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PORTO

PROBIOTIC POTENTIAL OF *Leuconostoc lactis* RK18 ISOLATED FROM FERMENTED FOOD AND ITS BACTERIOCINOGENIC ACTIVITY AGAINST FOODBORNE PATHOGENS

by

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Thesis presented to Escola Superior de Biotecnologia of the Universidade Católica Portuguesa to fulfil the requirements of Master of Science degree in Applied Microbiology

by

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To my parents and to my adorable Blue

Always in my heart,

forever

Abstract

Fermentation is one of the oldest techniques for the production of foods. Lactic acid bacteria (LAB) have been progressively used in food industry since, besides improving nutritional and technological features, they also contribute to the safety of food products. Consequently, this work aimed to isolate lactic acid bacteria from several food products and to evaluate their potential antimicrobial activity and probiotic characteristics. From 202 LAB isolated from 20 fermented food products, only three isolates were selected and identified as *Enterococcus faecium* RS7 (fermented shrimp), *Enterococcus faecium* P12 (pork sausage) and *Leuconostoc lactis* RK18 (khmer fermented rice fresh noodles) based on their antimicrobial activity against *Enterococcus faecalis* and *Listeria monocytogenes* strains. These selected isolates were further tested for probiotic characteristics. Despite the beneficial characteristics apparently presented by the three isolates due to their antimicrobial activity, only *Ln. lactis* RK18 met the safety requirement and therefore it was the only isolate selected for further tests. *Leuconostoc lactis* RK18 did not present any of the virulence factors nor virulence genes tested (with exception of aggregation substance protein gene *asa1*), and it was also susceptible to all antibiotics recommended by European Food Safety Authority. Regarding beneficial characteristics, it was found that anti-listerial activity of *Ln. lactis* RK18 was due to the production of a Class IIa bacteriocin (<6.5 kDa in size), which remained stable at average temperatures (30 ºC to 80 ºC) and at pH values ranging from 4 to 6, and although susceptible to some detergents, it showed greatly resistance to several enzymes. Despite being very sensitive to acidic environments, when incorporated into a complex food matrix such as *alheira*, *Ln. lactis* RK18 was able to survive through simulated gastrointestinal tract (GIT) conditions and also to adhere (but not invade) to human colon adenocarcinoma cell lines Caco-2 *in vitro*. Even though exposure to GIT conditions had influenced the adhesion ability of *Ln. lactis* RK18 cells, this potential probiotic and merely 10% of its treated cell-free supernatant, were able to prevent the ability of *L. monocytogenes* CEP 104794 to adhere and invade Caco-2 cells.

Overall, *Ln. lactis* RK18 appeared to be a safe strain, with no risk to human health, which harbored important features to be successfully considered as a potential biopreservative and probiotic culture. Nevertheless, further experiments should be performed for the validation of its application in the food industry.

Keywords: Antimicrobial activity; Fermented food products; Lactic acid bacteria; Probiotic

Resumo

A fermentação é uma das mais antigas técnicas na produção de alimentos. As bactérias do ácido lático (BAL) têm sido muito usadas na indústria alimentar, uma vez que melhoram as características nutricionais e tecnológicas, e contribuem para a segurança dos alimentos. Assim, o objetivo deste trabalho foi o isolamento de BAL de vários alimentos e de avaliar a sua potencial atividade antimicrobiana e características probióticas. De 202 BAL isoladas de 20 alimentos fermentados, apenas três isolados foram selecionados e identificados como *Enterococcus faecium* RS7 (camarão fermentado), *Enterococcus faecium* P12 (linguiça de porco) e *Leuconostoc lactis* RK18 (khmer "noodles" de arroz fermentado fresco), seleção essa baseada na sua atividade antimicrobiana contra estirpes de *Enterococcus faecalis* e *Listeria monocytogenes*. Os isolados selecionados foram testados quanto a características probióticas. Apesar da atividade antimicrobiana apresentada pelos três isolados, apenas *Ln. lactis* RK18 preencheu o requisito de segurança e, portanto, foi o único isolado selecionado para outros testes. *Leuconostoc lactis* RK18 não apresentou nenhum dos fatores ou genes de virulência testados (exceto o gene da proteína de substância de agregação *asa1*), e também foi suscetível a todos os antibióticos recomendados pela Autoridade Europeia de Segurança Alimentar. Relativamente às características benéficas, a atividade anti-listeria de *Ln. lactis* RK18 foi devida à produção de uma bacteriocina Classe IIa (tamanho <6,5 kDa), a qual permaneceu estável a temperaturas moderadas (30 ºC a 80 ºC) e em valores de pH entre 4 a 6 e, embora sensível a alguns detergentes, apresentou grande resistência a várias enzimas. Apesar de muito sensível a ambientes ácidos, quando incorporado numa matriz alimentar complexa como a alheira, *Ln. lactis* RK18 sobreviveu às condições simuladas do trato gastrointestinal (TGI) e aderiu (mas não invadiu) às linhas celulares de adenocarcinoma do cólon humano Caco-2 *in vitro*. Embora a exposição às condições do TGI tenha influenciado a capacidade de adesão de *Ln. lactis* RK18, este potencial probiótico e apenas 10% do seu sobrenadante, preveniram a adesão e invasão de células intestinais por *L. monocytogenes* CEP 104794. Em conclusão, *Ln. lactis* RK18 parece ser uma estirpe sem risco para a saúde humana, com características importantes que a tornam uma potencial cultura bioconservante e probiótica. No entanto, mais experiências devem ser realizadas para a validação da sua aplicação na indústria alimentar.

Palavras-chave: Atividade antimicrobiana; Alimentos fermentados; Bactérias do ácido láctico; Probióticos.

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Contents

I.   Introduction

In this section a brief introduction about fermented food products, lactic acid bacteria and their role in the food industry as biopreservative or probiotic cultures will be presented.

1.1. Fermented foods: a brief history

Fermentation dates back thousands of years as a food process technique and is actually considered a dietary strategy for human health. It is believed that it originated in the Indian Sub-continent, but the fermentation processes origins are lost in antiquity (Ray and Joshi, 2014). There are reports of the manufacture of fermented foods, such as cheese making, 8000 years ago, and wine brewing and leavened bread, developed between 4000-2000 b.c. (Fox, 1993; Prajapati and Nair, 2003). The industrial revolution led to the development of large-scale fermentation processes intended for commercial production of beer, wine, spirit beverages, dairy, vegetables and meat fermentations. Now, these extensive productions are dependent almost entirely on starter cultures that replace the undefined strain mixtures traditionally used for the manufacture of these products, accelerating the fermentation process and thus being more effective (Holzapfel, 1997). To achieve maximum culture performance and product quality and consistency, a limited number of strains have been extensively used in industries leading to the presence of side effects since it can result in unsatisfactory strain performance, for example the inefficiency of lactococcal fermentations via interaction with bacteriophages proliferation in cheese manufacture (Ray and Joshi, 2014).

The art of fermentation, via biotransformation of raw materials into final products by the action of live microorganisms, diverged into two methods, ethanol fermentation by yeasts (e.g. *Saccharomyces cerevisiae*) or lactic acid fermentation performed by a broad spectrum of bacteria known as lactic acid bacteria (LAB) (Papadimitriou *et al.*, 2016).

Currently, fermentation processes are used to achieve desirable properties such as improvement of safety, shelf life extension, enhancement of functionality, nutritional and sensory properties (Papadimitriou *et al.*, 2016; Rezac *et al.*, 2018). Nonetheless, fermented foods are generally perceived as a food containing only live microorganisms, which is an erroneous perception. Different fermentation treatments are performed depending on the final product. Overall, production organisms are inactivated by heat treatment, in bread and some beer production, or are physically removed, in wine and beer production (Rezac *et al.*, 2018). Still, heat treatment in

fermentation processes is intended to improve food safety and shelf-life extension and the absence of this step does not mean, necessarily, that the final product will contain high numbers of viable microorganisms simply due to inhospitable environmental conditions that grants the reduction of microbial populations over time (Rezac *et al*., 2018). The number of viable microorganisms is variable depending on how products were processed. Nevertheless, the absence of live bacteria in fermented foods does not exclude their functional role (Rezac *et al*., 2018).

1.2. Fermented Foods Safety

Safety of fermented foods is mostly ensured by the presence of lactic acid bacteria. They have an important role in the enhancement of food shelf-life and in the organoleptic characteristics of the product (Gonzalez, 2019). Overall, LAB are considered as Generally Regarded as Safe (GRAS status) (FAO/WHO, 2002), but the microbiological risk for human health is present when fermented foods are not handled safely.

In 2017, the World Health Organization (WHO, 2017) reported that nearly five thousand people perish each year due to the consumption of contaminated foods. Foodborne outbreaks have progressively grown but the real number of cases is still unknown. According to Centers for Disease Control and Prevention (CDC) there have been record of outbreaks related to fermented food consumption, such as, *Escherichia coli* O157:H7 linked to leafy green and romaine lettuce (2018); *Salmonella* linked to cucumbers (2016) and sprouts (2018); and *Listeria* linked to soft cheese (2017) and deli-sliced meats and cheeses (2019) (CDC). *Listeria monocytogenes* causes listeriosis and it is a rare but potentially fatal foodborne pathogen. The incidence of listeriosis in the European Union (EU) has been growing over the years (Jordan and McAuliffe, 2018). *Listeria monocytogenes* is ubiquitous in the environment being found in the water, soil and faeces, granting the possibility of contamination in food-processing environments. It has remarkable capability to evade host immunity system and survival in the human body, crossing the intestinal and blood-brain barrier, thereby being life threatening and causing severe infections such as encephalitis and meningitis to an immune-compromised host (Jordan and McAuliffe, 2018).

