in food production facilities, with the bacterium being able to overcome them and survive, persist and even spread in these environments [11,15].

1.2. Pathogenicity of *Listeria monocytogenes*

The pathogenicity of *L. monocytogenes* is strongly related to its ability to survive in adverse conditions and due to its virulence factors [14]. The adaptability to stresses, such

as low pH or low temperatures, along with their ability to form biofilms, are examples of factors that contribute to the survival of these bacteria in harsh environments. Furthermore, this pathogen can secrete diverse extracellular vesicles that have a relation to its life cycle and infectious process of host cells [16]. The virulence factors that allow the survival and spread of *L. monocytogenes* in adverse conditions, such as biofilms or flagellum motility and the virulence factors related to its life cycle, like LLO, are of most relevance, and should be carefully studied.

1.2.1. Infectious process and virulence factors involved

L. monocytogenes has the ability to facilitate the internalization of host cells and overcome significant barriers within the human host, including the intestinal epithelium, the blood-brain and placental barrier, and thus its able to spread to other organs and systems [17], [18]. This infection process involves several steps such as adhesion and invasion of host cells, internalization, vacuole lysis, intracellular multiplication, and intercellular spread to neighbouring cells [19] (Figure 1).

Upon ingestion through contaminated food, *L. monocytogenes* may successfully survive exposure to high acidity, bile salts, inflammatory responses, and host proteolytic enzymes [20]. After this initial survival, *L. monocytogenes* uses surface proteins known as internalin to adhere to and enter both phagocytic and non-phagocytic host cells [17]. The pathogen's ability to persist within phagocytes, despite their mechanisms for bacterial destruction, contributes to its pathogenicity [21]. Once internalized, *L. monocytogenes* escapes from the phagosomal vacuole with the help of listeriolysin O (LLO) and phosphatidylinositol-specific phospholipase, allowing it to replicate in the host cell's cytoplasm [19]. The organism uses the host cell's actin-based motility machinery to move intracellularly, spreading from cell to cell without exposure to extracellular immune surveillance [22]. Atin polymerization protein (ActA) on the bacterial surface is identified as the key factor that enables intracellular movement within the cytoplasm [23].

Once *L. monocytogenes* is internalized by neighbouring cells, it becomes confined within a double-membrane vacuole from which it escapes with the assistance of LLO and non-specific phosphotidylcholine phospholipase C, allowing it to restart its life cycle[17].

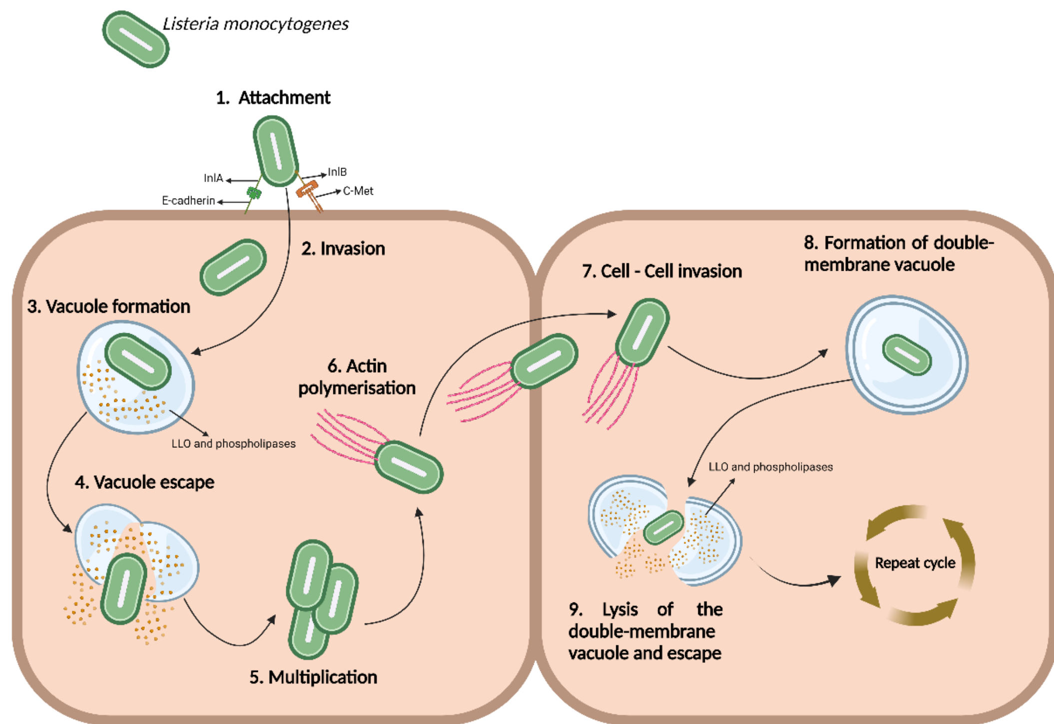

Figure 1. Schematic representation of the infectious process of *L. monocytogenes*. This pathogen enters host cells via receptor-mediated internalization and subsequently enclosed within a vacuole. The integrity of the vacuole compromised by the secretion of two phospholipases, PlcA and PlcB, as well as the pore-forming toxin known as listeriolysin O. This disruption leads to the release of bacteria into the cytoplasm, where they undergo multiplication and initiate actin polymerization. The polymerization of actin facilitates the traversal of bacteria into adjacent cells. Once inside the neighbouring cell, the bacteria reside within a vacuole composed of two membranes from which they escape, thereby perpetuating the infection cycle.

1.2.1.1. Internalin

The first part of the intracellular life cycle of *L. monocytogenes* is adhesion and invasion. These steps have a crucial importance for the disease caused by this pathogen in host. These two phases, adhesion and invasion, are mediated mainly by two internalin subfamilies, the first being composed of large proteins, such as InlA and InlB, and the second by smaller proteins, such as InlC [14].

The adhesion to the host and internalisation into a vacuole formed by the cell is facilitated by InlA and InlB, which are encoded by *inlAB* operon [14,19,24]. The InlA assists in the binding of *L. monocytogenes* and the E-cadherin, a protein expressed on the surface of enterocytes, onto the host which is also the first step of the invasion [14,25,26]. Alternatively, the InlB, which instead of binding to E-cadherin, it binds to the cellular receptor Met, a tyrosine kinase protein. Due to this ability of InlB to bind to this receptor, the range of host-cell targeted by this pathogen gets broader, thus targeting cells types such as hepatocytes, fibroblasts, and epithelioid cells [14,27].

InlC from the subfamily of smaller proteins is produced after internalisation of *L. monocytogens* and is responsible for interact with IkB kinase preventing a proinflammatory pathway [14,28,29].

1.2.1.2. Listeriolysin O (LLO) and phospholipases

When *L. monocytogenes* enters the host cell, it becomes trapped in a single-layer membrane vacuole and is later in the infectious process, surrounded by a double-membrane vacuole [14,30]. The escape of these vacuoles depends on Listeriolysin O (LLO) and phospholipases and is essential for their life cycle, as failure to do so stops the infection.

LLO is a pore forming cytotoxin encoded by the *hly* gene [14,26] and it was one of the first virulence factors to be identified in *L. monocytogenes* due to its ability to cause haemolysis [26,31]. This toxin is responsible for lysing the vacuole membranes and releasing the pathogen into the cytoplasm of the host [14]. The phospholipases that help in this process can be divided in two types the phosphatidylinositol-specific phospholipase C (PI-PLC or PLC-A) encoded by the *plcA* gene and non-specific phosphotidylcholine phospholipase C (PC-PLC or PLC-B) encoded by the *plcB* gene [24,32]. PLC-A or PI-PLC facilitates the exit of *L. monocytogenes* from the vacuole into the cytoplasm of the host cell. To achieve this purpose, this phospholipase plays a complementary role with LLO in the lysis of the primary and secondary vacuole created after the pathogen internalization [24,26]. PLC-B or PC-PLC is a broad range phospholipase that is particularly required for the lysis of the double-membrane secondary vacuole and the primary vacuole in conditions of LLO deficit [24]. Both phospholipases assist *L. monocytogenes* in disrupting the autophagy process that targets dysfunctional organelles and invading pathogens [33].

1.2.1.3. Actin polymerising protein (ActA)

The surface protein ActA, which is encoded by the *actA* gene, allows the recruitment and polymerization of actin, thus allowing bacterial motility within host cells [26,34]. The polymerisation of the actin into filaments with an asymmetric distribution, whick means that the filaments are more concentrated at one polar end of the pathogen. This distribution is responsible for directing *L. monocytogenes* in the direction of the host cell membrane improving intra- and intercellular motility, as well as the cell-to-cell dissemination [14,24,26].

1.2.2. Biofilm formation and flagellum motility

L. monocytogenes has various other virulence factors related to its resistance and propagation, in which the biofilm formation ability and flagellum motility are of relevance [11,16]. This microorganism is capable of attaching to various surfaces, such as stainless steel, polystyrene or glass [11]. Motility is often associated with initial adhesion and colonization and is mediated by the flagellum. The biosynthesis of this depends on temperature, with the flagellum being most active at 30 °C, which leads to abnormal motility at this temperature [16]. This motility is also related to the biofilm development. Biofilms allow the survival of the pathogen in the natural ecosystem, increasing its resistance along the food chain production, and even to the processes of washing and sterilization [11,16]. Thus, the development of biofilms in food represents a serious food safety concern, because it facilitates the entry of the pathogen into the digestive system[35].

1.2.2.1. Flagellum motility

Flagellum motility is important for many food pathogens, as it improves adherence and colonization of surfaces [16,36]. In *L. monocytogenes,* the flagellum plays a crucial role in the initial surface attachment and subsequent formation and development of the biofilm, as it gives an ability to seek and reach otherwise unattainable nutrients[11,16,37].

The flagella expression is regulated by the temperature and makes *L. monocytogenes* abnormally motile below 30°C, but at 37°C it is non motile [38].

1.2.2.2. Biofilm formation

Bacteria can establish multifaceted communities, named biofilms, that facilitate the survival against harsh conditions and the environment [16]. Biofilms in other words are a community of bacterial cells attached to each other or/and a surface and involved by a self-produced matrix of extracellular polymeric substances (EPS) (proteins, polysaccharides, and extracellular DNA) [24,39].

As a result of biofilm formation *L. monocytogenes* can survive and succeed in various surfaces, such as stainless steel, polystyrene or glass. However, the effectiveness of biofilm formation depends on temperature, bacterial strain, incubation time and surface material. For instance, the adequate temperature for the formation of this matrix is 37°C, but *L. monocytogenes* is also capable of forming biofilms at 4°C and 12°C in glass with more efficiency than in stainless steel or polystyrene [11,40].

Biofilm apart from helping the growth in various surfaces, it also plays a role in the survival of this pathogen along food production chain, including to stress induced by washing and sterilization processes, fatty acids, heavy metals, or by hampering the permeation of antimicrobial agents and antibiotics. Some studies even related an increase in biofilm formation with the presence of lactose when compared with the presence of glucose. Thus suggesting that the lactose utilisation capacity of this pathogen may contribute to the survival in food environment [16,21,24,41]. The ability of L monocytogenes to form films on various surfaces is one of the reasons why it is extremely difficult to eradicate them during food storage and processing.

1.2.3. Resistance to antimicrobial agents and antibiotics

Resistance to antimicrobial agents is an important feature of *L. monocytogenes*. In general, antibiotic resistance is increasing due to selective pressure caused by overprescribing of drugs in clinical settings, lack of public knowledge and awareness along with access to antibiotics without prescription, that causes an overflow of antibiotics and leftover antibiotics, decreasing their efficiency [14,42,43].

In the last decades, increasing reports on resistance among *L. monocytogenes* strains have been presented, such as resistance to gentamicin, ampicillin, streptomycin, erythromycin, kanamycin, sulfonamide and rifampin in clinical situations [14,42,44,45]. In strains isolated from food and animal sources resistance to ciprofloxacin, tetracycline, sulfonamide and nalidixic acid were also found [14,46]. Some studies have even established a correlation between exposure to sublethal dosages biocides and heavy metals to antibiotic resistance [24,47].

Most of the resistance encountered is usually due to acquired mechanisms, such as plasmids and conjugation; however, *L. monocytogenes* also has intrinsic resistance such as a lack of affinity for the antimicrobial agent or the role of efflux pumps [48].

Efflux pumps are transmembrane-spanning protein complexes that can transport molecules from the inside of the bacterial cell to the outside of the bacterium. Furthermore, efflux pumps enhance the resistance to antibiotics and antimicrobial agents . In *L. monocytogenes*, efflux pumps, such as Lde, can transport fluoroquinolones, thus conferring resistance to these substances. Another example is the MdrL which allows resistance to ethidium bromide, cefotaxime and heavy metals. [14,24,45,48–50].

Other alternative that contributes to the increase of resistance is the acquired mechanism such as plasmids and the conjugation process. Conjugation is the process of transferring genetic material, which occurs between living bacterial cells that are in direct contact [14,45]. *Enterococci* and *Streptococci* are considered reservoirs of

resistance genes for *L. monocytogenes* due to being presented in the gastrointestinal tract of humans [14,21].

Furthermore, this pathogen has another underlying mechanism that allows for increased resistance. One of them, as mentioned earlier, are biofilms, whose extra polymeric matrix keeps the population of bacteria more protected. Another mechanism is persister cells, these cells are a part of the bacterial population that is dormant in a state of non-division, which potentiates them to resist bactericidal antibiotics and adverse environmental conditions and, in some cases, the host's defence system [24,51].

1.2.4. Persistence in adverse conditions

L. monocytogenes like many other organisms can respond to the environment changes, which is crucial to its survival. Additionally, due to the pathogen ability to acquire resistance to antimicrobial agents or to biofilm formation, it can survive diverse environment changes, however it also has specific mechanisms to deal with adverse conditions such as osmotic pressure shifts, temperature changes and pH extremes [26,52].

The demand for minimally processed foods that preserve the freshness and nutritional quality is increasing, and due to this, various food products are processed with a combination of sublethal stress treatments [26]. These sublethal stresses can enhance the survival of pathogen when exposed to subsequent lethal stresses along the food value chain. Several studies have proven that sublethal stress used in food preservation is responsible for inducing adaptive tolerance to lethal stress treatments[26,53].

So, understanding the mechanisms how *L. monocytogenes* can adapt and overcome the stresses caused by these adverse conditions or sublethal stress may help in finding solutions to this concern.

L. monocytogenes is known for being able to withstand high concentrations of salt (NaCl), as it can grow even in media supplemented with 12% NaCl and tolerate up to 20% [11,54]. Elevated concentrations of NaCl decreases electrochemical potential in the cell membrane interfering with ATP production and can inhibit bacterial growth by diminishing the water activity in the surrounding environment, increasing plasmolysis and hence resulting in decreased intracellular turgor pressure and ultimately inhibiting bacterial growth [11,55,56]. The process by which *L. monocytogenes* responds to osmotic shifts is called osmoadaptation, which consists of two responses, a primary and a secondary. Both the mechanisms help the bacteria in maintaining turgidity and assist in the stabilization of protein structure and function [11,24,57]. The first response of the bacteria to elevated concentration of NaCl is an uptake of potassium ions (K^+) and

glutamate. What allows the first intake of potassium ions are two K^+ transporters, a high affinity KdpABC transporter system, and a low affinity system encoded by the *lmo0993* gene [11,57,58]. The second step is the replacement of the accumulated ions by compatible solutes or osmoprotectants, such as glycine betaine, carnitine proline, proline betaine, acetylcarnitine [11,24,57].

Another environment that can be found in food preservation is an ambient with low pH [11,59]. *L. monocytogenes* meets acid environments in the gastrointestinal tract of the host and in food products. Furthermore, this pathogen can adapt to low pH conditions as it can find them on food preservation methods such as acid sanitation [11,60]. Normally, low pH increases the concentration of hydrogen protons, which causes the inhibition of microbial growth [11,61]; however, this bacterium has three different mechanisms that can used to adapt to this kind of environment.

The acid tolerance response (ATR) is one of the mechanisms, becoming active when it occurs a pre-exposure to a sublethal pH between 5 or 6, and it decreases the susceptibility to lethal pH [11,24].

Another mechanism, which is classified as one of the major mechanisms responsible for the maintenance of the intracellular homeostasis, is the glutamate decarboxylase (GAD) system [62]. This mechanism is responsible for the conversion of cytosolic glutamate to a neutral compound, the γ-aminobutyrate (GABA)[63]. When GABA is synthesized the intracellular level of proton decreases, and increases the pH inside *L. monocytogenes*. Another advantage of this mechanism is that GABA needs to be excreted and this provokes a neutralization of the pH in the environment [63].

Yet another cell system that protects bacteria from low pH is the arginine deiminase (ADI) pathway [64]. ADI, together with two other enzymes, carbamoyltransferase and carbamate kinase are the tree enzymes used in this system that allows the defence from environment with low pH. ADI converts arginine to citrulline and ammonia, then carbamoyltransferase catalyzes the conversion of citrulline to carbamoylphospate and ammonia. The level of ammonia produced as a by-product of the system combines with intracellular protons maintaining the intracellular level of the cytoplasmic pH, thereby protecting the *L. monocytogenes* cell from adverse acidic extracellular environments. Additionally, the carbamate kinase allows synthesizes of adenosine triphosphate using carbamoylphosphate and adenosine diphosphate [24,65].

L. monocytogenes also has an adaptability to different temperatures. High temperatures are known as a method used in food preservation and production to prevent pathogen growth [11,54,66]. *L. monocytogenes* however resists temperatures above 45°C making the efficacy of this method questionable [11,67]. To achieve total

inactivation of this pathogen by thermal treatment, it is needed a minimum temperature of 55°C for at least 10 minutes [11,68].

The resistance to thermal treatment depends on various factors, such as cells age, previous growth and stress conditions, composition of food and strain serotype [11,69]. In this line, it is known that cells in the stationary growth phase are generally more resistant to thermal stress [11,69] and that the composition of food can be related to the heat resistance, being shown that certain components in some food products can protect the bacteria [11,70]. Furthermore, cross-adaptation should also be considered since heat tolerance can be induced by the exposure of acid or oxidative stresses [26,71]. These factors play an important role in heat resistance, however *L. monocytogenes* has specific methods of response to thermal treatment. In response to the heat stress this bacterium increases the transcription of heat shock genes that codes for three classes of heat shock proteins (HSPs) [26,72]. From these classes, the class I and III are direct responses to heat shock and the class II proteins are a more overall stress response of *L .monocytogenes* [26,73]. The function of these HSPs is to repair partially denatured proteins and prevent intracellular aggregation [11,74]. Under normal temperature, the expression of these proteins is inhibited by repressors, in turn when the temperatures rise those repressors are inhibited allowing the expression of the proteins [26,72].

Due to the mechanisms mentioned above, *L. monocytogenes* can adapt to high temperatures, but this pathogen can also overcome low temperatures. Although *L. monocytogenes* has an ideal growth temperature around 37°C it has also the capacity to survive and grow in low temperatures, such as 0.4°C or 4°C [11,75]. This ability to grow in such temperatures allows this pathogen to be found in food products storage in refrigeration [11,76]. The mechanisms used by this bacterium to adapt to these low temperatures, include a decrease in the metabolism of the bacterial cells, changes in cell membrane composition, expression of cold shock proteins, and uptake of cryoprotective compounds from the environment [11,54]. These temperatures reduces the membrane lipid fluidity, and to counteract this effect *L. monocytogenes* changes its membrane lipid composition, increasing the concentration of unsaturated fatty acids, which prevents formation of a gel-like state that may result in leakage of cytoplasmic content and helping the nutrient transport [11,54]. Another mechanism used by this bacterium are the cold shock proteins (CSP), which is a mechanism similar to the one used in the high temperature adaptation. These proteins assume the role of molecular chaperones, allowing replication, transcription, translation, and protein folding at low temperatures [24,54,77]. Beyond that, the intake of cryoprotectants, such as glycine betaine, and carnitine found in various foods helps the survival of this bacterium [24,54]. Furthermore, increasing the intracellular solutes levels help to reduce the loss of

intracellular water at low temperatures [24,60]. An investigation about this adaptation observed that a mutant *L. monocytogenes* strain for glycine betaine and carnitine uptake systems failed to accumulate any of the two and, thus, were not able to survive in low temperatures [24,78].

All of these mechanisms allow *L. monocytogenes* to survive and grow in conditions very often used in food processing and storage, which makes them a target for study.

1.3. Alternatives to combat *L. monocytogenes*

As mentioned earlier, *L. monocytogenes* can survive and adapt to antibiotics often used in the treatment of listeriosis, as well as to antimicrobial agents and adverse conditions used in the storage and processing of food products. So, finding alternative methods to reduce this pathogen presence is an objective of many studies nowadays. Alternatives to overcome *L. monocytogenes* in food products have been reported, such as the use of bacteriophage as biocontrol, bacteriocins or natural compounds, such as essential oils [14].

Bacteriophages are viruses that can kill bacteria and can be classified as candidates for biocontrol of *L. monocytogenes* [14], these bacteriophages may specifically provoke the lysis of this pathogen without affecting the microflora of the consumer and other bacteria in the food [14,79]. Based on these works some commercial bacteriophage-based products have already been developed and are being used in countries like the Netherlands and the US [14,79]. Studies regarding bacteriophages and *L. monocytogenes* were already made and their effect was even studied against the formation of biofilms of this pathogen and as a biocontrol agent in foods [80].

Other alternatives are bacteriocins that consists of ribosomal synthesised antimicrobial peptides synthesized by bacteria capable of forming pores on the membranes compromising the integrity of the target cell [14,81]. There is studies regarding bacteriocins and *L. monocytogenes* and even some already study the resistance of this pathogen against the this alternative [82,83]

Beyond that, essential oils may also have an use potential due to its antimicrobial activity, these oils are extracted from plants and spices [84]. These essential oils have the advantage of being alternatives to synthetic/chemical antimicrobials that are losing popularity nowadays due to being harmful to the health of the consumer. Essential oils are a mix of 20-60 natural compounds at different concentrations that results in highly volatile soluble liquids. Due to their constituents they have a wide range of bioactivities, such as antimicrobial, or antioxidant activities [84,85]. The components of the essential oils (natural compounds) can be divided in several groups, such as terpenes, terpenoids,

aldehydes, phenols, being the first two mentioned the most abundant [85]. Nowadays there is an abundant number of review studies, [84,85] that allow to see what kind of essential oils were already studied and their effects in diverse pathogens like *L. monocytogenes* allowing thus a better understanding in the matter. Nonetheless, these oils are not perfect and have some problems, such as having differences in composition dependent on the climate, harvesting stages, planting, and preparation methods, plant age and genetics of the same [86]. To be used as a food preservative in substitution of chemical antimicrobials, the product needs to be consistent, which is not a feature of the essential oils.

In recent years isolated natural compounds often found as being components of essential oils, emerged as a possible alternative.

1.3.1. Natural compounds

The natural compounds are an alternative that surfaced in recent years in response to the concern of options in food safety and combat to food pathogens, such as *L. monocytogenes*. Unlike the essential oils whose composition can easily vary due to climate, plant age and other factors, the isolated natural compounds do not have this concern. Another positive side of using specific compounds is that essential oils have a variety of compounds in their composition that can alter the flavour and other characteristics of the food, however using specific or isolated compounds can diminish the possible interactions with the food. Less interactions may lead to a lower possibility of alteration of flavour or other characteristics [85,87]. Another advantage of the natural compounds is that it can combine with other substances, such as antibiotics and restore or increase their effect [88,89]. In addition, several studies on natural compounds have reported antibacterial properties and ability to inhibit virulence factors, such as biofilm formation and quorum sensing, in various compounds studied [89,90]. Besides the bactericidal or bacteriostatic activity of compounds, other ways to combat pathogens are being studied, considering diverse approaches, with one possible path being the interference with bacterial virulence. These kinds of strategies that reduce the virulence impact of the microorganism instead of causing the death of the pathogen are more common now. These strategies focus on inhibiting adherence to surfaces, tissue invasion, toxin production and/or interference with gene regulation of other virulence factors. This strategy may even present the advantage of contributing to the development of the immune system [91].

These antibacterial and antivirulence properties were also observed in studies on *L. monocytogenes*. A review by Kawacka et al., 2020 [92] focused on the plant derived compounds and their activity against *L. monocytogenes*. Several compounds were found

to have some sort of antibacterial effect, such as eugenol, cinnamaldehyde, thymol, citral, geraniol, citronellol, limonene, carvacrol [92]. Most of these examples also have anti-virulence properties, with compounds such as cinnamaldehyde, carvacrol and thymol significantly lowering adhesion and invasion in Caco-2 cells, motility, haemolysis of sheep red blood cells and phospholipase activities [93]. Additionally, to this antibacterial and anti-virulence properties there are also studies about natural compounds and their synergism with other substances, such as antibiotics, but also with various treatments like heat treatment and bacteriocins, which is reviewed in the study performed by Kawacka et al., 2020 [92]. Although several natural compounds have already been studied against several bacterial species, there are still some compounds still lack research in *L. monocytogenes* or in some specific areas of the pathogenicity of this bacterium, like the four compounds which are the focus of this study.

1.3.2. Resveratrol

Resveratrol (3,5,4'-trihydroxystilbene) is a naturally occurring polyphenolic antioxidant belonging to the stilbene family that in recent years attracted attention due to its health benefits, such as anti-inflammation, anti-carcinogenesis, anti-obesity, anti-diabetes type 2, anti-aging, cardiovascular protection, neuroprotection, and antimicrobial properties [94]. Focusing on the antimicrobial properties of resveratrol, this compound showed to have antifungal and antibacterial activity against various species, such as *Bacillus cereus, Staphylococcus aureus, Enterococcus faecalis Listeria monocytogenes, Escherichia coli, Klebsiella pneumoniae, Pseudomonas aeruginosa* and many other species (reviewed by Vestergaard et al., 2019 [94]). This same work also reviewed the anti-virulence properties of this compound and summarized it. In short, resveratrol has been shown to inhibit biofilm formation, antimotility, anti-toxin properties and interference in the quorum sensing in different bacteria. In *L. monocytogenes* resveratrol already showed antibacterial, antibiofilm, anti-quorum sensing properties and synergy with antibiotics [94,95]. However, there is a lack of studies that relate some virulence factors and characteristics of L. monocytogenes with resveratrol, thus giving an opportunity to study the interactions of this compound with this bacterium.