1.2.1. Lactic acid bacteria

Industrialized fermented foods produced through the activity of lactic acid bacteria (LAB) have great success due to the benefits conferred by the consumption of these products in immune health. The high expectations in the expansion of fermented products commercialization led to the demand of rapid and controlled conversion of sugar into lactic acid as well as the improvement of organoleptic characteristics such as flavor, texture and aroma, adding value to the final product. These measures can be accomplished knowing the optimal growth conditions of LAB and their properly described and characterized metabolism, genome and functional genomics tools (Flahaut and de Vos, 2015).

Taxonomically, LAB are heterogeneous and it is believed that they have diverged from a common ancestral. The most common genera of LAB related to fermented food products are *Lactobacillus*, *Lactococcus*, *Streptococcus*, *Enterococcus*, *Pediococcus*, *Leuconostoc*, *Oenococcus*, *Tetragenococcus*, *Carnobacterium* and *Weissella* (Papadimitriou *et al*., 2016; Rezac *et al*., 2018).

Given the involvement of LAB in commercial fermentations of food products, this group of bacteria is among the most explored microorganisms concerning their physiology, biochemistry, genetics and evolution proving to be the most "domesticated" group. In this matter, LAB adapted to survive, proliferate and exert their functional roles in food environments (Papadimitriou *et al*., 2016).

Morphologically, LAB have been recognized as Gram-positive, microaerophilic or anaerobic, non-spore-forming bacteria, catalase-negative and most species are non-motile (Narvhus, and Axelsson, 2003; Papadimitriou *et al*., 2016). Although the GRAS status (Flahaut and de Vos, 2015), is generalized to all microorganisms in the LAB group, there are LAB species considered commensals and pathogens (Narvhus, and Axelsson, 2003; Papadimitriou *et al*., 2016). The difference lies on the ability to adapt and survive to diverse conditions in different niches of the host (commensals) and the capacity to prevail and evade host immunity system (pathogens) (Papadimitriou *et al*., 2016). However, under optimal conditions, commensal bacteria may develop virulence capacities (Papadimitriou *et al*., 2016). These opportunistic LAB pathogens have been found in various genus including enterococci (*Enterococcus faecalis* and *Enterococcus faecium*) commonly related to nosocomial infections. This shows that LAB can act as a reservoir for transferable resistance genes commonly associated with intestinal pathogenic organisms, and

it demonstrates that not all LAB are considered fermented food related (Narvhus, and Axelsson, 2003; Papadimitriou *et al.*, 2016). One of the major attributes of LAB as starter cultures is their bactericidal activity against spoilage and pathogenic bacteria. This is achieved by the acidification of food matrices during lactic acid fermentation (Papadimitriou *et al.*, 2016). Since LAB are relatively acid tolerant, acidification of the medium can cause a stress to pathogenic bacteria, damaging both the cell wall and the cell membrane, allowing the action of exerted lactic acid, viewed as an antimicrobial agent against competing microorganisms (Papadimitriou *et al.*, 2016). There is a possible outcome of acidic conditions as it can affect the metabolism of LAB. Low pH causes cession of LAB growth much faster than depletion of nutrients (Papadimitriou *et al.*, 2016). Prolonged exposures to acidification environments result in the denaturation of proteins, affecting the metabolism, leading to lack of energy and cell death (Papadimitriou *et al.*, 2016). A side effect of acidification is that starvation may be induced indirectly by lactic acid autoacidification as it can obliterate the nutrient uptake. Commercially wise, under this condition, low pH can enhance the metabolism of citrate, resulting in aroma compounds or production of pyruvate (Papadimitriou *et al.*, 2016). This shows that the environment can determine the metabolization and LAB capacities.

Studying the stress physiology and conditions, such as acidification, high and low temperatures, osmolarity, exposure to enzymes and depletion of nutrients is important to understand stress behaviours at a species level, since it may differ not only between genus.

Lastly, LAB are recognized as being part of human and animal microbiome (Papadimitriou *et al.*, 2016), were they play a major role bringing a sophisticated balance that can be crucial for health and disease (Papadimitriou *et al.*, 2016). For example, LAB can produce and excrete vitamins, proteinaceous compounds (bacteriocins), bioactive molecules and other constituents *in situ* that are increased via fermentation and, therefore, increase their availability, preventing several immunity responses and prevent diarrhoeas after antibiotic ingestion, acting as a barrier against pathogenic bacteria or even reducing cholesterol levels (Papadimitriou *et al.*, 2016).

1.2.2. The genus *Leuconostoc*

The genus *Leuconostoc* has constantly been the object of morphological, chemical and phylogenetic changes within the genus. Currently, there is known the existence of 13 species

belonging to this genus, including *Leuconostoc mesenteroides* and *Leuconostoc lactis* (Muñoz *et al.*, 2011; Liu, 2016).

Leuconostoc spp. are ubiquitous microorganisms, especially found in fermented foods (meat, vegetable, and dairy products), but also in plant-matter and human clinical sources (Liu, 2016). They are Gram-positive bacteria, catalase-negative, non-motile, mesophilic (approximately, 25 ºC to 30 ºC), obligatory heterofermentative cocci and aerotolerant (Liu, 2016). Selective medium containing antibiotics vancomycin and tetracycline can be used to isolate *Leuconostoc* from fermented dairy products (Liu, 2016) and, usually, they grow very well in de Man Rogosa and Sharpe (MRS) broth and poorly in milk (Liu, 2016).

Leuconostocs are able to contribute to human health improvement by producing and excreting certain vitamins (*Ln. lactis* is found to produce B11, K1 and K2 vitamins) and antimicrobial compounds against spoilage and pathogenic microorganisms such as bacteriocins. Leuconostocs have been studied more frequently due to their contribution to the enhancement of nutritional value and the quality of fermented foods (Liu, 2016; Hwang *et al.*, 2018). Despite the association of human infections to *Leuconostoc* species, this is related to opportunistic strains that affect susceptible immunocompromised individuals and, therefore, overall the genus is considered as GRAS (Liu, 2016).

1.2.3. Antimicrobial proteinaceous compounds: bacteriocins

It has been known that different organisms, such as animals, plants, insects and bacteria, can produce antibacterial substances, which includes organic acids, antibiotics and bacteriocins (Ray and Joshi, 2014; Yang *et al.*, 2014).

Bacteriocins are defined as a proteinaceous inhibitors or antimicrobial peptides that are produced by bacteria. In general, these compounds act as antibacterial through the inhibition of target cell wall synthesis or through depolarization of the cell membrane (Ray and Joshi, 2014; Yang *et al.*, 2014). The competition for both living space and nutrients, and insufficient amounts of environmental resources can act as a set up to the production of these antibacterial compounds. These bacteriocins have greatly variations depending on the producing strain, but have large antimicrobial diversity ranging in specificity from related bacteria, narrow spectrum of activity (e.g. lactococcins only inhibit lactococci), to non-related microorganisms, broad spectrum of activity (e.g. lantibiotic nisin) (Ray and Joshi, 2014; Yang *et al.*, 2014). This suggests that the

production of bacteriocins by bacteria acts as an inherent defence system in the environment which can maintain population numbers trough reduction of bacterial competitors to obtain nutrients and resources (Yang *et al.*, 2014). Despite the killing ability of bacteriocins, these substances are sensitive to proteases meaning that, in general, are harmless to the human body and the environment.

In the industry, the growing concern over the presence of chemical additives in food has a heavy impact on food safety. Lactic acid bacteria are known to be the most studied bacteriocin producers, giving the high variety of bacteriocins of different structures, sizes, physical and chemical properties and inhibitory spectrum (Yang *et al.*, 2014). Bacteriocins produced by LAB are divided into three main groups (schematic overview in Figure 1.1.). The most common class I bacteriocin is nisin A produced by *Lactococcus lactis* subsp. *lactis*, frequently used as a preservative in processed cheeses (Ray and Joshi, 2014; Yang *et al.*, 2014). Class II bacteriocins are subdivided into four classes. Briefly, class IIa bacteriocins are recognized as anti-listerial bacteriocins, class IIb require two peptides for forming a fully active poration complex, class IIc are circular bacteriocins and class IId are non-pediocin like (Yang *et al.*, 2014). The most common are the pediocins produced by *Pediococcus* spp. that are used as main starter cultures in fermented meats (Ray and Joshi, 2014).

Lastly, class III bacteriocins are subdivided into Group A - bacteriolytic bacteriocins, such as enterolisin A produced by *Enterococcus faecalis* LMG 2333, and Group B - non-lytic bacteriocins, such as caseicin 80 produced by *Lactobacillus casei* B80 (Yang *et al.*, 2014).

Class I	Class II	Class III
• Small peptide (<5 kDa) • Modified peptides • Recognized as lantibiotic	• Bigger peptides (<10 kDa) • Sub-divided into class IIa; class IIb, class IIc and class IId • Nonmodified peptides, non-lanthionine	• Large molecular weight (>30 kDa) • Heat-labile proteins • Subdivided into Group A and Group B

Figure 1.1. Classification of bacteriocins from Gram-positive bacteria (Harzallah and Belhadj, 2013).

1.3. Criteria for selecting probiotic bacteria to be used in the food industry

There are key criteria for selecting probiotic candidates for commercial application describing major steps as initial screening, such as safety, technological, functional as well as desirable physiological criteria (Harzallah and Belhadj, 2013). A schematic overview is shown in Table 1.1..

Table 1.1. Key criteria for the selection of probiotic bacteria for commercial use (Adapted from Harzallah and Belhadj, 2013)

Criteria	Properties
Safety	Origin Phenotype and genotype stability Virulence factors (i.e. antibiotic susceptibility, virulence genes, biogenic amine-forming capacity, hemolytic activity, DNase and gelatinase activity)
Technological	Viability during process and storage Large scale production Lipase activity
Functional	Resistance to GIT conditions Bile tolerance Adhesion to mucosal surface
Beneficial	Antimicrobial Activity Survival at different stress conditions (e.g. Temperature) Bacteriocin activity spectrum Bacteriocin characterization (i.e. class/group definition)
Desirable	Antagonist activity against GIT pathogens Lysozyme tolerance Cholesterol metabolism Auto and Co-aggregation Hydrophobicity

Overall, the criteria for evaluation of probiotic bacteria to be used in food should proceed the following important assays: i) initial screening and selection of probiotics to evaluate the phenotype and genotype stability, ii) acid and bile tolerance (survival and growth under these conditions), iii) production of antimicrobial substances (such as bacteriocins), iv) antibiotic resistance patterns, v) ability to inhibit the growth of pathogenic bacteria and vi) intestinal adhesion properties (Harzallah and Belhadj, 2013). Subsequently, the characterization of the proteinaceous compounds produced by putative probiotic is important to estimate its beneficial

role, evaluating the proteinaceous antimicrobial compound activity (AU/ml) at a wide range of temperatures and pH values, diverse enzymes, detergents, surfactants and protease inhibitors.

Safety criteria involves the evaluation of the identification by specificity of probiotic bacteria and the assessment of virulence factors, including antibiotic resistance, virulence genes, hemolytic activity, biogenic amine-forming capacity (BA) and hydrolytic enzyme activity (gelatinase and DNase), among others.

Antibiotic susceptibility and virulence genes assay should be followed to exclude possible harmful bacteria to the host regarding the specificity of the probiotic strain.

The transmission of genetic material via horizontal gene transfer from antibiotic resistant bacteria to other normal susceptible bacteria, and among closely or distantly related species (e.g. from *Enterococcus* strains), may contribute to the dissemination of antibiotic resistances (Flórez *et al*., 2016). Antibiotic resistant non-pathogenic bacteria or opportunistic bacteria constitute and indirect hazard in microbial systems including food related niches. Therefore addressing the possibility of transferable antibiotic resistance genes via horizontal transfer between foodborne commensal bacteria is crucial for public health.

There are stated guidelines (EFSA guidelines, 2012) to assess antibiotic susceptibility and the minimal inhibitory concentrations (MICs, µg/ml).

Regarding the presence of virulence genes there are a few that should be assessed according to the specificity of the probiotic strain, since they can be expressed depending on external environmental factors, that could increase the capacity to cause infection, facilitating adhesion and colonization. Enterococci are known to possess virulence determinants, that code for aggregation substances (*agg* and *asa1* genes), cell wall adhesins (*efaAfm* and *efaAfs* genes) and cytolysin (*cyl*) (Eaton *et al*., 2001; Barbosa *et al*., 2010; Biswas *et al*., 2016) (role of virulence genes products are listed in Table 1.2.).

Biogenic amines (BA) are organic compounds that can be produced by bacteria, such as those involved in fermentation, via decarboxylation of corresponding amino acids in foods through substrate-specific of corresponding enzymes. Biogenic amines can cause adverse health effects if the capacity of metabolizing amines becomes saturated (Bover-Cid and Holzapfel 1999; Doeun *et al*., 2017). The most common BA are, among others, tyramine, histamine, cadaverine and putrescine.

Table 1.2. Role of virulence genes products

Gene	Gene type	Role of product in virulence	Reference
ace		Adhesion of collagen which mediates the association of bacteria to host	Medeiros *et al.* (2014)
vanA *vanB*		Vancomycin-resistant genes	Biswas *et al.* (2016)
agg	Aggregation substance	Aggregation protein involved in adherence to eukaryotic cells	Eaton *et al.* (2001)
gelE	Gelatinase	Extracellular peptidase that hydrolyzes bioactive compounds	Eaton *et al.* (2001)
esp	Enterococcal surface protein	Cell wall-associated protein involved in immune evasion	Eaton *et al.* (2001)
efaAfs *efaAfm*	Cell wall adhesins	Cell wall adhesins expressed in serum by *E. faecalis* and *E. faecium*, respectively	Eaton *et al.* (2001)
cylA	Cytolysin	Activation of cytolysin	Eaton *et al.* (2001)
cylB	Cytolysin	Transport of cytolysin	Eaton *et al.* (2001)
cylM	Cytolysin	Post translational modification of cytolysin	Eaton *et al.* (2001)
cylL$_L$ *cylL$_S$*	Cytolysin	Cytolysin precursor	Eaton *et al.* (2001)
hyl	Hyaluronidase	Acts on hyaluronic acid and increases bacterial invasion	Biswas *et al.* (2016)
asa1	Aggregation substance	Facilitates the transfer of transmissible conjugative plasmids	Biswas *et al.* (2016)
hdc1	Biogenic amines	Linked to histidine production	Barbieri *et al.* (2019)
tdc	Biogenic amines	Linked to tyramine production	Barbieri *et al.* (2019)
odc	Biogenic amines	Linked to ornithine production	Barbieri *et al.* (2019)

Polyamines, putrescine and cadaverine, are involved in growth and cell proliferation, and can be potential carcinogens when converted to nitrosamines in the presence of nitrites. Tyramine is an aromatic BA that alone can induce migraines and when combined with putrescine leads to heart failure or brain haemorrhage. The presence of histamine can cause allergen-type reactions (e.g. vomiting, hypertension, rash) and, when combined with the polyamines, enhances their toxicity (Naila *et al.*, 2010).

Gelatinase is a protease capable of hydrolysing gelatin, collagen, casein, haemoglobin and others bioactive peptides, conducting to virulence. DNase is an extracellular enzyme able of digest Deoxyribonucleic acid (DNA). The haemolytic activity is associated to the ability of microorganisms to lyse red blood cells causing the release of haemoglobin in blood.

As a technological property, lipase activity should be accounted since the biological role of lipase enzyme interferes in the immunity response of host and is able to hydrolyse lipids. The production of this enzyme enhances the degradation of surface molecules of the host facilitating the colonization and persistence of bacteria, and it is associated to many bacterial infections (Chakchouk-Mtibaa *et al*., 2018).

Tolerance to gastric juices, bile tolerance and adhesion to mucosal surfaces are assessed as functional roles, because probiotic strains must survive the passage through the digestive system and survive, proliferate and colonize the active location (Harzallah and Belhadj, 2013).

Lastly, physiological criteria, such as cholesterol metabolism, auto and co-aggregation, may be assessed since these are viewed as beneficial criteria for putative probiotics.

1.3.1. LAB as probiotic bacteria

As referred previously, the high ability to survive under stress conditions, production of antimicrobial compounds and, consequently, the influence in human health infers probiotic characteristics in lactic acid bacteria. Probiotics are generally defined as live microorganisms that can confer health benefits on the host, when administered in adequate amounts, and are intended to assist the body's host gut microbiota (WGO, 2011), which may enhance immunity and disease resistance (FAO/WHO, 2002).

There is no existing scientific consensus to the legal definitions of the term "probiotic", however there are guidelines reported by FAO/WHO Consultancy (2002) that should be used as a starting-point regarding the new probiotic strains that are to be released to the market (WGO, 2011). The minimum criteria intended for probiotic products are that probiotics should be alive, specified by genus, species and strain and produced in adequate dosages to endure high numbers through the end of shelf-life. Also, probiotics must be safe for ingestion and effective in *in vivo* studies (WGO, 2011).

Despite the general health benefits imposed by probiotics, the effects on human health is attributed to the strain tested since studies conducted at species level are recognized as not sufficient to support the potential beneficial roles. Regarding to the dosage, it varies greatly depending the viability and endurance of the strain and rely also in the product formula (WGO, 2011). Despite the recognition of GRAS (WGO, 2011) for consumption and usage in food

fermentations, specific established criteria to analyse safety of LAB must be succeed during the selection and evaluation of probiotics (Ray and Joshi, 2014; Flahaut and de Vos, 2015).

In the food industry, LAB must resist technological stresses during preparation, production and storage of probiotic formulas to uphold high viable counts (Papadimitriou *et al*., 2016). Also, LAB needs to be able to survive several environmental challenges and implement their beneficial role. Consequently, probiotics used in pharmaceutical and food industry must be prior subjected to controlled *in vivo* studies to report the behavior of probiotic bacteria under stress environments.

1.4. Objective

This work aimed to isolate lactic acid bacteria from several food products and to evaluate their potential antimicrobial activity and probiotic characteristics. From 202 isolated lactic acid bacteria from 20 fermented food products, *Leuconostoc lactis* RK18 was the only isolate chosen to evaluate its safety and functional characteristics as well as to further characterize its anti-listerial bacteriocin.