1.3.3. *p*-Coumaric acid

Coumaric acid is a phenolic acid of the hydroxycinnamic acid family that is often related to the secondary metabolism of diverse plants, fruits, vegetables, cereals, and mushrooms [96]. This compound also exhibits various bioactivities, including antioxidant, anti-inflammatory, antimutagenic, anti-ulcer, antiplatelet, anti-cancer activities and antimicrobial activity [97]. Although coumaric acid presents antimicrobial

activity, there is some level of contradicting results. Some studies like Lou et al., 2012 [98] obtained anti-bacterial activity against *Streptococcus pneumonia, Staphylococcus aureus, Bacillus subtilis, Escherichia coli, Shigella dysenteriae* and *Salmonella* Typhimurium, with the authors describing the compound's mechanism of action as the interaction with the cell membranes and DNA, disrupting the bacterial cell membranes and inhibiting cellular functions leading to cell death. However, another study found the antimicrobial properties against *S. aureus* were weaker and there was no inhibition on *E. coli*. Nevertheless, researchers also studied the capabilities of anti-virulence of coumaric acid showing that it was capable of inhibiting different virulence factors such as quorum sensing in *Enterococcus faecalis* [99], motility and biofilm formation on *Salmonella* Enteritidis [100] and even inhibited the RecA protein functions and SOS response in *L. monocytogenes* [101].

Coumaric acid has some anti-virulence properties against some pathogens, however, studies between coumaric and *L. monocytogenes* are few, so the effects of this compound on the pathogenicity of this pathogen is still unknown.

1.3.4. Camphor

Camphor is a terpenoid compound obtained from Camphor tree (*Cinnamomum camphora),* which is an ornamental plant with high value in Asia. Camphor has already been used as treatment to inflammation, infection, congestion, muscle pain, and irritation in various regions of Asia [102]. Due to these characteristics and concern for food safety, camphor and its antimicrobial and anti-virulence properties began to be investigated. However, research about the compound itself is little when compared to the base camphor essential oils. The studies regarding camphor itself are about the antifungal effect on *Candida* species [103]. Against *Candida*, camphor has a strong effect inhibiting the growth, biofilm formation and the efflux pumps [103,104].

Against *L. monocytogenes* there are no studies regarding the effect of camphor, making it this way the first study regarding camphor and this pathogen.

1.3.5. Linalool

Linalool (3,7-dimethyl-1,6-octadien-3-ol) is a monoterpene acyclic tertiary alcohol used in diverse utilities as a fragrant compound in perfumes, lotions, soaps, shampoos as well as detergent and cleaners [105]. This compound has diverse bioactive properties, such as anticancer, neuroprotective, antidepressant, hepatoprotective and antimicrobial activities [18]. Linalool is also recognised as safe by the Food and Drug Administration [17]. In recent years the use of linalool in food safety emerged due to its antimicrobial and anti-virulence capabilities [105].

This compound showed to have antimicrobial activity against both bacteria and fungi, namely *S. aureus, L. monocytogenes, Salmonella* sp., *E. coli, Pseudomonas* sp. and *Candida albicans*, *Aspergillus brasiliensis* [105]. Beside the antibacterial effect, linalool also showed anti-virulence properties against diverse foodborne pathogens, like *L. monocytogenes* and *P. aeruginosa,* in which biofilms and quorum sensing were inhibited, respectively [106,107]. However, apart from this biofilm inhibition and a study that observed that linalool targeted cell membranes and ribosomes in *L. monocytogenes* [108], little more of the virulence and resistances of this pathogen against this compound were studied. This gives an opportunity to study the effect on response and adaptation to adverse conditions and other virulence factors such as LLO.

2. Objectives

Foodborne pathogens and food safety have become a growing concern, with the emergence of more resistant pathogens and the decrease in methods to combat them. Thus, new approaches to overcome this concern are needed, which may include the use of natural compounds. A foodborne pathogen known to contaminate food and cause disease is *L. monocytogenes*. Given the risks associated with the consumption of food contaminated with *L. monocytogenes*, the study of the potential of the natural compounds as antimicrobial or antivirulence agents will be of the utmost importance. Thus, this study aims to analyse the impact of four specific natural compounds (resveratrol, p-coumaric acid, camphor, and linalool) on the virulence of *L. monocytogenes*.

Considering the global aim, the following specific objectives were established:

i. To determine sub-inhibitory concentrations for each natural compound that may be used in the study of their anti-virulence effect on *L. monocytogenes*;

ii. To study the effect of natural compounds in virulence factors of *L. monocytogenes*, such as biofilm, motility and haemolytic activity;

iii. To evaluate the effect of the natural compounds on increasing the susceptibility of *L. monocytogenes* to adverse conditions;

iv. To study the potential modulation of antibiotics susceptibility in *L. monocytogenes* by the natural compounds under evaluation.

3. Materials and methods

3.1. Natural compounds

In this work the anti-virulence properties of linalool (Sigma-Aldrich, United States of America), camphor (Sigma-Aldrich, Germany), *p*-coumaric acid (Sigma-Aldrich, United Kingdom) and resveratrol (TCI, Belgium) was evaluated. All compounds except linalool were conserved in a stocks solutions solved in dimethyl sulfoxide (DMSO).

3.2. Microorganisms and culture medium

The activity of the natural compound was evaluated against *Listeria monocytogenes* LMG 13305 serotype 4b from BCCM/LMG collection (Belgium). The microorganism was conserved in cryogenic tubes at -80 °C in adequate medium with 20% glycerol. The strain was cultured in Tryptic soy agar (TSA, VWR International, Belgium) at 37 ºC for 24 h prior to every assay.

3.3. Minimal inhibitory concentration (MIC) determination

To determine the MIC of the various natural compounds under test against *L. monocytogenes* LMG 13305, the microdilution method described by Carvalho et al., 2023 [109] was used. Thus, for each compound a solution was made in DMSO concentration and diluted in Tryptic soy broth (TSB, VWR International, Belgium) assuring a maximum solvent final concentration of 2% (v/v).

In the case of linalool, a concentration range of 10 to 0.039 mg/mL was evaluated, for resveratrol the concentration ranged from 0.625 to 0.002 mg/mL, in the case of coumaric acid ranged from 5 to 0.019 mg/mL and lastly camphor ranged from 1.25 to 0.005 mg/mL. To produce these concentrations, successive dilutions (1:2) with TSB were made in 96-well plates.

A bacterial inoculum was prepared by direct suspension of an isolated colony to a turbidity of 0.5 McFarland in 0.85% (w/v) NaCl, followed by dilution in TSB to obtain a final cell concentration of 5×10^5 colony forming units (cfu/mL), in a final volume of 100 µL. A growth control was also made with 50 µL of TSB and 50 µL of bacterial inoculum and a negative control with only 100 µL of TSB.

The 96-well plates were incubated at 37 °C for 24 hours (h) and the MIC value was assumed to be the lowest concentration with no bacterial growth to the naked eye. This experiment was performed at least three independent times.

3.4. Growth curves evaluation

In this work, non-bactericidal concentrations of the natural compounds were required to ensure that the expected anti-virulence effect was not caused by cell death, but rather by the effect of the compounds. Therefore, sub-MIC concentrations were evaluated in growth curves determination. Thus, firstly a culture was prepared by suspending a colony, from a 24-hour preculture of *L. monocytogenes,* in 10 mL of TSB, which was then incubated at 37 °C for 16 hours with shaking at 250 rotations per minute (rpm). After incubation, the optical density of the culture was measured at 600 nm (OD_{600nm}) and the cell suspension was adjusted in TSB medium to obtain an OD_{600nm} of 0.1, which corresponds to about 2×10^8 cfu/mL.

Solutions of each compound, resveratrol, coumaric acid and camphor were prepared to obtain a final concentration corresponding to 1/8 x MIC, 1/16 x MIC and 1/32 × MIC for linalool.

For the growth curve, solutions were added to the inoculum resulting in a final concentration of 10^7 cfu/mL in a total volume of 1 mL. The maximum concentration of DMSO present was 0.5% (v/v).

A growth control and solvent control (DMSO) were also performed, where the solvent control had a concentration of 0.5%(v/v), the maximum percentage of DMSO used in the assays. The cultures were incubated at 37 °C for 24 h and cell counts were made at 0, 3, 6, 9, 12 and 24 h by the drop plate method. Thus, successive dilutions (1:10) in a 96-well plate were made of each sample, then 10 µL of each dilution were then applied to TSA plates, in triplicate, and incubated at 37 °C for 24 h for following counting. The assay was made at least three independent times.

3.5. Quorum sensing inhibition

To better understand how the natural compounds under study can inhibit quorum sensing, a biosensor strain, the bacterium *Chromobacterium violaceum* ATCC 12472, and its production of violacein were used [110].

Thus, an overnight culture of *C. violaceum* was prepared using Luria-Bertani (LB) medium by suspending an isolated colony of *C. violaceum* from a 24 hour culture in LB agar at 30 ºC, and was incubated at 30°C for 16 hours with agitation at 250 rpm. After this step, the $OD_{600\ nm}$ was measured, and the inoculum was adjusted in LB medium to an $OD_{600\ nm}$ of 0.02. The adjusted inoculum was added to a 48-well plate, together with the compound's solutions to reach a final concentration of 1/16 and 1/32 × MIC for linalool and 1/8 × MIC for the resveratrol, coumaric acid and camphor. Simultaneously, a growth control, containing only LB and inoculum, and a solvent control with a maximum concentration of 0.06%(v/v) were prepared in the

48-well plate. For all the assays, wells were made in triplicate in a final volume of 1 mL with a final cell concentration of 1×10^6 cfu/mL. The plate was then incubated at 30 °C for 48 hours.
The inhibition of quorum sensing was correlated with the production of violacein, thus 750 µL of each well was centrifuged at 6800 ×g for 3 minutes, followed by the removal of the supernatant, 750 µL of DMSO were added to extract the produced violacein. After extracting the violacein by vortexing, another centrifugation was made at 8000 ×g for 5 minutes obtaining a pellet of cells and a supernatant with violacein. From the supernatant, 200 µL was taken and added in triplicate to a 96-wells plate. The cell pellet was used to assess if the inhibition of quorum sensing occurred without the inhibition of the *C. violaceum*. For that, the pellet was resuspended in 750 µL distilled water and 200 µL was transferred to other 96-well plate, in triplicate. The absorbance of both 96-well plates was measured in a microplate reader (Bio-Rad, xMark, USA) with a wavelength of 585 nm for the violacein production measurement and at 600 nm for cellular density analysis. This assay was made in triplicate for at least three independent times.

3.6. Evaluation of motility of *Listeria monocytogenes*

In order to verify the potential inhibitory role from the natural compounds studied in this work in the motility of *L. monocytogenes,* this feature was evaluated accordingly with the previously described [111]. Firstly, TSA plates containing 0.3% (w/v) of agar and natural compounds in the concentrations being tested were prepared. A solvent control with a 0.128 % (v/v) of DMSO and a motility control were also made.

As previously described, an overnight culture was prepared, and its optical density was adjusted to a $OD_{600\ nm}$= 1. From this suspension, 5 µL was pipetted to the centre of the previously prepared TSA plates. The plates were the incubated at 30°C for 72 hours and measurements of the halo diameter (mm) taken at 24, 48 and 72 hours. This assay was made at least three independent times.

3.7. Inhibition of biofilm formation

To understand whether there was an inhibitory effect of the compounds under study on the formation of *L. monocytogenes* biofilms, the following assay based on [109,112] was performed, at least three independent times, with some modifications.

After an overnight culture, the inoculum was adjusted to a $OD_{600\ nm}$ = 0.1 (~10^8 cfu/mL). The adjusted inoculum was added to 48-well plate together with the

compound's solutions, thus reaching a final concentration 1/16 and 1/32 × MIC for linalool, and 1/8 × MIC for all remaining compounds. Simultaneously, a growth control containing inoculum and TSB and a solvent control with a maximum concentration of 0.06%(v/v) of DMSO were prepared. In all the assays, quadruplicates wells were made on a 500 µL final volume. The plates were incubated at 37 °C for 24 h. After the incubation, the wells were washed carefully two times with 500 µL of distilled water and then fixed with 500 µL of methanol for 20 min. Methanol was removed, the plates allowed to dry and crystal violet at 0.1 % (w/v) was added for 10 min. After the removal of crystal violet, the wells were washed with distilled water three times and 500 µL of acetic acid 33 % (v/v) was added to dissolve the stain. The plate was then measured at 570 nm in the plate spectrophotometer (Bio-Rad). This assay was made at least three independent times.