II.   Material and Methods

2.1. Lactic acid bacteria used in this study

2.1.1.   Isolation of LAB from different food products

Twenty different fermented food products (Table 2.1.), purchased at local supermarkets or already stored in the laboratory, were analysed between September and October, 2018.

Twenty-five grams of each sample were added to 225 ml of sterile Buffered Peptone Water (BPW, Biokar, Beauvais, France) and homogenized in a stomacher for 2 minutes. Several decimal dilutions were prepared in sterile Ringer's solution (Biokar) for lactic acid bacteria (LAB) enumeration on De Man Rogosa & Sharp agar (MRS, Biokar) and M17 agar (Biokar) and incubated at 30 ºC for 3 to 5 days, under microaerophilic conditions (ISO 15214:1998).

2.1.2. Selection of LAB isolates

Colonies were randomly selected (10%) and cultured in MRS or M17 agar for 24 to 72 h at 30 ºC under the same atmospheric conditions.

Cellular characteristics (Gram stain), morphological characteristics (colour and type of colony, elevation and opacity), catalase and oxidase tests were evaluated to select potential LAB isolates with antimicrobial activity.

2.1.3. Growth and storage conditions

Selected LAB isolates were cultivated on MRS or M17 agar (Biokar) at 30 °C for 24 to 72 h and stored at -20 °C in broth culture medium containing 30% (v/v) glycerol (Sigma, Steinheim, Germany) and sub-cultured twice before use in assays.

Table 2.1. Description and origin of fermented food products used in this study

Samples	Description	Origin
Tâmaras	Fruit	
Watercress Baby Leaf*	Ready-to-eat food salads	Purchased at local supermarkets
Wild Arugula*		
Black Bean Pasta	Korean-Chinese noodle dish	Purchased at an Asian supermarket
Kimchi	Korean Fermented Cabbage	
"Pickled Lettuce"	Chinese canned vegetable pickles	
Fermented Shrimp	Fresh cleaned shrimp, salt, ground roasted rice powder and galangals are mixed and fermented in a jar for up to 10 days	
Fermented Cucumber	Cucumber, garlic, spices are mixed are mixed and fermented in a jar with saline solution for up to one week	
Fermented Fish	Cleaned fish, salt and roasted rice powder are mixed and fermented in a jar for longer than a week	
Beef Sausage	Beef, sugar, steamed rice, garlic, galangal, roasted rice powder and red wine are mixed, wrapped and fermented for up to one week	
Fish Sausage	Fish meat, sugar, steamed rice, pepper, salt, garlic and spices are mixed, wrapped and fermented for up to 48 hours	Samples stored in laboratory (Cambodian fermented food) References from Peng *et al.* (2017)
Pork Sausage	Pork, sugar, ground star anise powder, salt and white rice wine are mixed, wrapped and fermented up to one week	
Fermented Cabbage	Cleaned and dried Cambodian cabbage and saline solution are mixed and fermented up to one week	
Fish Paste	Overnight soaked rice passes through a mechanical pressure and cooking processes to become sticky and the processed into noodles	
Khmer Fermented Rice Fresh Noodles	Soaked fish is dried for 1 hour, mixed with a saline solution and fermented in a jar for up to a month	
Watercress*	Ready-to-eat food salads	Purchased at local supermarkets
Coriander*		
Parsley*		
Green Cos*		
Green Batavia*		

* - modified atmosphere packaged samples

2.2. Study of antimicrobial activity of isolated LAB

2.2.1. Antimicrobial activity screening

Antibacterial activity was tested by the agar spot test (Van Reenen *et al.*, 1998). Seven target microorganisms (Table 2.2.) were grown on TSAYE (Tryptic Soy Agar (TSA, Biokar) with 6 g/l of Yeast Extract (YE, Lab M, Lancashire, United Kingdom)) and incubated at 37 °C for 24 h and, subsequently, one colony of each isolate was transferred to 10 ml of TSBYE (Tryptic Soy Broth (TSB, Biokar) with 6 g/l of YE) and incubated overnight at 37 °C. Suspensions of each target bacterial culture were spread onto TSAYE plates and 10 µl drops of each LAB culture, grown twice in MRS or M17 broth (Biokar), were spotted on the lawns of target organisms (Table 2.2.) and incubated overnight at 37 °C. Inhibition was recorded as positive if a translucent halo zone was observed around the spot and *Pediococcus acidilactici* HA-6111-2 (from Culture Collection of *Escola Superior de Biotecnologia*) was used as anti-listerial control strain (Albano *et al.*, 2009).

2.2.2. Antimicrobial activity characterization

For LAB isolates showing antimicrobial activity, the nature of the inhibition was determined by the qualitative agar-diffusion technique according to Tomé *et al.* (2006). Culture broths of each LAB were centrifuged at 7000 rpm for 10 min at 4 °C (Centrifuge 5427 R, Eppendorf, Hamburg, Germany), the clear supernatants were sterilized by membrane filtration (0.2 µm; Sartorius, Goettingen, Germany) and their pH adjusted to ≈ 5.0 and 6.0 with sterilized solution of sodium hydroxide (1M NaOH, José M. Vaz Pereira, Lisbon, Portugal). To determine whether the inhibition was due to hydrogen peroxide production or to proteinaceous compounds, neutralized supernatants were treated with catalase (500 IU/ml; Sigma) and proteinase K (0.1 mg/ml, sterile; Sigma), respectively, for 1 h at 37 ºC. Culture grown (C), cell free supernatant (CFS), neutralized cell-free supernatant (CFSn), neutralized cell-free supernatant treated with catalase (CFSnC) and neutralized cell-free supernatant treated with proteinase K (CFSnK), were spotted against target microorganisms listed in Table 2.2.. *P. acidilactici* HA-6111-2 was used as anti-listerial control strain (Albano *et al.*, 2009). Inhibition by a proteinaceous substance (possible bacteriocin production) was assumed if a translucent halo zone was observed around all the spots except for cell-free supernatant treated with proteinase K.

Table 2.2. Target microorganisms used in the study of antimicrobial activity

Microorganism	Source
Enterococcus faecalis ATCC 29212	American Type Culture Collection (ATCC; Manassas, EUA)
Staphylococcus aureus ATCC 29213	
Enterococcus faecium DSMZ 13590	Leibniz Institute DSMZ – German Collection of Microorganisms and Cell Cultures (DSMZ; Brunsvique, Germany)
Listeria monocytogenes NCTC 1194	Isolates from Culture Collection of *Listeria Research Center of Escola Superior de Biotecnologia* (LRCESB)
Listeria monocytogenes CECT 911	
Listeria monocytogenes CECT 936	
Listeria monocytogenes CEP 104794	

2.3. Identification of selected LAB by 16S rRNA sequencing

Lactic acid bacteria isolates that were putative bacteriocin producers were selected and identified by 16S rRNA sequencing. Deoxyribonucleic acid (DNA) was extracted according to the protocol for total DNA purification from Gram-positive bacteria of the GRS genomic DNA Kit (Grisp, Porto, Portugal). Polymerase Chain Reaction (PCR) amplification of the 16S rRNA gene fragments was performed using primers 27F (5'-AGAGTTTGATCCTGGCTCAG-3') and 1492R (5'-GGTTACCTTGTTACGACTT-3') as described by Vaz-Moreira *et al.* (2011). PCR amplifications were performed in a Thermocycler (Bio-Rad, Alfragide, Portugal) with 50 µl mixtures using 10 mM of dNTPs, 10X of Taq buffer $(NH_4)_2SO_4$, 25 mM of $MgCl_2$, 100 µM of each primer, 5U of Taq polymerase and 2 µl of bacterial DNA and under the following conditions: initial cycle of 95 ºC for 5 minutes; 30 cycles of 94 ºC for 1 min (denaturation), annealing temperature of 55 ºC for 1 min and 72 ºC for 1 min (extension); a final extension step of 72 ºC for 10 minutes followed by cooling at 4 ºC. For each PCR reaction a negative control (sample without template) and a positive control (sample with DNA from strain *P. acidilactici* HA-6111-2) were included. All amplification products (10 µl) were combined with 3 µl of loading buffer (Bio-Rad) and applied to a submerged horizontal 1.2% (w/v) agarose gel (Seakem® LE Agarose, Rockland, ME, USA) in 1x TAE Buffer (40 mM Tris, 20 mM acetic acid and 1 mM EDTA, pH 8.3; Bio-Rad) containing 1x of Xpert Green DNA Stain (Grisp, Porto, Portugal). Electrophoretic separation was performed at 90 V for 45 minutes and, on each gel, a

molecular weight marker (NZYDNA ladder VI, 50 to 1500 bp, Nzytech, Lisbon, Portugal) was included at two positions. Gels were photographed on a UV light transilluminator (ImageLab, Bio-Rad).

The purification of the PCR products was carried out using GRS PCR & Gel Purification Kit (Grisp) and used as templates for sequencing. Sequences obtained were aligned with the sequences in Gene Bank using the BLAST program (http://www.ncbi.nlm.nih.gov) (Altschul *et al.*, 1997).

2.4. Study of several criteria required for potential probiotics

Selected LAB, two *Enterococcus faecium* (RS7 and P12) and one *Leuconostoc lactis* (RK18), were assessed for the presence of several criteria required for a microorganism to be considered as a potential probiotic.