3.8. Cytotoxicity of natural compounds in humans erythrocytes

The potential cytotoxicity of the natural compounds under study was evaluated by assessing the haemolytic activity of the compounds in human erythrocytes.

Thus, the compound's cytotoxicity was assessed in 96 U-well plates with human erythrocytes. The human erythrocytes were collected from one healthy volunteer, separated by centrifugation, washed three times with PBS and a stock suspension of 20%(v/v) in PBS was made. This suspension was stored at 4 °C for a maximum of 4 days. For each natural compound and for the solvent control, a range of concentrations in PBS was tested (the same ranges used for MIC determination) by performing double dilutions in the 96-well plates. After dilutions were made, 100 µL of human erythrocytes at a concentration of 2% (v/v) were added to the 100 µL of natural compound in each well for a final volume of 200 µL. The plates were then incubated at 37 °C for 24 hours.

At the end of the incubation time, the plates were centrifuged at 1000 ×g for 5 minutes. This centrifugation step allowed a supernatant to be extracted into another flat bottom 96-well plate to read the absorbance at 543 nm. A positive control (1%(v/v) Triton X 100) and a negative control (PBS) were also made.

3.9. Study of the inhibitory capabilities of natural compounds in the haemolysis of humans erythrocytes caused by *Listeria monocytogenes*

One of the aims of this work was to see if natural compounds could inhibit the expression of LLO though the haemolysis of human erythrocytes. For this purpose, haemolytic assay based on [113] was performed with some modifications.

An overnight culture and solutions of each natural compound were prepared, such as previously described. The overnight culture was then used to initiate a new culture in TSB containing the subinhibitory concentration of each natural compound, and a final cell concentration of 10^6 cfu/mL. After this step, a 10-hour incubation at 37 °C was carried out. After this period of incubation, the cultures were centrifuged at 9000 ×g, with a temperature of 4 °C for 10 minutes, and 100 µL of the supernatant of each culture was collected and added to 100 µL of 2% (v/v) human erythrocytes in a 96-well plate, after which an incubation at 37 °C for 30 minutes occurred.

After this brief incubation, the plates were centrifuged at 1000 ×g for 5 minutes and the supernatant was transferred to other 96-well plates to be read the absorbance at 543 nm. A solvent control, positive control (unexposed bacteria), negative control (only medium with compounds) and a positive total haemolysis control (1% (v/v) Triton X 100) were also made. The assay was performed in triplicate and three independent times.

3.10. Evaluation of tolerance to adverse conditions

As some natural compounds may affect the tolerance of *L. monocytogenes* to adverse conditions, such as osmotic stress, low pH, high and low temperature, the following assays were performed, at least three times independently [111,114].

As common steps for preparation of the assays, an overnight culture was prepared such as previously described and adjusted to $OD_{600\ nm}$=0.1. From this suspension, 30 µL were added to glass tubes with 2970 µL of TSB with 1/16 and 1/32 × MIC for linalool and 1/8 × MIC for resveratrol, coumaric acid and camphor for a final concentration, and adjusted to the different stresses to be analysed. A growth control and a solvent control (DMSO 0.06%(v/v) were included. For all the assays described below, the sample analysis was performed by assessment of viable counts by taking a sample, performing decimal successive dilutions with NaCl 0.85% (v/v) TSB and using the drop plate method in TSA plates.

3.10.1. Osmotic stress

In the osmotic stress assay, the tubes containing TSB and compounds were further supplemented with 12% (w/v) NaCl. The tubes were incubated at 37 °C for 24 hours and measurements were taken at the beginning of the incubation period (0 hours) and at the end of incubation (24 hours).

3.10.2. Low pH

For the low pH conditions, the TSB medium used was acidified with hydrochloric acid to a pH of 2.4. The tubes in this case were incubated for at 37 °C 15 minutes and samples were taken at 0, 5, 10 and 15 minutes.

3.10.3. High temperature

In this assay, the tubes with the culture and the compounds were incubated at 55 °C for 45 minutes, in a water bath. Samples were collected at 0, 15, 30 and 45 minutes.

3.10.4. Low temperature

The effect of low temperature in presence of the natural compounds under study was tested by incubating cryogenic tubes with a final volume of 1 mL of TSB with 1/16 and 1/32 × MIC for linalool and 1/8 × MIC for resveratrol, coumaric acid and camphor of final concentration, and a final cell concentration of 10^6 cfu/mL. Then, the tubes were incubated at 4 °C for 196 days with periodic measurements. Furthermore, the effect of osmotic stress at low temperature and in presence of the compounds was also studied. Beyond that, a parallel set of assays was further supplemented with 12% NaCl (w/v) were performed. Growth and solvent controls were also included.

3.11. Modulation of antibiotic MICs in the presence of natural compounds against *L. monocytogenes*

To assess whether natural compounds had a modulating effect on antibiotic susceptibility in *L. monocytogenes* [115], MIC of six antibiotics (Ampicillin (AMP) (NzyTech), Cefotaxime (CTX) (Sigma-Aldrich), Gentamicin (GEN) (Sigma-Aldrich), Erythromycin (ERY) (Sigma-Aldrich), Tetracycline (TET) (Sigma-Aldrich) and Rifampicin (RIF) (Sigma-Aldrich)) were evaluated in the presence and absence of subinhibitory concentrations of natural compounds, specifically 1/4 and 1/8 × MIC for all compounds, meanwhile linalool was also tested at 1/32 × MIC concentration. For this, the method described in 3.1 was applied with some adjustments. Briefly, the MIC of antibiotic was determined by performing double successive dilutions of each

antibiotic in a volume of 25 µL and adding 25 µL of a solution of each compound to each well, finally 50 µL of the adjusted inoculum was added to achieve a final cell concentration of 5×10^5 cfu/mL, in a final volume of 100 µL. After the incubation, the MIC in presence of subinhibitory concentrations of the natural compounds was determined. Positive and negative growth controls were performed.

3.12. Statistical analysis

The statistical analysis was performed by using the GraphPad Prism v8.0.2 software. Using the one-way ANOVA statistical test and t-student. The t-student test, with a 95% confidence interval and considering as statistically significant the values of $p<0.05$.

4. Results and discussion

4.1. Study of the antimicrobial activity of the natural compounds on *Listeria monocytogenes*

One of the central points of this work was to understand the antimicrobial activity of a group of natural compounds on the virulence factors of *L. monocytogenes*. For this, the MIC was first determined to better understand what is the minimal concentration that can inhibit the bacterial growth. Thus, allowing as well to determine the sub-inhibitory concentrations of each natural compound to be used in the following assays.

The results of the MIC determination are shown in Table 1. Regarding the phenolic compounds, in the case of resveratrol a MIC value of 0.156 mg/mL was obtained, a value close to other MIC values obtained in other works that studied the effect of resveratrol in *L. monocytogenes* [116]. For the other phenolic compound tested, coumaric acid presented a MIC value of 2.5 mg/mL, such as previously described in other works involving coumaric acid and *L. monocytogenes* [117].

Table 1. Values of the minimal inhibitory concentration (MIC) in mg/mL for different compounds for *L. monocytogenes* LMG 13305.

Natural compounds	(MIC) (mg/mL)
Resveratrol	0.156
Coumaric Acid	2.500
Camphor	>1.250
Linalool	>10.00

For camphor, a higher value than 1.25 mg/mL was observed, which can be in accordance with another study in *L. monocytogenes* where the MIC value of camphor was 2.5 mg/mL [118]. Concerning linalool, a value higher than 10 mg/mL was obtained, but in other studies, the MIC values of linalool obtained were very different, being lower than the one obtained in this work [119]. This difference may be related to the volatility of the compound in question. To validate the subinhibitory effect of the selected concentrations to be used on the following studies, and thus assuring that the potentially observed effect and inhibition of virulence is caused by the compounds and not by cell death, the effect of sub-MIC of the compounds in *L.*

monocytogenes was evaluated through growth curves (Figure 2). Analysing the graph, the only natural compounds that show any significant influence in bacterial growth was 1/16 × MIC of linalool at 6, 9 and at 24 hours ($p<0.05$) and 1/8 × MIC of coumaric acid at 24 hours ($p<0.01$). Resveratrol and camphor at 1/8 ×MIC did not have any significant difference in bacterial growth of the strain.

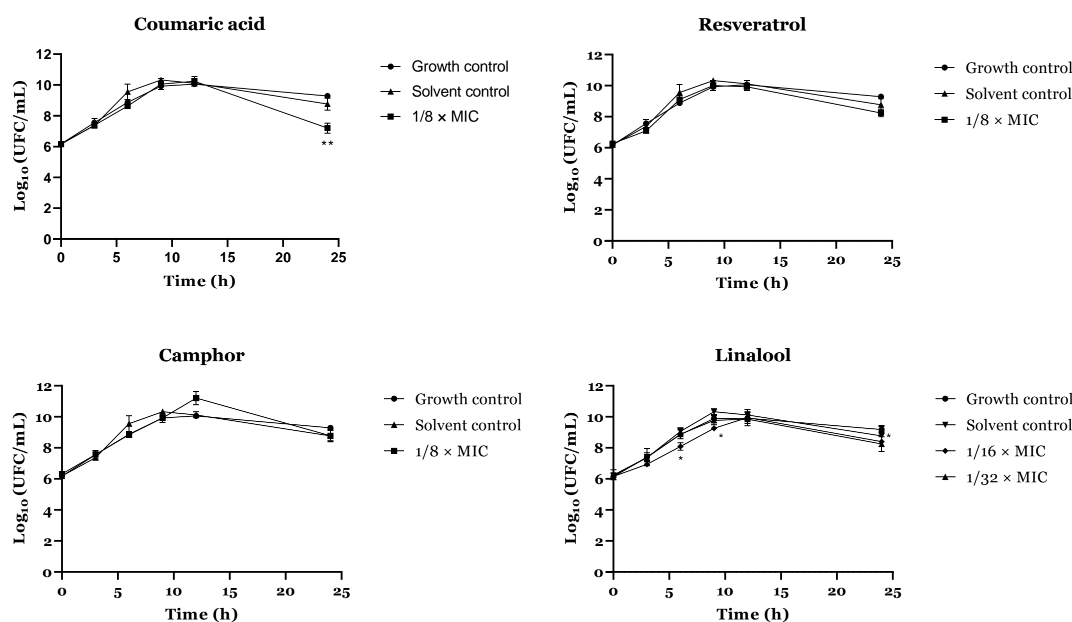

Figure 2. Growth curves for *L. monocytogenes*, incubated with four different natural compounds, with respective controls. The results are presented as the mean ± standard deviation. Asterisks represent significant differences determined by t student test in comparison to growth control. * ($p<0.05$); ** ($p<0.01$).

4.2. Natural compounds on the virulence of *Listeria monocytogenes*

In recent years, several studies have shown that natural compounds are a possible alternative approach against bacterial infection and the food safety concern. Natural compounds have shown in various works noticeable antimicrobial and anti-virulence properties [89,90,92], gaining popularity as an alternative or adjuvants to antibiotics [88,89].

In this work, one of the objectives was to evaluate how natural compounds could affect the virulence factors of *L. monocytogenes* and function as anti-virulence substances. One of the main focuses of this alternative is the attenuation of several virulence factors, such as quorum sensing, motility and biofilm formation, giving to the immune system a better opportunity to respond when infected [91].

4.2.1. Quorum sensing inhibition

One of the factors that can be affected by anti-virulence substances is quorum sensing, this factor is a mechanism used by several bacteria to coordinate community behaviour and may be related to other virulence factors, such as luminescence, sporulation, motility, biofilm formation and the secretion of virulence factors [107,120,121].

To evaluate the effect of natural compounds on quorum sensing, the bacterium *Chromobacterium violaceum* was used. This bacterium was used as a biosensor due to the production of the violacein pigment. When quorum sensing is inhibited, violacein production is also inhibited [110]. Thus, all subinhibitory concentrations of natural compounds were tested, with different profiles being obtained (Figure 3).

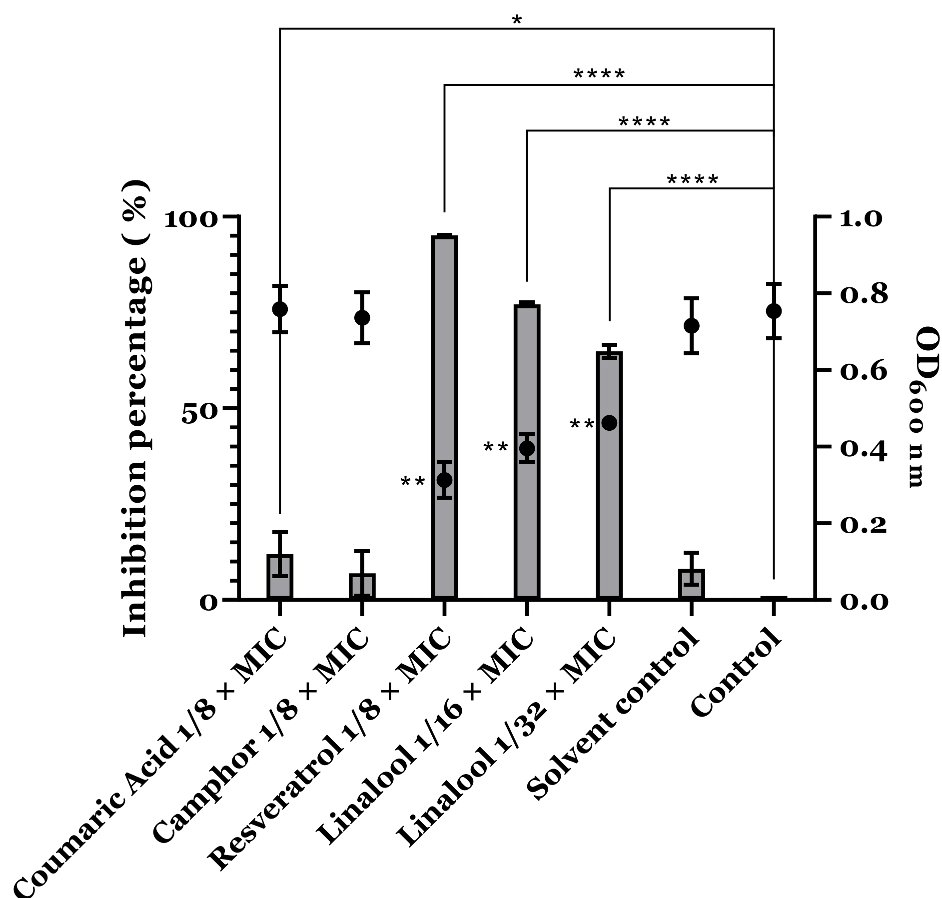

Figure 3. Quorum sensing inhibition, represented by violacein percentage of inhibition, and microbial density (OD_{600nm}) by exposure to four natural compounds. The results are presented as the mean ± standard error of the means. Asterisks represent significant differences, in comparison to the control (t student test). * ($p<0.05$); ** ($p<0.01$); ****($p<0.0001$).

As can be seen in figure 3, the highest percentages of inhibition of quorum sensing were obtained from exposure to resveratrol 1/8 × MIC and both concentrations of linalool. The linalool concentrations showed a significant inhibition of violacein

production of 64.9 and 77.1% ($p<0.0001$) and resveratrol of 95.1% ($p<0.0001$). From the obtained values of linalool, an increase in the inhibition in violacein production can be observed along with the rise in linalool concentration.

In turn, 1/8 × MIC coumaric acid and 1/8 × MIC camphor showed inhibition values compared to the ones obtained to solvent control, with coumaric acid showing 12.0% of violacein production reduction and camphor 6.9%, without statistical significance in relation to the control ($p<0.05$). The solvent control (DMSO) showed no statistically significant inhibition when compared to control.

The anti-quorum sensing ability of resveratrol has been recognized in other studies and it has even been used as a positive control in several studies. In fact, the 95.1% of inhibition obtained in this study confirms the quorum sensing inhibition properties that were also shown in other studies [122]. However, a significant decrease in cell density ($p<0.01$) can also be observed along with the high inhibition value obtained. Thus, a possible explanation for this high inhibition of violacein production may be related to the death or inhibition of the growth of *C. violaceum*. Although, there is a lethal effect of the compound, it cannot be the only justification for the inhibition noticed. The mechanism for which resveratrol affects *C. violaceum* is still uncharacterised; however, Wang et al. (2006) suggested that the anti-quorum sensing activity is due to resveratrol's ability to mimic some molecules used in this system [94,123].

Linalool was another case where a significant decrease in cell density occurred ($p<0.01$), happening for both concentrations, but being more noticeable with 1/16 × MIC (the higher concentration). One reason that can justify the elevated inhibition was the reduction in cell density that occurs due to growth inhibition or death of the microorganism, although the cell death is not proportional to the inhibition. Additionally, another reason that can justify the remarkable inhibition in violacein production is that linalool has the capability to interfere synergistically with AHL synthase or receptors LuxR of the *C. violaceum* [124].

The coumaric acid had a small inhibition of 12.0% of quorum sensing. This value can be justified by a study carried out by Bodini et al. (2009) [125] that, although coumaric acid reveals an inhibition in quorum sensing in *C. violaceum*, concluded that, depending on the strain of *C. violaceum* and the concentration used, this acid may have a stimulating or inhibitory effect on quorum sensing. Another study about coumaric acid in *E. faecalis* showed that this natural compound has the capabilities to inhibit the production of autoinducer-2 by *E. faecalis* [99]. The autoinducer-2 are quorum sensing molecules common to both Gram-negative and Gram-positive

bacteria. [126]. So, the mechanism by which coumaric acid inhibits quorum sensing may be related to this molecule.

4.2.2. Evaluation of *Listeria monocytogenes* motility

Motility is an important virulence factor of *L. monocytogenes* associated with surface colonisation, spreading, quorum sensing and biofilm formation [127]. Being an important virulence factor and an advantage to the bacteria makes it an important target of this study. Thus, all natural compounds at subinhibitory concentrations were tested in TSA plates with 0.03%(w/v) of agar for 72 hours. At 24 hours, only linalool 1/16 × MIC showed a significant decrease in halo diameter ($p<0.05$) when compared to the control halo. This concentration of linalool also showed the highest inhibition in halo diameters at 48 hours ($p<0.0001$) and 72 hours ($p<0.001$). The lowest concentration of linalool also exhibited a significant inhibition the halo diameter at 48 ($p<0.05$) and 72 ($p<0.01$) hours, but it was less noticeable when compared to the highest concentration (Figure 4).

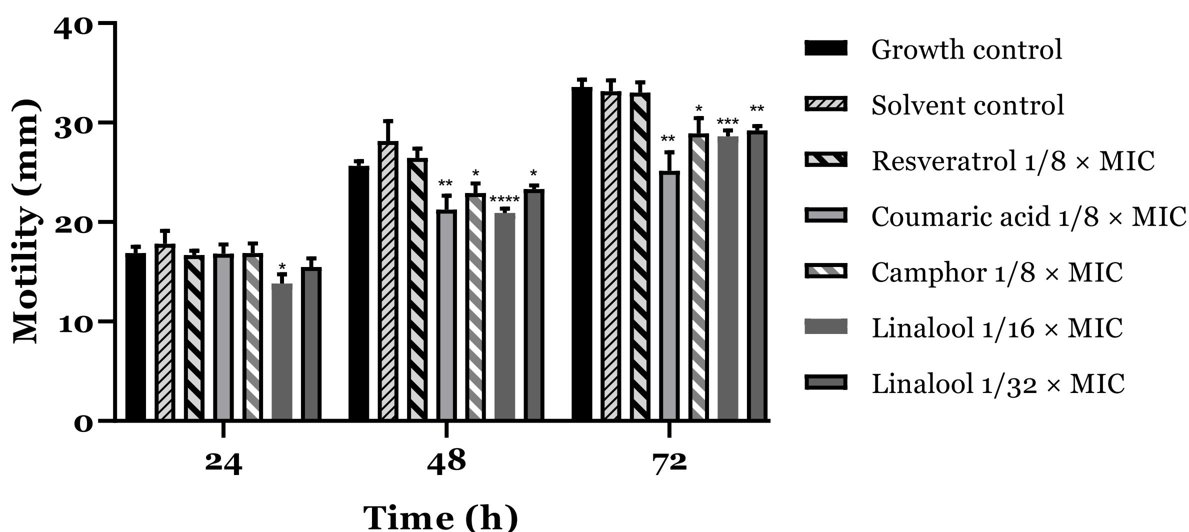

Figure 4. Inhibitory effect of sub-inhibitory concentrations of four natural compounds in *L. monocytogenes* motility at 24, 48 and 72 hours. The results are presented as the mean ± standard deviation. Asterisks represent significant differences, in comparison to the control (t student test). * ($p<0.05$); ** ($p<0.01$); *** ($p<0.001$); ****($p<0.0001$).

For the other compounds, camphor and coumaric acid displayed significant results. Camphor showed a significant decrease in the motility halo ($p<0.05$) at all times, except at 24 hours, when compared to the control. Coumaric acid showed motility inhibition similar to camphor, nonetheless the diameter of the inhibition halo was more noticeable ($p<0.01$)). Resveratrol and the solvent control (DMSO) did

not demonstrate any significant decrease in the motility halo when compared to the control at all times tested.

The effect of linalool on the motility of *L. monocytogenes* is lacking in research, but studies on essential oils where main constituent is linalool and studies on other bacteria may provide some information about the inhibition obtained in this assay. A study on the essential oil of *C. camphora*, whose main constituent is linalool, showed an inhibition in the motility of *C. violaceum* [124]. Another study conducted on *V. harveyi* obtained a decrease in swarming and swimming movement when exposed to linalool [128]. These works are examples of the linalool influence in motility, such as it was obtained in this assay, however the mechanisms by which it affects motility are yet to be discovered.

Coumaric acid was one of the natural compounds that showed motility inhibition, in line with a study showing that coumaric acid could inhibit *S.* Enteritidis swimming motility as a function of varying concentration. In this study it is also concluded that the inhibition comes from the high binding affinities of coumaric acid to three crucial flagellum-mediated motility proteins [100]. Nonetheless, information related to the motility of coumaric acid and L. *monocytogenes* is scarce.

The other compound that showed inhibition of motility in *L. monocytogenes* was 1/8 × MIC camphor. As no information was found on inhibition involving camphor on motility, further testing will be needed to understand these results.

Although resveratrol demonstrated an effect on quorum sensing and is a promising compound, in the motility assay had no influence. This absence of inhibition has also been seen in another study, where resveratrol inhibited the biofilm formation but had no effect on motility of *L. monocytogenes* [129].

4.2.3. Inhibition of biofilm formation

Biofilm is one of the most important virulence factor of *L. monocytogenes,* since it allows the colonization of surfaces, such as stainless steel, polystyrene or glass and also gives protection against sterilization, fatty acids, heavy metals, and limits the permeation of antimicrobial agents and antibiotics [11,40], being biofilms one of the features responsible for this bacterium' survival in food during storage. Biofilms also present a strong connection with quorum sensing due to production of signal molecules called autoinducers that are responsible for regulating different physical activities and that can be a switch for biofilm formation [103]. Considering the role of biofilm in the dispersion and persistence of *L. monocytogenes*, the effect of the

four natural compounds under study was evaluated regarding its potential for inhibition of biofilm formation (Figure 5).

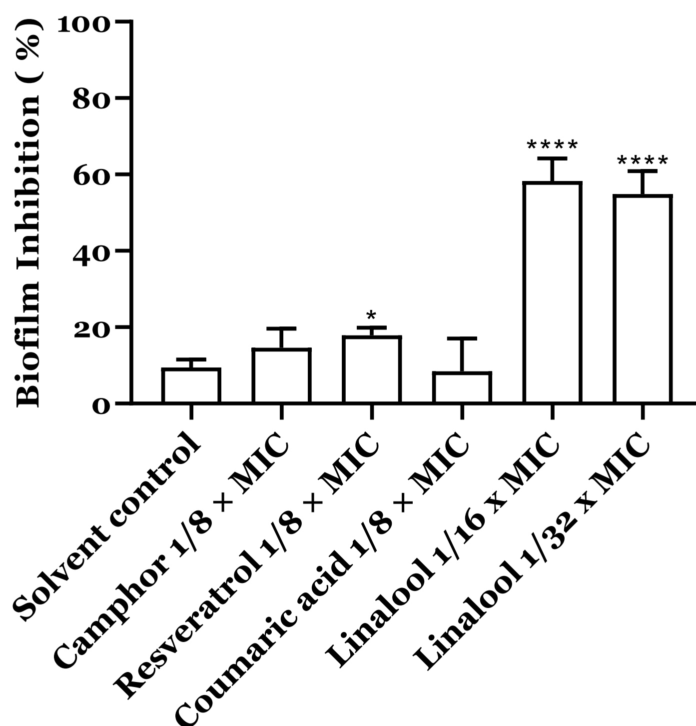

Figure 5. Inhibition of *L. monocytogenes* biofilm formation by four natural compounds at subinhibitory concentrations. The results are presented as the mean ± standard error of the means. Asterisks represent significant differences, in comparison to the control (t student test). *(p<0.05); ****(p<0.0001).

As can be seen in Figure 5, only resveratrol (p<0.05) and linalool (p<0.0001) showed a significant inhibition of biofilm formation. Looking at resveratrol, this compound inhibited in 17.91% (p<0.01) the biofilm formation when compared to the solvent control. Similar results were obtained in other studies regarding the inhibition of biofilm by resveratrol in *L. monocytogenes*. Liu et al. (2021) analysed the effect of resveratrol in motility and biofilm formation of *L. monocytogenes* showing no inhibition on the motility, but the ability to inhibit the biofilm formation by *L. monocytogenes* [129]. Another study by Ferreira et al. (2016) [116] also obtained a inhibition in biofilm formation when exposed to different concentrations of resveratrol. In both studies mentioned, the inhibition of the biofilm formation was higher when compared to that obtained in this work, a reason for this can be related to the concentration of resveratrol used. The concentration used in this assay is inferior to the concentrations used in these works, although the use of the subinhibitory concentration without bactericidal effect allows to exclude lethal effects.