2.4.1. Safety criteria of potential probiotic

2.4.1.1. Antibiotic susceptibility testing

The minimal inhibitory concentrations (MICs, µg/ml) of antibiotics ampicillin, vancomycin, chloramphenicol, streptomycin, kanamycin (all from Fluka, Steinheim, Germany), erythromycin, gentamicin and tetracycline (kindly supplied by the company Labesfal, Tondela, Portugal), were determined by the agar microdilution method, according to Clinical and Laboratory Standards Institute (CLSI, 2012) for *Enterococcus faecium* isolates and by broth microdilution method according to Klare *et al.* (2005) for *Leuconostoc lactis* RK18 isolate. Each antibiotic was prepared in order to obtain concentrations that ranged the breakpoints defined by European Food Safety Authority (EFSA, 2012). Briefly, each inoculum of LAB was prepared from an overnight culture on M17 (*E. faecium* RS7 and P12) or MRS plates (*Ln. lactis* RK18), by suspension in sterile Ringer's solution (Biokar) in order to obtain turbidity equivalent to 0.5 McFarland standards. For *E. faecium* isolates, Mueller-Hinton agar (MHA, BioMérieux, Marcy l'Etoile, France) plates with several concentrations of each antibiotic were spotted with each *E. faecium*

suspension. After 24 hours of incubation at 37 ºC it was observed the presence or absence of growth. Broth microdilutions of each antibiotic were performed in 96-well microtiter plates (Sarstedt, Sintra, Portugal) in LSM medium (90% of Iso-Sensitest broth and 10% of MRS broth; Klare *et al.*, 2005) for *Ln. lactis* RK18. After 24 hours of incubation at 30 ºC, presence or absence of turbidity in each well was observed.

Minimal inhibitory concentration was recorded as the first concentration for which no growth was verified. Isolates grown on MHA (BioMérieux) and LSM broth (Klare *et al.*, 2005) with no antibiotic were used as control and quality control strains *Enterococcus faecalis* ATCC 29212 and *Escherichia coli* ATCC 25922 were used to monitor the accuracy of MICs. Each experiment was performed in duplicate. Isolates were classified according to their susceptibility (as sensitive, intermediate or resistant) by values recommended by EFSA (2012).

2.4.1.2. Virulence factors

2.4.1.2.1. Determination of biogenic amine-forming capacity

Bover-Cid and Holzapfel (1999) developed an improved screening plate method for the detection of amino acid decarboxylase-positive microorganisms (especially lactic acid bacteria). The three LAB isolates were tested using this method, in order to identify their potential to produce the biogenic amines tyramine, histamine, putrescine and cadaverine. Before the screening test, *Enterococcus* isolates were sub-cultured seven times in Brain Heart Infusion (BHI, Biokar) broth and *Ln. lactis* RK18 in MRS broth (Biokar) with 0.1% (w/v) of each precursor amino-acid (all from Sigma): tyrosine free base for tyramine, histidine monohydrochloride for histamine, ornithine monohydrochloride for putrescine and lysine monohydrochloride for cadaverine, and supplemented with 0.005% (w/v) of pyridoxal-5-phosphate (Fluka, Steinheim, Germany), to promote the enzyme induction. Then, isolates were spotted in duplicate on the medium with each amino acid (Table 2.3.) and incubated at 37 ºC for *E. faecium* RS7 and P12 and at 30 ºC for *Ln. lactis* RK18 for 4 days. Plates without amino acid were used as controls. Positive reaction was confirmed if a purple colour occurred or tyrosine precipitate disappeared around the colonies. Two replicates were performed for each isolate.

Table 2.3. Composition (g/l) of decarboxylase media according to Bover-Cid and Holzapfel (1999)

Component	Medium
Tryptone	5
Yeast extract	5
Sodium chloride	2.5
Glucose	0.5
Tween 80 ®	1
$MgSO_4$	0.2
$MnSO_4$	0.05
$FeSO_4$	0.04
Ammonium citrate	2
Thiamine	0.01
K_2P_4	2
$CaCO_3$	0.1
Pyridoxal-5-phosphate	0.05
Amino acid	10
Bromocresol purple	0.06
Agar	20
pH	5.3

2.4.1.2.2. Production of hydrolytic enzymes: gelatinase and DNase

The production of extracellular enzymes, gelatinase and DNase, were assessed according to Tiago *et al*. (2004) and Ben-Omar *et al*. (2004), respectively. Gelatinase activity was assayed by using the Modified Luria-Bertani (MLB, Sigma) broth supplemented with 50.0 g/l of gelatin. Tubes were incubated at 30 ºC for 7 days and then placed into the refrigerator for approximately 30 min. The production of sufficient gelatinase turned the medium liquid even when placed in the refrigerator, indicating a positive result.

DNase activity was tested using DNase agar (Pronadisa, Madrid, Spain) as described by Ben-Omar *et al*. (2004). A clear halo around the colonies indicated a positive result.

All experiments were performed in duplicate and *Staphylococcus aureus* ATCC 25213 was used as a positive control.

2.4.1.2.3. Hemolytic activity

Production of haemolysin was determined by streaking isolates onto Columbia Agar plates (Oxoid, Hampshire, United Kingdom). Plates were incubated at 37 ºC (for *Enterococcus* spp. isolates) and at 30 ºC (for *Ln. lactis* RK18) for 24 hours after which plates were examined for haemolysis activity. Greenish and translucent zones around the colonies indicated α-haemolysis and β-haemolysis, respectively. The absence of clear zones around the colonies indicated the absence of haemolytic activity, γ-haemolysis. As controls, *E. faecalis* F2 (from a collection of Tracy Eaton, Division of Food Safety Sciences, Institute of Food Research, Norwich, United Kingdom) and *E. faecalis* DS16 (from a collection of C. B. Clewell, Department of Oral Biology, School of Dentistry, University of Michigan, Ann Arbor, USA) were used.

2.4.1.2.4. Presence of virulence genes

Eighteen virulence genes were investigated: surface adhesin genes (*esp, ace*, *efaAfs* and *efaAfm*), aggregation protein gene (*agg*), extracellular metallo-endopeptidase gene (*gelE*), cytolysin genes (*cylA, cylB cylM, cylL$_L$* and *cylL$_S$*), vancomycin resistance genes (*vanA* and *vanB*), hyaluronidase gene (*hyl*), aggregation substance precursor (*asa1*) and genes related to biogenic amines (*hdc1, tdc* and *odc*). Information about PCR amplification conditions and concentrations, product size and positive controls of each virulence gene tested are listed in Table 2.4..

For each PCR reaction, samples without template were used as negative control and DNA from each specific strain according to the studied gene was used as positive control. Electrophoretic separation (at 80 V) was performed as described above in the section 2.3.

Table 2.4. Virulence genes primers and PCR conditions

Gene	*Primer* (5' to 3')	PCR	PCR conditions	Size (bp)	Positive Controls	Source
ace	GAATTGAGCAAAAGTTCAATCG GTCTGTCTTTTCACTTGTTTC	0,5 µl dNTPs (10 mM); 2.5 µl buffer NH4; 2.5 µl MgCl2 (25 mM); 0.25 µl primer F/R (10 pM); 0.4 µl Taq polimerase (5U)	95 °C (1 min); 30 x [94 °C (1 min), 55 °C (1 min), 72 °C (1 min)]; 72 °C (10 min); 4 °C	1008	*E. faecalis* DS16; *E. faecalis* F2; *E. faecalis* P1; *E. faecalis* P36; *E. faecalis* 29212	Martín-Platero *et al.* (2009)
vanA	TCTGCAATAGAGATAGCCGC GGAGTAGCTATCCCAGCATT			377	*E. faecalis* vanA; *E. faecalis* vanB; *E.* DSMZ 12956	
vanB	GCTCCGCAGCCTGCATGGACA ACGATGCCGCCATCCTCCTGC			529	E. faecalis vanB	
agg	AAG AAA AAG AAG TAG ACC AAC AAA CGG CAA GAC AAG TAA ATA	0.25 µl dNTPs (10 mM); 2.5 µl buffer NH4; 2.5 µl MgCl2 (25 mM); 1.25 µl primer F/R (10 pM); 0.25 µlTaq polimerase (5U)	94 °C (1 min); 35 x [94 °C (1 min),55 °C (1 min), 72 °C (2 min)]; 72 °C (7 min); 4 °C	1553	*E. faecalis* P1	Eaton and Gasson (2001)
esp	TTG CTA ATG CTA GTC CAC GAC C GCG TCA ACA CTT GCA TTG CCG AA			933	*E. faecalis* P36	
gelE	ACC CCG TAT CAT TGG TTT ACG CAT TGC TTT TCC ATC			419	*E. faecalis* P1	
efaAfs	GAC AGA CCC TCA CGA ATA AGT TCA TCA TGC TGT AGT A			705	*E. faecalis* F2	
efaAfm	AAC AGA TCC GCA TGA ATA CAT TTC ATC ATC TGA TAG TA			735	*E. faecalis* F10	

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

Table 2.4. Virulence genes primers and PCR conditions (continuation)