When considering other Gram-positive bacterium, such as *S. aureus*, the inhibition of biofilm by resveratrol has been associated with the disturbance of quorum sensing, synthesis of surface proteins and capsular polysaccharides [130]. Considering the obtained results, it could be suggested that quorum sensing inhibition may justify the observed inhibition on biofilm formation due to the correlation between these two virulence factors, in a similar way to the study made with *S. aureus* [130].

Linalool compound showed the ability to inhibit biofilms, with the highest concentration showing an inhibition of 58.32% ($p<0.0001$) and the lowest concentration of 54.91% ($p<0.0001$), when compared to solvent control.This compound already showed an inhibition effect on biofilm of *L. monocytogenes* and in others pathogens, such as *S. flexneri, Candida albicans,* and *P. aeruginosa* [131–133]. These authors suggested that the mechanism behind this inhibition could be the formation of hollows and holes in cell membranes and the reduction of the biofilm thickness making it more fragile [131].

4.3. Cytotoxicity of natural compounds in human's erythrocytes and study of the effect of natural compounds in haemolysis induced by *Listeria monocytogenes*

L. monocytogenes is a pathogen able to invade and grow within eukaryotic cells, such as phagocytic and epithelial nonphagocytic cells [134]. The internalization of *L. monocytogenes* is possible because this bacterium produces the toxin Listeriolysin O (LLO).[135]. LLO is a cholesterol-dependent cytolysin capable of pore forming that is crucial to vacuole escape, aiding the intracellular replication and later facilitating the cell to cell spread process [24]. This toxin is also able to induce haemolysis *in vitro* [135]. Thus, the study of the effect of natural in haemolysis induced by *L. monocytogenes* was evaluated.

The cytotoxicity test allowed to evaluate whether the subinhibitory concentrations of the natural compounds previously evaluated, namely those that presented anti-virulence properties, were cytotoxic to human erythrocytes. Figure 6 clearly shows that the subinhibitory concentrations previously tested in the evaluation of virulence factors inhibition either have no haemolytic activity or have low activity. The highest percentage of haemolysis was observed for camphor at its MIC value, followed by linalool that showed high percentages of haemolysis with a maximum of 84.0% even at sub inhibitory concentrations. However, none of the

subinhibitory concentrations used to evaluate the effect of the compounds on the virulence factors of *L. monocytogenes* presented haemolysis capacity above 10%, thus showing that the chosen concentrations do not present cytotoxic activity.

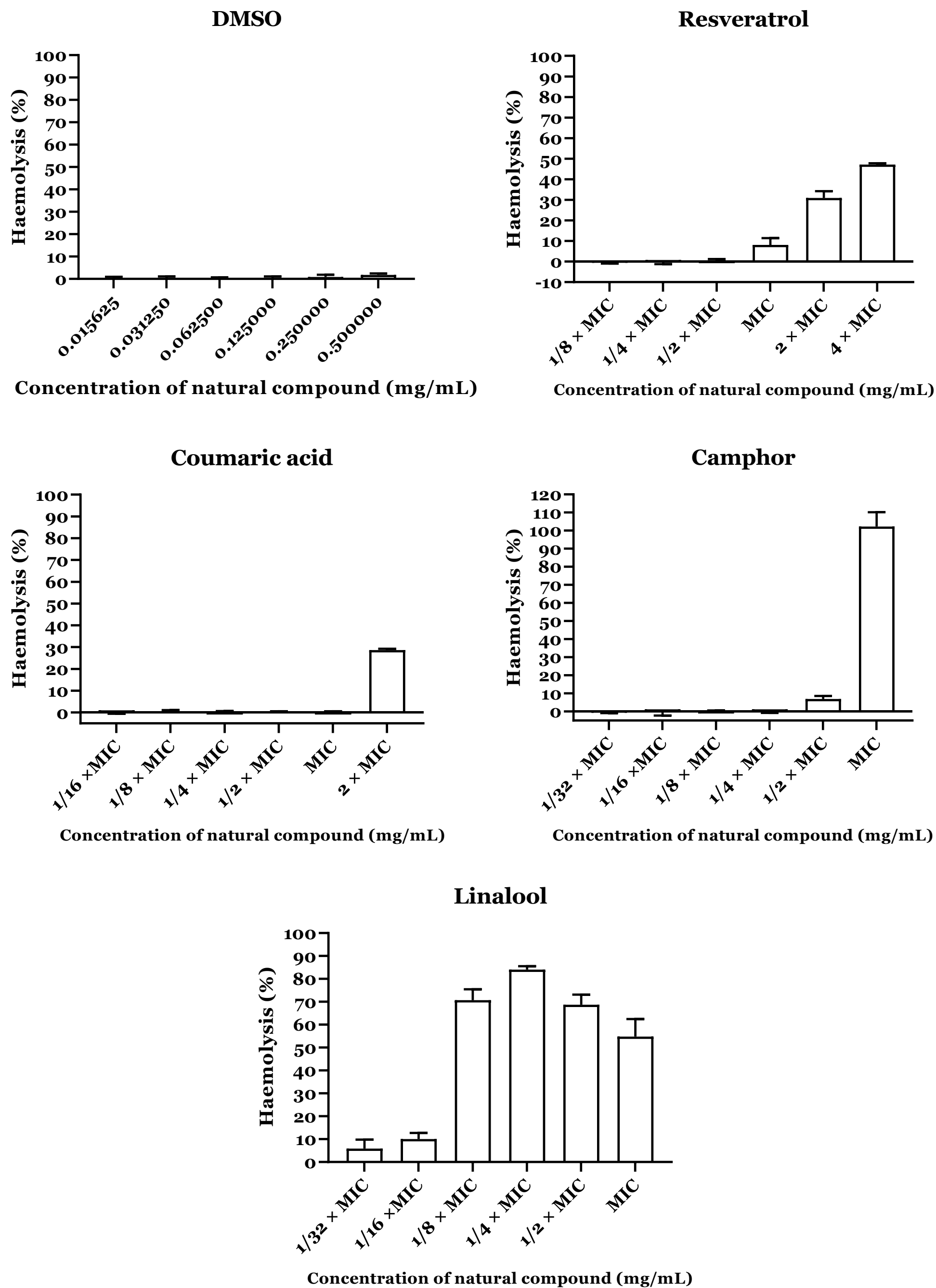

Figure 6. Evaluation of the cytotoxicity of sub-inhibitory concentrations of four natural compounds and the solvent control in humans erythrocytes.

A variation of the haemolytic activity assay enables us to examine if the natural compounds had a role in haemolytic capabilities of *L. monocytogenes* and in turn in the effect of the LLO expression. For this, a pre-incubation of the bacteria with the

sub-inhibitory concentrations of the natural compound was carried out. The bacteria were then incubated with human erythrocytes to assess whether the bacteria's haemolytic activity had been altered. As can be seen in Figure 7, the pre-exposure of *L. monocytogenes* to coumaric acid and linalool leads to a significant decrease in erythrocyte haemolysis when compared to the control.

When exposed to coumaric acid, *L. monocytogenes* was capable of inhibiting the haemolysis caused by this pathogen in 84.2% presenting a haemolytic activity of 15.8% ($p<0.0001$), (Figure 7).

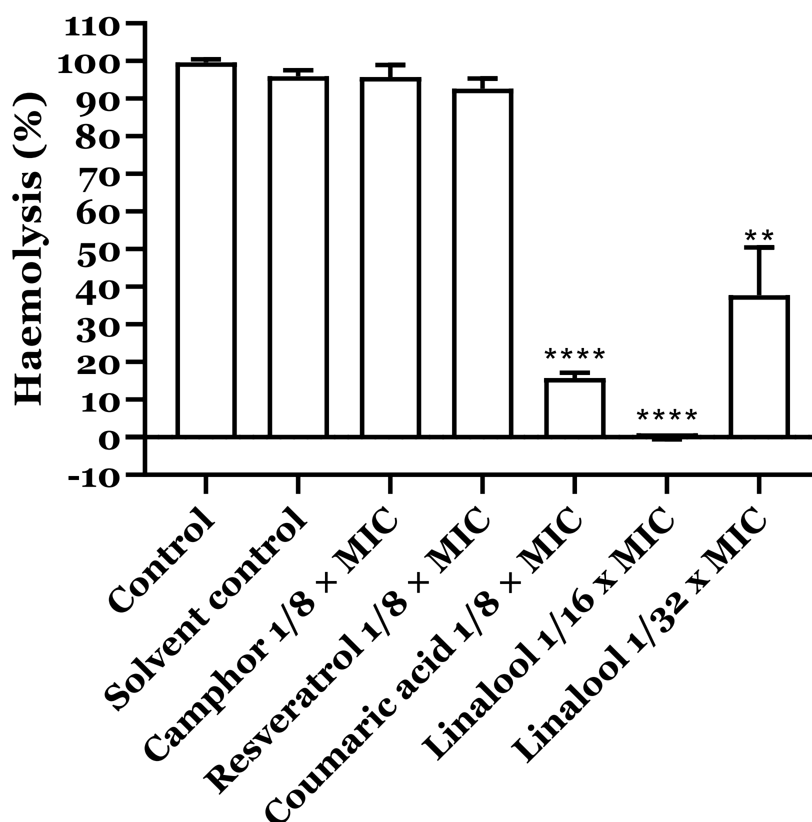

Figure 7. Haemolysis of humans erythrocytes by *L. monocytogenes* pre-exposed to natural compounds. The results are presented as the mean ± standard error of the means. Asterisks represent significant differences, in comparison to the control (t student test). **($p<0.01$); **** ($p<0.0001$).

Although there are no studies linking coumaric acid and LLO, there are studies on the effect of phenolic compounds on LLO. One study about phloretin, a polyphenolic compound, demonstrated that this compound inhibits LLO at a transcriptional or translational level, blocking the escape of vacuole and reducing this way the invasion of host cells [136]. In another study, which involved fisetin, there was also inhibition of LLO, through its engaging in the loops of LLO.[137]. Being these compounds and coumaric acid both phenolics compounds, there may be a connection in the inhibition presented. However, it is important to mention that

these compounds belong to different subclass of phenolics compounds, so more studies are needed to better understand the mechanism of action of coumaric acid and whether it has any similarity with other phenolic compounds.

Linalool was the other compound that managed to inhibit the haemolysis in both concentrations. The higher concentration was capable of total inhibition of haemolysis ($p<0.0001$). Alternatively, the lower concentration presented a 62.2% inhibition ($p<0.0001$). This difference in results can point to a connection between the concentration used and the inhibition caused when exposed to linalool. A study by Gao et al (2021) refers to the ability of linalool to target ribosomes, cellular and metabolic processes, which may explain the inhibition obtained in this assay with both linalool concentrations [108].

4.4. Evaluation of tolerance to adverse conditions

Listeria monocytogenes is a pathogenic bacterium known for its ability to survive and grow under adverse conditions, usually found in food production chains, food storage and also in different hosts [11,26]. Some of the adverse conditions in which *L. monocytogenes* can survive include osmotic stress, high temperatures, low pH, and low temperatures [11,26]. Most of these conditions can be used in food preservation or even found in the gastrointestinal tract, where the bacteria must deal with low pH. Based on these characteristics mentioned, it is important to assess whether natural compounds can influence the tolerance of the bacteria to several adverse conditions, namely to osmotic stress, low pH, and low and high temperatures.

4.4.1. Osmotic stress

L. monocytogenes can survive in various adverse conditions, one of them is osmotic stress. It is known that this bacterium can grow on average, up to 12 to 20% of NaCl [54]. Usually, high concentrations of NaCl suppress the growth of the bacteria by altering the intracellular pressure or due to decreased electrochemical potential in the cell membrane [56]. *L. monocytogenes* in response to this type of environment accumulates compatible solutes to counterbalance the effect of pressure. This accumulation is possible due to the presence of $K+$ transporters and compatible solute transporters [57].

Thus, to evaluate whether natural compounds can influence this ability or even potentiate the deleterious effect of osmotic stress, *L. monocytogenes* was exposed for 24 hours to a solution of sub-inhibitory concentrations of each natural compound supplemented with 12% NaCl. The results obtained show that, of all the studied

compounds, only linalool was able to decrease the tolerance of *L. monocytogenes* to this adverse condition (Figure 8). A previous study on linalool suggested that there could be several targets for this compound in *L. monocytogenes*, such as cell membranes, cell walls, nucleoids, and ribosomes [108]. As these cellular components are involved in the adaptation to osmotic stress, it is possible that *L. monocytogenes*, when under the effect of linalool, cannot adapt to high concentrations of NaCl. However more studies are needed to better understand the effect of linalool.