Gene	*Primer* (5' to 3')	PCR	PCR conditions	Size (bp)	Positive Controls	Source
cylA	TGG ATG ATA GTG ATA GGA AGT TCT ACA GTA AAT CTT TCG TCA	0.25 µl dNTPs (10 mM); 2.5 µl buffer NH4; 2.5 µl MgCl2 (25 mM); 1.25 µl primer F/R (10 pM); 0.25 µlTaq polimerase (5U)	95 °C (1 min); 35 x [94 °C (1 min),55 °C (1 min), 72 °C (2 min)]; 72 °C (7 min); 4 °C	517	*E. faecalis* F2	Semedo *et al.* (2003)
cylB	ATT CCT ACC TAT GTT CTG TTA AAT AAA CTC TTC TTT TCC AAC			843	*E. faecalis* F2	
cylM	CTG ATG GAA AGA AGA TAG TAT TGA GTT GGT CTG ATT ACA TTT			742	*E. faecalis* F2	
cylLL	GAT GGA GGG TAA GAA TTA TGG GCT TCA CCT CAC TAA GTT TTA TAG			253	*E. faecalis* DS16	
cylLS	GAA GCA CAG TGC TAA ATA AGG GTA TAA GAG GGC TAG TTT CAC			240	*E. faecalis* DS16	
hyl	ACAGAAGAGCTGCAGGAAATG GACTGACGTCCAAGTTTCCAA	0.5 µl dNTPs (10 mM); 2.5 µl buffer NH4; 2.5 µl MgCl2 (25 mM); 0.35 µl primer hyl e asa1 F/R (10 pM); 0.4 µl Taq polimerase (5U)	95 °C (1 min); 30 x [94 °C (1 min), 56 °C (1 min), 72 °C (1 min)]; 72 °C (10 min); 4 °C	276	*E. faecalis* vanB	Vankerckhoven *et al.* (2004)
asa1	GCACGCTATTACGAACTATGA TAAGAAAGAACATCACCACGA			375	*E. faecalis* DS16; *E. faecalis* F2; *E. faecalis* P1; *E. faecalis* P36; *E. faecalis* 29212	
hdc1	AGATGGTATTGTTTCTTATG AGACCATACACCATAACCTT	2.5 µl Tris HCl; 0.5 µl dNTPs (10 mM); 5 µl buffer KCl; 2.5 µl MgCl2 (25 mM); 0.75 µl primer hdc F/R (10pM), 2 µl primer tdc F/ (10 pM) e 1 µl primer odc F/R (10 pM); 0.4 µlTaq polimerase (5U)	95 °C (1 min); 30 x [95 °C (30s), 52 °C (30s), 72 °C (2 min)]; 72 °C (10 min); 4 °C	367	*E. faecalis* DS16; *E. faecalis* F2; *E. faecalis* P1; *E. faecalis* P36	De las Rivas *et al.* (2005)
tcd	GAYATNATNGGNATNGGNYTNGAYCARG CCRTARTCNGGNATAGCRAARTCNGTRTG			924		
odc	GTNTTYAAYGCNGAYAARCANTAYTTYGT ATNGARTTNAGTTCRCAYTTYTCNGG			1446		

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

2.4.2. Technological Criteria

2.4.2.1. Lipase activity

Briefly, the production of lipase was assayed in MLB broth supplemented with 2.0 g/l of $CaCl_2$ and 10 g/l of Tween 80. After 7 days of incubation at 30 ºC, a positive reaction was indicated by a clear halo around the colonies.

All the tests described below were only performed for *Leuconostoc lactis* RK18 isolate, since it was the only isolate that showed neither virulence factors nor antibiotic resistances.

2.4.3. Beneficial Criteria

2.4.3.1. Characterization of the bacteriocin produced by *Leuconostoc lactis* RK18

2.4.3.1.1. Maximum bacteriocin production (AU/ml) during growth of *Ln. lactis* RK18

To determine de maximum bacteriocin production during its growth, the optimal growth temperature of *Ln. Lactis* RK18 was firstly determined: several aliquots were taken from an overnight culture in MRS broth (Biokar), grown at each temperature defined, at every three hours after inoculation until twenty-one hours of growth in three different temperatures: 25 ºC, 30 ºC and 37 ºC. Several decimal dilutions were prepared in sterile Ringer's solution, onto MRS agar by the drop count technique (Miles and Misra, 1938) and incubated for 24 h at 30 ºC for enumeration. The colonies of all viable cells were counted and the colony forming units (cfu/ml) calculated.

Then, one percent (v/v) of an overnight *Ln. lactis* RK18 culture was inoculated in MRS broth (100 ml) and incubated at 30 ºC. Changes in pH and Optical Density (O.D., 600 nm) were recorded every hour until 48 h. Every three hours, aliquots of 10 ml and 1 ml were taken to test bacteriocin activity (AU/ml) against *L. monocytogenes* from 4 serogroups - *L. monocytogenes*

CEP 104794 (serogroup 1/2a), *L. monocytogenes* CECT 936 (serogroup 1/2b), *L. monocytogenes* CECT 911 (serogroup 1/2c) and *L. monocytogenes* NCTC 1194 (serogroup 1/4b) in the treated cell-free supernatant (Van Reenen *et al.*, 1998) and to determine viable cell counts (cfu/ml) of *Ln. lactis* RK18, respectively.

To determine the bacteriocin activity (AU/ml), cells from 10 ml aliquots were harvest by centrifugation (7000 rpm, 10 min, 4 ºC; Centrifuge 5427 R, Eppendorf) and the cell-free supernatants (CFS) adjusted to pH 5.0-6.0 with 1M NaOH and incubated at 80 ºC for 10 minutes (CFSnh) (Van Reenen *et al.*, 1998). Then, each treated CFS was successive diluted on PBS (VWR Chemicals, Ohio, USA) and 10 µl aliquots of each dilution were spotted onto a soft agar plate (BHI with 0.7% w/v agar) seeded with approximately 10^6 cfu/ml of each target *L. monocytogenes* strain. Plates were incubated at 30 ºC during 24 h to 48 h. *Pediococcus acidilactici* HA-6111-2 was used as control. Antimicrobial activity was expressed as arbitrary units (AU) per ml and according to Van Reenen *et al.* (1998) one AU is defined as the reciprocal of the highest dilution showing a clear zone of growth inhibition.

2.4.3.1.2.   Effect of pH, enzymes, temperature, detergents, surfactants and protease inhibitors on bacteriocin activity

The evaluation of the following tests was performed on treated cell-free supernatant (CFS) of *Ln. lactis* RK18 prepared as described in previous section 2.4.3.1.1.

The effect of enzymes was determined by incubating 1 ml of each CFS neutralized and heated at 80 ºC (CFSnh) for 2 hours in the presence of 1 mg/ml and 0.1 mg/ml of each enzyme: proteinase K, papain, pepsin and catalase (all from Sigma).

The effect of temperature on bacteriocin activity was tested by incubating CFSn at 4, 25, 30, 60, 80 and 100 ºC for both 1 h and 2 h. Bacteriocin activity was also tested after 15 min at 121 ºC.

Finally, 1% (w/v) Tween 20, Tween 80, Triton X-100 (Bio-Rad Laboratories, Richmond, CA, USA), EDTA (Panreac Quimica SA, Barcelona, Spain), Ox-bile (Pronadisa), Urea (Merck, Darmstadt, Germany), SDS, NaCl, Sodium Carbonate and Sodium deoxycholate (all for Sigma) were added to CFSn. Untreated CFS and detergents in water at the same concentrations were used as controls. All samples were incubated at 30 ºC for 5 h.

For the evaluation of the effect of pH on the bacteriocin activity, CFS was adjusted from pH 2.0 to 12.0 (at increments of two pH units) with sterile 1M NaOH or 1M Hydrochloric Acid (HCl). After 1 h of incubation at room temperature, the samples were readjusted to pH 5 to 6, incubated at 80 ºC for 10 min.

For all the conditions, antimicrobial activity was monitored by agar-spot test method (Van Reenen *et al*., 1998) and 4 serogroups of *L. monocytogenes*, mentioned in the section 2.4.3.1.1., were used as target strains.

2.4.3.1.3. Cell lysis of target microorganisms

Twenty percent (v/v) of bacteriocin-containing cell-free supernatant, neutralized and filtered, was added to 100 ml cultures of each target organism (when reached early exponential stages). Every hour, optical density at 600 nm was read for 12 h. Viable cell counts (cfu/ml) were determined every 2 h between 0 (time at which inoculum of each *L. monocytogenes* strain was added at flasks containing TSBYE) and 12 h by plating onto TSAYE and incubated at 37 ºC for 24 h. As control, each target culture without added bacteriocins, under the same conditions (incubated at 37 ºC for 12 h), were used.

2.4.3.1.4. Adsorption studies and partial purification of *Ln. lactis* RK18 bacteriocin

Adsorption of *Ln. Lactis* RK18 bacteriocin was conducted according to the method described by Yang *et al*. (1992). Briefly, after pH adjusted to 6.0, bacteriocin producing cells (cultured for 15 h to 18 h at 30 ºC) were harvested by centrifugation (7000 rpm, 15 min, 4 ºC; Centrifuge 5427 R, Eppendorf), washed with sterile 0.1M phosphate buffer (pH 6.5), ressuspended in 10 ml of 100mM NaCl (pH 2.0) and agitated for 1 h at 4 ºC, to allow delaminating bacteriocin from the cells. Then, cells were harvested, and cell-free supernatant was neutralized and tested for bacteriocin activity as described by Van Reenen *et al*. (1998).

The supernatant resulting from the first centrifugation was kept at 4 ºC for partial purification. Then, ammonium sulphate was added gradually to the supernatant stored to reach 40%, 60% and 80% of saturation and each solution was kept at slow stirring during 4 h at 4 ºC. After

centrifugation (12000 rpm, 20 min, 4 ºC; Centrifuge 5427 R, Eppendorf) precipitated proteins in the pellet and floating on the surface were collected and dissolved in 25mM ammonium acetate buffer (pH 6.5) following the method described by Sambrook *et al*. (1989). All samples were stored at -20 ºC.

2.4.3.1.5. Molecular size of *Ln. lactis* RK18 bacteriocin

The samples collected in the previous section 2.4.3.1.4. and stored at -20 ºC were separated by tricine-SDS-PAGE as described by Schägger and Von Jagow (1987). A low molecular weight marker with sizes ranging from 6.5 kDa to 270 kDa (Grisp) was used.

Samples were added to the acrylamide gel in duplicate and, after running, the gel was split in two. One half of the gels was fixed with 20% isopropanol and 10% acetic acid, and the other half was stained with Coomassie Brilliant Blue R250 (Bio-Rad) to visualize the position of the peptide band and the other half was not stained and extensively pre-washed with the sterile distilled water to determine the position of the active bacteriocin. The non-stained gel was overlaid with 10^6 cfu/ml of *L. monocytogenes* CEP 104794, firstly embedded in BHI agar (0.7% agar w/v; Biokar).

2.4.3.1.6. Bacteriocin activity spectrum

Antimicrobial activity by bacteriocin production of *Leuconostoc lactis* RK18 strain was screened for a large number of microorganisms (Table 2.5.) according to the method described by Van Reenen *et al*. (1998). The bacteriocin activity was tested following the method described in section 2.4.3.1.1.. Treated cell-free supernatant (CFSnh) was screened against Gram-positive and Gram-negative bacteria listed in Table 2.5.. The confirmation of antimicrobial activity was assumed if a translucent halo zone was observed around the spots.

Table 2.5. Target organisms, differentiated as Gram-positive and Gram-negative bacteria and their source, used for bacteriocin activity spectrum test

Microorganisms	Species	Source
Gram-positive	*Bacillus cereus* *Bacillus subtilis* *Bacillus stearothermophilus* *Listeria monocytogenes* SCOTT A *Listeria innocua* 2030c *Staphylococcus aureus* 18N (Methicillin-resistant *Staphylococcus aureus*-MRSA) *Staphylococcus aureus* 2037 M1 (Methicillin-sensitive *Staphylococcus aureus*-MSSA)	ESB culture collection
	Enterococcus faecalis ATCC 29212 *Staphylococcus aureus* ATCC	ATCC
	Enterococcus casseliflavus DSMZ 20680 *Enterococcus faecalis* DSMZ 12956 *Enterococcus faecium* DSMZ 13590 *Enterococcus flavescens* DSMZ 7370 *Enterococcus gallinarum* DSMZ 20628	DSMZ
	Listeria monocytogenes L 7946 *Listeria monocytogenes* L 7947	McLauchlin *et al.* (1997)
	Listeria monocytogenes NCTC 1194 *Listeria monocytogenes* CECT 911 *Listeria monocytogenes* CECT 936 *Listeria monocytogenes* CEP 104794	LRCESB
Gram-negative	*Acinetobacter baumanii* R *Acinetobacter baumanii* S-1 *Acinetobacter baumanii* S-2 *Acinetobacter calcoaceticus* S *Acinetobacter calcoaceticus* R *Klebsiella pneumoniae* *Proteus mirabilis* *Proteus vulgaris* *Pseudomonas aeruginosa* *Salmonella* Braenderup *Salmonella* Enteritidis *Salmonella* Enteritidis 417536 *Salmonella* Enteritidis 545047 *Salmonella* Tiphymurium *Yersinia enterocolitica*	ESB culture collection
	Escherichia coli ATCC 25922	ATCC
	Yersinia enterocolitica NCTC 10406	NCTC

ESB – culture collection of Escola Superior de Biotecnologia; DSMZ – German Collection of Microorganisms and Cell Culture; LRCESB – Isolates from Culture Collection of *Listeria* Research Center of Escola Superior de Biotecnologia*;* ATCC – American Type Culture Collection; NCTC – National Collection of Types cultures – Culture Collection of Public Health England. S – sensitive to several tested antibiotics; R – resistant to several tested antibiotics.

2.4.4. Functional Criteria

2.4.4.1. Preparation of *Ln. lactis* RK18 inoculum

One colony of *Ln. Lactis* RK18, grown in MRS agar (Biokar) at 30 ºC for 24 h, was transferred to 10 ml of MRS broth and incubated overnight at 30 ºC. One percent (v/v) was taken from the last culture to 10 ml of fresh MRS broth and incubated in the same conditions.

2.4.4.2. Ability to resist to pH 2.5, pH 2.5 with pepsin and bile salts

The ability of the isolates to resist to acidic pH and to the presence of bile salts was investigated in MRS broth as control and in MRS broth i) adjusted to pH 2.5 (with 1M HCl), ii) adjusted to pH 2.5 and supplemented with 1000 U/ml pepsin (Sigma) and iii) to neutral pH but with 0.3% (w/v) bovine bile salt (Pronadisa). Briefly, to each condition, 1% (v/v) of an overnight culture of *Ln. lactis* RK18 was added and incubated at 30 ºC. Every hour, samples were taken until 4 hours of incubation. Enumeration was done as described in the section 2.1.1..

2.4.4.3. Survival through simulated gastrointestinal conditions

The survival through simulated gastrointestinal tract (GIT) conditions was performed according to Barbosa *et al*. (2014). *Leuconostoc lactis* RK18 cells was grown as mentioned in section 2.4.3.1, harvested by centrifugation (7000 rpm, 10 min, 4 ºC; Centrifuge 5427 R, Eppendorf) washed twice and ressuspended in sterile Ringer's solution to obtain approximately 10^{11} cfu/ml. Then, aliquots of 0.5 ml of inoculum were mixed in sterile glass flasks with 5 g of a fermented meat product (*alheira*) or 5 ml of skim milk (11% w/v, Sigma). After 10 min in contact with each food matrix, 49.5 ml of BPW adjusted to pH 2.5 with 1M HCl and with 1000 units/ml of a filter sterilized solution of pepsin were added and each glass flask was incubated at 30 ºC. As control, 0.5 ml of inoculum without previous matrix exposure was also added to 49.5 ml of BPW at the same conditions. To simulate the conditions of the stomach, samples were taken at time 0 (time of inoculation) and every 30 min until a total of 60 min. Subsequently, a filtered sterilized solution of 1M NaOH was added to increase the pH from 2.5 to 7.0 and a sterile solution of bile salts (Pronadisa) was also added to reach a final concentration of 0.3% (w/v). Samples were kept at 30 ºC and taken at time 0 and every 30 min for a total of 60 min. Each experiment was

conducted in duplicate and three independent assays were performed. For each assay, five controls were used: an aliquot of 0.5 ml of inoculum was placed into glass flasks with: 49.5 ml of BPW at pH 7.0; 49.5 ml of BPW at pH 2.5; 49.5 ml of BPW at pH 7.0 with 1000 units/ml of pepsin; and 49.5 ml of BPW at pH 7.0, for which, after 60 min, a bile salt solution was added (0.3% (w/v) final concentration). Enumeration was done as described in the section 2.1.1..

2.4.4.4. Human colon adenocarcinoma cell lines Caco-2 cells assays

2.4.4.4.1. Preparation of Caco-2 cell lines

The human colon adenocarcinoma cell lines Caco-2 (American Type Culture Collection ECACC 86010202) were cultured in Dulbecco's modified Eagle's minimal essential medium (DMEM, Lonza, Verviers, Belgium) supplemented with 20% (v/v) of fetal bovine serum (FBS; Biowest, Nuaillé, France), 1% (v/v) of pyruvate (Lonza), 1% (v/v) of non-essential amino acids (Biosera, Boussens, France), and 50 U/ml of antibiotic (penicillin and streptomycin). Incubation was at 37 ºC in the presence of 5% CO_2- 95% air atmosphere. The media was replaced every second day. For each experiment, Caco-2 cells were seeded at 1.0×10^5 cells/well in 24-well microtiter plates (Sarstedt) with fresh DMEM media and incubated as described before, until reach the appropriate confluence (90%). Prior to each experiment, Caco-2 cells were washed twice with sterile PBS and media on each well was replaced.

2.4.4.4.2. Preparation of bacterial cultures

Leuconostoc lactis RK18 cells, grown overnight in MRS broth or previously exposed to GIT conditions in the presence of *alheira* matrix, were harvested by centrifugation (7000 rpm, for 10 min, 4 ºC; Centrifuge 5427 R, Eppendorf), washed three times with sterile PBS (VWR Chemicals) and diluted in DMEM (without FBS, antibiotics and NEAA) to reach the desired level (volume adjusted based on initial OD_{600} reading).

The same procedure was applied to *L. monocytogenes* CEP 104794 cells, grown overnight in TSBYE, and diluted to reach a final concentration of 10^5 cfu/ml.