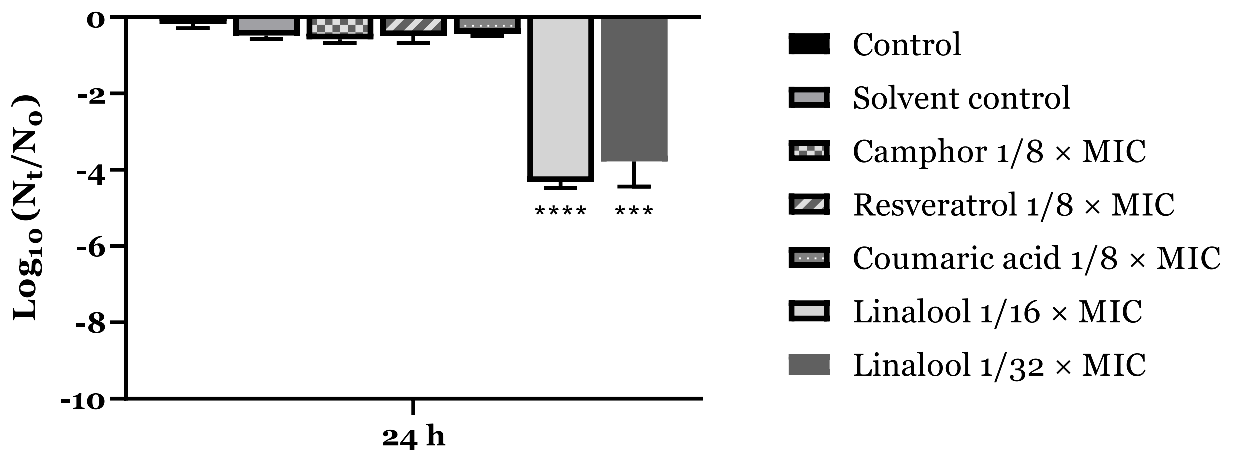

Figure 8. Evaluation of the effect of sub-inhibitory concentrations of four natural compounds in the tolerance of *L. monocytogenes* to osmotic stress (12% (w/v) NaCl). The results are presented as the mean ± standard deviation. Asterisks represent significant differences, in comparison to the control (t student test). *** (p<0.001); ****(p<0.0001).

4.4.2. Low pH

Low pH or acidic pH is another adverse condition that *L. monocytogenes* can adapt and overcome, since it is generally used as a method of food preservation [59]. Further, *L. monocytogenes* encounters these acidic conditions when reaching to the gastrointestinal tract [60]. Some studies also say that this pathogen, in addition to surviving in this type of environment, can also activate other virulence mechanisms leading to greater tolerance and protection against this condition [138]. Thus, studying the effect of these natural compounds on *L. monocytogenes* at low pH was part of this work. From the assay performed, where the bacterium was exposed to pH 2.4 for 15 min, significant differences were observed only in 10 min of exposure for both linalool concentrations.

As can be seen in figure 9the higher concentration of linalool had a more evident effect ($p<0.01$) than the lower one ($p<0.05$). It is also important to mention that linalool at 10 minutes evidenced more death cells when compared to the control and at 15 minutes most of the cells were dead both in control and with the tested compounds

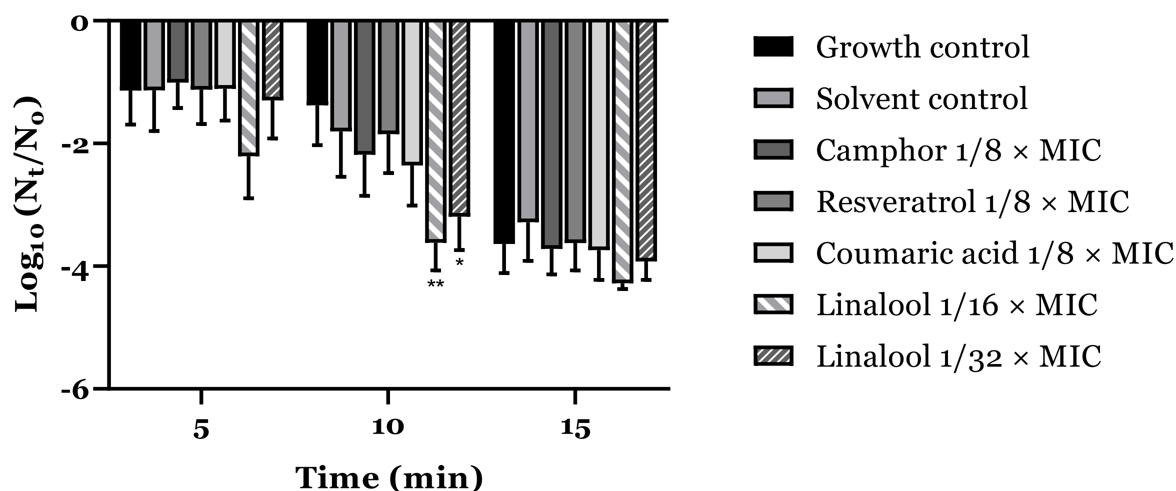

Figure 9. Evaluation of the effect of sub-inhibitory concentrations of four natural compounds on the tolerance of *L. monocytogenes* to acidic pH (2.4). The results are presented as the mean ± standard deviation. Asterisks represent significant differences, in comparison to the control (t student test). *(p<0.05); **(p<0.01).

Linalool, like in the osmotic stress assay, showed to have a significant outcome in the pH tolerance assay and this may have a connection. Several studies indicated that osmotolerance-associated genes in *L. monocytogenes* are also activated and have other functions during other isolated stress environmental conditions, such as low pH and low temperature [139]. Being linalool a compound that can targets ribosomes [108] it is possible that this molecule may influence the production of proteins. Another reason can be that linalool affects the glutamate decarboxylase system (GAD), which is an important mechanisms that *L. monocytogenes* uses to adapt to low pH and maintain intracellular homeostasis [62]. However, more studies are still needed to better understand the mechanism of action of resveratrol.

None of the other tested compounds showed a significant increase or decrease of tolerance to acid pH. However, there is a study with resveratrol that obtained an increase in tolerance of pH acid in *L. monocytogenes* after 2 hours of pre-exposure to resveratrol, however this was a study using a adapted cells [114].

In turn, coumaric acid at low pH shoed no significant effect. Several studies showed that coumaric acid have a more noticeable antimicrobial effect against Gram-negative than Gram-positive bacteria [98,140], this antimicrobial effect also tends to increase with a reduction in pH [140,141]. The results obtained in this work may be seen as contradictory, however it may be related to *L. monocytogenes* being Gram-positive. Other justification can be that due to fast death cell noticed at the 15 minutes mark there was no enough time for the compound to show any effect.

Of all the compounds, the one which proves to be a better applier under low pH conditions in order to render *L. monocytogenes* more susceptible was Linalool. However, more studies are still needed to better understand the mechanism of action of natural compounds on stress adaptation/tolerance in *L. monocytogenes*.

4.4.3. High temperature

Heat treatment with high temperatures is another method used in the food industry to preserve and prevent contamination with foodborne pathogens as well as in the food confection [66]. *L. monocytogenes*, however, has shown resistance to high temperatures above 45°C, making this method sometimes insufficient [67]. This heat resistance involves heat shock genes encoding three HSPs [52,72]. This resistance may also differ depending on the serotype, with one study showing that strains classified to serotypes 1/2b and 4b were more resistant to elevated temperatures that the ones from serotype 1/2a [70].

In this work, the effect of the compounds under study on the tolerance to a temperature of 55°C for 45 minutes was evaluated. Looking at the results obtained (Figure 10), only linalool showed a significant reduction of survival of *L. monocytogenes* in all the times tested. It is also noticeable that the higher linalool concentration ($p<0.0001$) had a higher effect on the tolerance than the lower linalool concentration ($p<0.001$). This effect of linalool once again may be justified due to its capability of targeting cell membranes and cell walls [108] making *L. monocytogenes* more susceptible to the heat provided. The HSPs used by this pathogen have the objective of protect cellular proteins and enzymes against denaturation [73], being linalool a compound that targets the ribosomes, the pathway to form this HSPs may be affected.

Resveratrol in this assay did not showed any significant effect, but the study that stated that *L. monocytogenes* had an increase pH tolerance when exposed to resveratrol also acquired an increase in the survival of *L. monocytogenes* at 55°C after adaptation to the subinhibitory concentration of resveratrol [114].

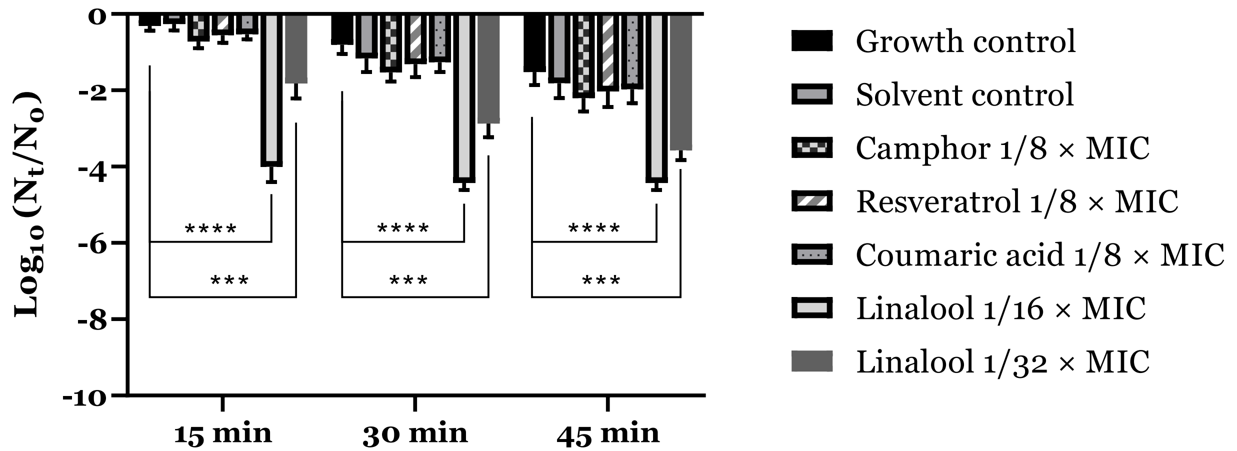

Figure 10. Evaluation of the effect of sub-inhibitory concentrations of four natural compounds on the tolerance of *L. monocytogenes* to high temperature (55 ºC) The results are presented as the mean ± standard deviation. Asterisks represent significant differences, in comparison to the control (t student test). *** (p<0.001); **** (p<0.0001).

4.4.4. Low temperature

Finally, the ability of *L. monocytogenes* to survive and thrive at low temperatures when exposed to sub-inhibitory concentrations of the natural compounds was tested. This foodborne pathogen has the ability to survive, proliferate and grow at temperatures down to 0.4°C [75,142], making *L. monocytogenes* a pathogen frequently present in food products under refrigeration conditions [76]. Low temperatures normally affect the cell by reducing membrane fluidity, however *L. monocytogenes* responds to this stress using at least three mechanisms: cell membrane changes and adaptation, production of CSPs and uptake of cryoprotective compounds from the environment [54].

Thus, to see if the compounds under study were able to diminish the survival ability of *L. monocytogenes* to low temperatures, an incubation at 4°C for 196 days occurred. The compounds and the pathogen were also incubated with and without 12% (w/v) of NaCl.

From the results obtained (Figure 11), it can be said that camphor had no visible effect on the tolerance of this pathogen to low temperatures with or without NaCl at any point in time during the assay.

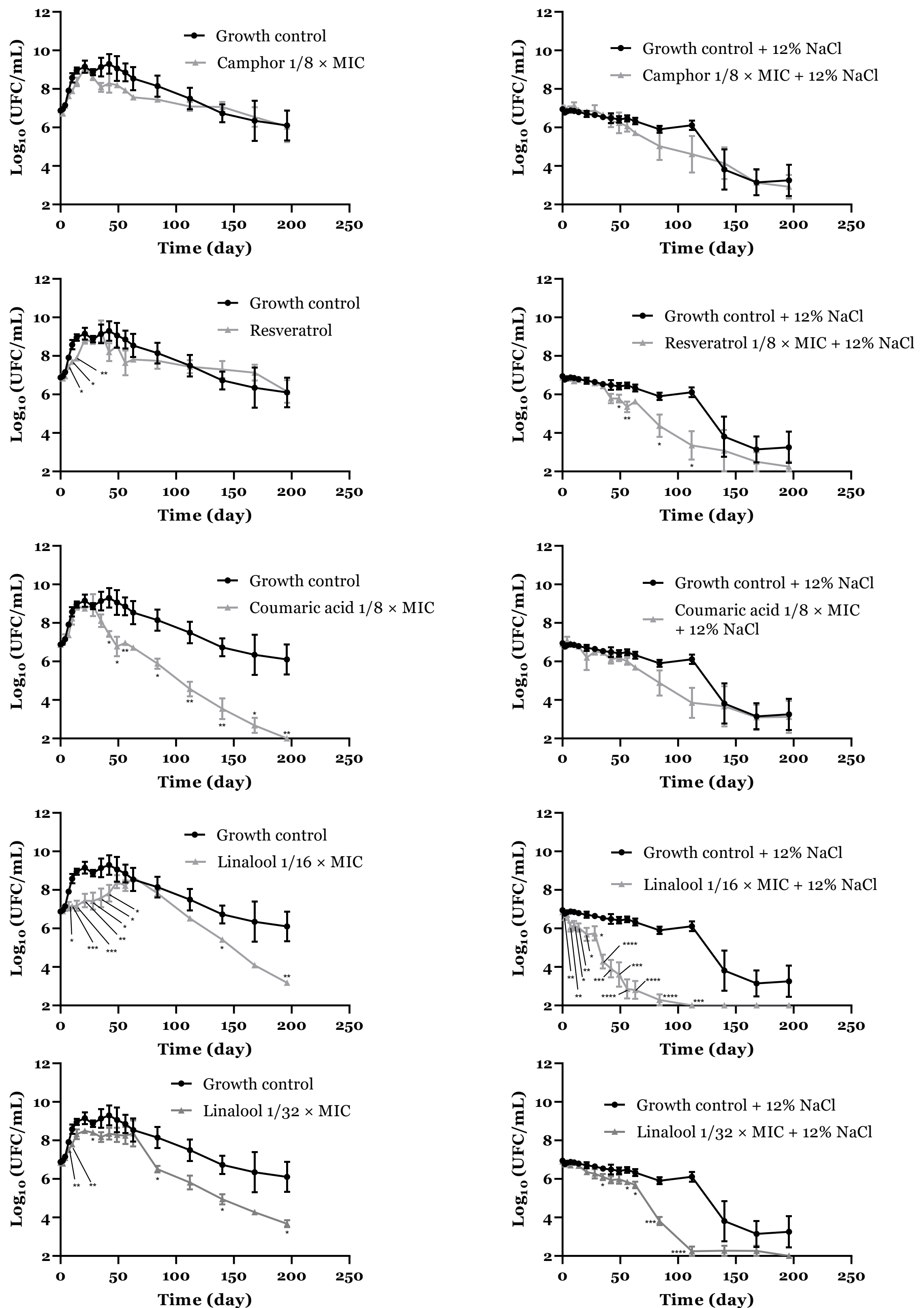

Figure 11. Evaluation of the effect of sub-inhibitory concentrations of four natural compounds on the tolerance of *L. monocytogenes* to low temperature (4 ºC) with or without 12% (w/v) NaCl. The results are presented as the mean ± standard deviation. Asterisks represent significant differences, in comparison to the control (t student test). * ($p<0.05$); ** ($p<0.01$); ***($p<0.001$); ****($p<0.0001$).

Resveratrol, on the other hand, showed some inhibition of *L. monocytogenes* growth after one week of exposure without NaCl (7,10 and 14 days), however, the inhibition of growth later disappeared after this period. Resveratrol supplemented with NaCl, instead of showing any inhibition in the first weeks of exposure, showed it after 49 days of exposure. After the 49 days, the values in cfu/mL obtained were always lower when compared to the values obtained in the control. This shows that resveratrol potentiated the inhibitory effect of low temperature and high osmolarity on *L. monocytogenes*.

Coumaric acid, like camphor, did not shown any significant decrease in growth when together with NaCl. However, contrarily to resveratrol, coumaric acid showed a significant and evident growth inhibition ($p<0.05$) on *L. monocytogenes* without NaCl supplementation. From day 42 there was a noticeable constant decrease in the concentration of *L. monocytogenes* and eventually the death of this pathogen occurred after 196 days of exposure, such decrease and cell death did not occur in the control.

A similar effect was observed in a study using coumaric acid against *Alicyclobacillus acidoterrestris* in apple juice. When this bacterium was exposed to this compound at 4 °C an acceleration of cell degradation occurred. This study also noted the ability of coumaric acid to damage cell integrity, decrease intracellular ATP, hyperpolarization of cell membrane, degradation of whole cell protein, and malformation of cell morphology [143]. Another study also verified this ability to disrupt bacterial cell membranes and binding to bacterial genomic DNA to inhibit cellular functions [98]. This cell membrane hyperpolarisation effect may justify why the coumaric acid assay with NaCl achieved less inhibition when compared to the assay without NaCl.

Linalool was the compound with the most significant inhibitory effect against *L. monocytogenes* in this assay. Looking at the results without NaCl, the 1/16 × MIC concentration showed a stronger effect overall when compared to the lower linalool concentration. The higher concentration showed an inhibition from day 10 to day 42, regaining it later in the assay. This prolonged inhibition did not occur at the lower concentration and may be a concentration-related effect.

The effects of linalool with NaCl have an even greater impact on the growth and survival of *L. monocytogenes* at low temperature. The concentration of 1/16 × MIC showed an inhibition as early as day 2 and increase the inhibition until the pathogen died on day 112, while the control after 196 days still had viable cells.

The 1/32 × MIC concentration supplemented with NaCl showed a similar effect, however the inhibition was not as strong in the initial weeks, also this concentration only caused the death of all cells on day 196. This suggests that the effect was concentration linked. Comparing the results obtained with and without NaCl, a stronger effect occurs with the supplementation of NaCl, this may suggest a synergistic effect between linalool and NaCl.

L. monocytogenes normally respond to cold temperature stress by making changes in the cell membrane and through the production of CSPs [54]. Furthermore linalool targets cell membranes, walls and ribosomes [108]. So, a possible reason for the strong effect demonstrated by linalool in this assay, may result from its action on the membranes of *L. monocytogenes*, thus not allowing the adaptation of the membranes and walls to occur as supposed. Another important point to note is that linalool together with NaCl had an even stronger effect. This could be caused by overloading the cell membrane with both effects or by disrupting the protein pathway involved.

4.5. Modulation of antibiotic MICs in the presence of natural compounds against *L. monocytogenes*

Microbial resistance to antibiotics and other antimicrobial agents is a known problem and it has been recognised by the WHO as a major global health threat [144]. Due to the misuse of antibiotics in humans and animals, the spread of antibiotic resistance had an increased among foodborne pathogens [145], namely *L. monocytogenes*. The line of treatment of listeriosis often include antibiotics, such as tetracyclines, erythromycin, ampicillin, and gentamicin [145,146]. However, resistant strains of *L. monocytogenes* to commonly used antibiotics have increased. Some studies even link the effect of environmental stress to increased antibiotic resistance in *L. monocytogenes* [146]. Thus, there is a need to find new substitutes or adjuvants for antibiotics. With this in mind, the focus of this experiment was to understand if natural compounds at sub-inhibitory concentrations could modulate the MIC of antibiotics and be used as possible adjuvants.

To achieve this purpose the MICs of antibiotics were determined alone and then compared to MICs of antibiotics supplemented with subinhibitory concentrations of natural compounds. For every compound, the 1/4 × MIC concentration was also evaluated together with the other subinhibitory concentrations already used in previous assays. When a diminution of at least four times in the MIC value of the antibiotic occurred in presence of the compounds, it was considered that an effect occurred. Looking at the results (Table 2), the MIC values for the antibiotics AMP, CTX, TET, ERY and RIF supplemented with subinhibitory concentrations of natural compounds showed

no significant reduction, thus meaning, there was no modulatory effect. Furthermore, camphor and coumaric acid at both concentrations did not show any effect with any antibiotic either. Nonetheless, some studies found a synergetic effect of coumaric acid with some antibiotics, such as ampicillin and erythromycin, this effect was however found mostly in Gram-negative bacteria [147,148]. This may be related to the fact that coumaric acid is more noticeable in Gram-negative bacteria than Gram-positive ones [98,140]. This observation may also help explain why in this assay with coumaric acid no effect was observed, possibly because these assays were performed on *L. monocytogenes*, which is a gram-positive bacterium.

On the contrary, resveratrol was capable of reducing by 4–fold the MIC value of gentamicin when ¼ × MIC was used. This MIC value change was also observed by another study by Nøhr-Meldgaard et al., 2018 [95], where the ability of resveratrol to inhibit ATP synthase in various bacterial pathogens was tested along with aminoglycosides to find out if there was an increase in the efficacy of these molecules. Of the pathogens tested, *S. aureus* showed to be the most affected (8-16-fold reduction) and *L. monocytogenes* least affected (1 -2-fold reduction) therefore demonstrating that resveratrol was able to increase the effect of gentamicin on *L. monocytogenes*. However, further studies are needed to better understand this increase in *L. monocytogenes*.

Linalool like resveratrol was able to enhance the effect of gentamicin, this compound reduced the MIC of gentamicin by 8–fold in higher concentrations. A synergistic effect between gentamicin and linalool against *S. aureus* was previously observed, being linked to the membrane permeabilising abilities of linalool, which facilitates the penetration of the antibiotic [149]. This study also reported a decrease in the MIC of tetracycline and erythromycin, but this did not occur in *L. monocytogenes* [149].

Table 2. Modulation of minimum inhibitory concentration of a pool of antibiotics in the presence and absence of subinhibitory concentration of natural compounds in *L. monocytogenes*.

		AMP	CTX	GEN	TET	ERY	RIF
MIC (ug/ml)	Control	0,5	4	2	0,5	0,125	0,03125
	+ Camphor 1/8 × MIC	0,5	4	2	0,5	0,125	0,03125
	+Camphor 1/4 × MIC	0,5	4	2	0,5	0,125	0,03125
	+Resveratrol 1/8 × MIC	0,5	4	1	0,5	0,125	0,03125
	+Resveratrol 1/4 × MIC	0,25	4	**0,5**	0,5	0,125	0,03125
	+Coumaric acid 1/8 × MIC	0,5	4	2	0,5	0,125	0,03125
	+Coumaric acid 1/4 × MIC	0,5	4	2	0,5	0,25	0,03125
	+Linalool 1/32 × MIC	0,5	4	1	0,5	0,125	0,03125
	+Linalool 1/16 × MIC	0,5	4	**0,25**	0,5	0,125	0,03125
	+Linalool 1/4 × MIC	0,5	4	**0,25**	0,5	0,125	0,03125

A reduction of at least 4-fold is highlighted at bold.

5. Conclusion and future perspectives

Foodborne pathogens, such as *L. monocytogenes,* are currently a food safety concern. This pathogen has diverse virulence factors that allows the survival in harsh environments and infect host cells and cause listeriosis. So, innovative, and effective methods are needed to control them. Isolated natural compounds are possible alternatives because of their antibacterial and anti-virulence properties. In this study, four different compounds (resveratrol, *p*-coumaric acid, camphor and linalool) were studied regarding their anti-virulence capabilities at subinhibitory concentrations.

From four the compounds, camphor only showed inhibition in the motility assay. Coumaric acid, on the other hand, has shown inhibitory effects not only in the motility assay, but also in the reduction of the haemolysis caused by *L. monocytogenes,* as well as in increasing the susceptibility to the low temperatures in absence of NaCl. This inhibition may point to the use of this natural compound against LLO or as a food preservative in cold storage.

Another natural compound tested was resveratrol, this compound presented evident inhibition in diverse assays, such as quorum sensing, biofilm formation and was capable of strengthening the inhibitory effect of low temperatures and high osmolarity on *L. monocytogenes*. In addition, it was also able to decrease the MIC of gentamicin, one of the antibiotics used to combat this pathogen, by four-fold. With further testing, resveratrol has been shown to have the potential to be used in the control of *L. monocytogenes*.

Finally, linalool unlike the other compounds showed some inhibition capacity in all the assays performed, being, in this way, the most promising compound tested in this work. Linalool was capable of affecting the tolerance of this pathogen to all adverse conditions tested in this work, such as osmotic stress, low pH, high and low temperature to which *L. monocytogenes* can adapt and survive. Furthermore, it was also able to decrease the MIC of gentamicin and affect virulence factors, such as biofilms, motility, quorum sensing and haemolysis caused by *L. monocytogenes*. The results obtained in this work with linalool are very promising for a potential use of this compound in the control of *L monocytogenes*.

However, mechanisms responsible for the inhibitory activity of most of the compounds studied are not yet fully elucidated. For example, the mechanism by which coumaric acid can affect haemolysis caused by this pathogen is not yet described, there are several studies on other phenolic compounds, but none for coumaric acid. Other

important mechanisms that need further studies are the ones used overall by linalool in the tolerance to adverse conditions. Studies that allow to understand if the inhibitory effect is one of the mechanisms used by *L. monocytogenes* to defend off these conditions, such as CSPs or HSPs, or if it is a pathway inhibition, are one of the next steps that could take place.

In this work, no study about the invasion and adhesion of *L monocytogenes* was made, thus to analyse if these natural compounds can have an effect on these abilities of this pathogen is another step that can be taken in the future. Other relevant work that can be taken to improve the knowledge on these natural compounds on *L. monocytogenes* is to evaluate how the pre-exposure to the compounds may affect the tolerance of the bacterium to antibiotics and adverse conditions. Alternatively, the synergetic effect between the compound can also be an interesting topic of study.

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Annex I

Oral communication

Joel Dias, Fernanda Domingues, Susana Ferreira (July 20th and 21st, 2022). Natural compounds in attenuating virulence of *Listeria monocytogenes*. XVII International CICS-UBI Symposium.

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SHORT COMMUNICATION AWARD

I herewith certify that Joel Dias was awarded with best short communication in the XVII International CICS-UBI Symposium, which was held the 20th and 21st July 2022.

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