2.4.4.4.3. Adhesion ability of *Ln. lactis* RK18 to Caco-2 cells

Adhesion assays were performed with cells at late post-confluence (15 days in culture) and according to Botes *et al*. (2008), with minor modifications. Briefly, wells with Caco-2 cells were inoculated with 1.0 x 10^5 viable cells of bacterial cell suspension (fresh cells or previously exposed to GIT conditions in the presence of *alheira* matrix), and incubated at 37 ºC (5% CO_2-95% air atmosphere). After 2 h of incubation, non-adherent bacterial cells were withdrawn from the wells and the monolayers washed three times with 1 ml sterile PBS (VWR) and then lysed with 1 ml of 0.2% (v/v) Triton-X 100 cold solution (Sigma, Saint Louis, USA) and vigorous pipetting. The Caco-2 cell lysates and respective LAB culture were serially diluted, spread plated onto MRS and enumerated after 48 h at 30 ºC. For each experiment, a positive control was performed with an adherent probiotic strain, *Lactobacillus rhamnosus* GG (ATCC), and wells without bacterial cells were used as negative control.

Triplicates of three independent assays were performed and the percentage of adherence and invasion was calculated as described by Schillinger *et al*. (2005): % Adhesion = [(cfu/ml$_{120}$) / (cfu/ml$_0$)] x 100, where cfu/ml$_0$ is the initial viable count of *Ln. lactis* RK18 and cfu/ml$_{120}$ refers to adhesion of viable bacterial cells at the end of the experiment.

2.4.3.4.1. Ability of *Ln. lactis* RK18 to invade Caco-2 cells

To evaluate cell invasion of Caco-2 cells by *Ln. lactis* RK18, wells with Caco-2 cells were inoculated with 1.0 x 10^5 cfu/ml of bacterial cell suspension (fresh cells or previously exposed to GIT conditions in the presence of *alheira* matrix) and incubated (37 ºC, 5% CO_2-95% air atmosphere) for 2 h. Subsequently, the medium was aspirated and fresh DMEM medium containing 40 µg/ml gentamicin (Sigma) was added to kill remaining extracellular bacteria and incubated for 1 h. The wells were washed three times with 1 ml sterile PBS (VWR) and treated as described in section 2.4.3.4.3..

At least three independent invasion assays were performed, and results were reported as percent invasion efficiency [(bacterial numbers recovered/bacterial numbers inoculated)*100].

2.4.3.4.2. Role of _Ln. lactis_ RK18 on the inhibition of foodborne pathogens adherence and/or invasion to Caco-2 cells

A. Prevention of _L. monocytogenes_ CEP 104794 adhesion to Caco-2 cells

In vitro adherence ability of _L. monocytogenes_ CEP 104794 to Caco-2 cells was evaluated as described previously (section 2.4.3.4.3.) for _Ln. lactis_ RK18.

The ability to prevent the adherence of _L. monocytogenes_ CEP 104794 to Caco-2 cells by _Ln. lactis_ RK18 (previously exposed to GIT conditions in the presence of _alheira_ matrix) was determined following the method described by Botes _et al_. (2008). Wells with Caco-2 cells were inoculated with 100 µl _Ln. lactis_ RK18 (approximately 1.0 x 10^5 cfu/well) and incubated (37 ºC, 5% CO_2-95% air atmosphere) for 2 h. Non-adherent cells were removed from each well by washing twice with sterile PBS (VWR), fresh DMEM medium and 100 µl _L. monocytogenes_ CEP 104794 (approximately 1.0 x 10^5 cfu/well) were added and incubated at the same conditions for further 2 h. After that period, each well was washed three times with 1 ml sterile PBS (VWR) and treated as described in section 2.4.3.4.3.. The Caco-2 cell lysates and respective _L. monocytogenes_ CEP 104794 / _Ln. lactis_ RK18 cultures were serially diluted, spread plated onto MRS and Palcam and enumerated after 48 h at 30 ºC and 24 h at 37 ºC, respectively. Triplicates of three independent assays were performed and the percentage of adherence was calculated as described previously.

B. Inhibition of _L. monocytogenes_ CEP 104794 invasion to Caco-2 cells

In vitro invasion ability of _L. monocytogenes_ CEP 104794 to Caco-2 cells was assessed as described previously (section 2.4.3.4.4.) for _Ln. lactis_ RK18.

Inhibition of the invasion of _L. monocytogenes_ CEP 104794 was evaluated, individually, by both _Ln. lactis_ RK18 cells and 10% (v/v) of its treated cell-free supernatant. Treated cell-free supernatant was obtained after centrifugation (7000 rpm, for 10 min, 4 ºC; Centrifuge 5427 R, Eppendorf) of _Ln. lactis_ RK18 culture (previously exposed to GIT conditions in the presence of _alheira_ matrix), pH neutralization and heating at 80 ºC for 10 min (CSFnh), which was diluted in DMEM medium (without FBS, antibiotics and NEAA) in order to obtain 10% (v/v) of CSFnh. In this particular case, instead of fresh DMEM, 10% (v/v) of CSFnh in DMEM was the medium replaced in each well containing Caco-2 cells prior to the experiment.

Briefly, wells with Caco-2 cells were inoculated with 1:1 *L. monocytogenes* CEP 104794 and *Ln. lactis* RK18 or with 100 µl *L. monocytogenes* CEP 104794 (approximately 1.0×10^5 cfu/well) with 0.9 ml of 10% (v/v) CSFnh and incubated (37 ºC, 5% CO_2-95% air atmosphere) for 2 h. Subsequently, the medium was aspirated and fresh DMEM medium containing 40 µg/ml gentamicin was added and incubated for further 1 h. The wells were washed three times with 1 ml sterile PBS and treated as described in section 2.4.3.4.3.. The Caco-2 cell lysates and respective cell content were serially diluted, spread plated onto MRS and Palcam in wells with *L. monocytogenes* CEP 104794 / *Ln. lactis* RK18 cultures or spread plated on Palcam in wells with *L. monocytogenes* CEP 104794 in 10% CFSnh. Enumeration was performed after 48 h at 30 ºC for MRS and 24 h at 37 ºC for Palcam. Triplicates of three independent assays were performed and the invasion efficiency was calculated as described above.

III.   Results and Discussion

3.1. Lactic acid bacteria isolated from fermented foods products

Twenty different food products were used to isolate lactic acid bacteria. The number of isolates and their origin are listed in Table 3.1..

Overall, the viable cell counts in MRS and M17 were, in average, ca. 5.5 log cfu/g and ca. 6.6 log cfu/g, respectively. Total viable cell counts in MRS agar varied from 3.9 log cfu/g to 6.2 log cfu/g. Total viable cell counts in M17 ranged 3.6 log cfu/g to 7.5 log cfu/g. No colonies were detected in MRS and M17 agar for 12 and 8 samples, respectively.

Ten percent of colonies from each sample were randomly chosen and then further selected based on cell morphology, Gram staining, colonial morphology and absence of catalase and oxidase enzymes. In total, two hundred and two Gram-positive, catalase, oxidase negative and cocci/bacilli isolates were selected to be tested for potential bacteriocinogenic capacity against seven foodborne pathogens.

3.2. Study of antimicrobial activity potential of isolated LAB

Inhibitory activity can be achieved by competition against microorganisms or by compounds that are produced and secreted by some bacteria. So, using this screening method, the observation of an inhibition zone, may result from competition, lactic acid with consequent pH decrease,

hydrogen peroxide or bacteriocin production. A screening for the antagonistic activity of the 202 LAB against seven Gram-positive foodborne pathogens was conducted (Table 3.2.). In order to test for possible bacteriocin production, cell-free extracts were subjected to neutralization, addition of catalase and digestion with proteinase K.

Only three isolates demonstrated antimicrobial activity (by competition), being active against *E. faecalis* ATCC 29212 and all *Listeria* strains. One out of three LAB isolates also inhibited *E. faecium* DSMZ 13590 by competition.

No antimicrobial activity against *Staphylococcus aureus* was recorded. The effectiveness of LAB in inhibiting Gram-positive bacteria is widely recognized and described by several authors (Albano *et al.*, 2007; Albano *et al.*, 2009; Abrams *et al.*, 2011; Peng *et al.*, 2017). Antimicrobial activity due to bacteriocinogenic activity was observed for the same 3 LAB isolates against *Enterococcus faecalis* and *Listeria monocytogenes* strains.

Table 3.1. Enumeration of lactic acid bacteria on MRS and M17 from 20 samples

	Samples	Date of sampling	Viable cell counts (log cfu/g)		Isolates (n)	
			MRS	M17	MRS	M17
1	*Tâmaras*	14/09/2018	-	-	-	-
2	Watercress Baby Leaf*	14/09/2018	5.5	7.5	10	14
3	Wild arugula*	14/09/2018	4.9	6.7	11	9
4	Black Bean pasta	18/09/2018	-	4.9	-	8
5	Kimchi	18/09/2018	-	-	-	-
6	"Pickle lettuce"	18/09/2018	-	-	-	-
7	Fermented Shrimp	20/09/2018	4.3	5.0	12	10
8	Fermented Cucumber	20/09/2018	-	-	-	-
9	Fermented Fish	20/09/2018	-	-	-	-
10	Beef Sausage	20/09/2018	-	-	-	-
11	Fish Sausage	20/09/2018	6.2	5.9	22	11
12	Pork Sausage	25/09/2018	4.4	4.9	10	10
13	Fermented Cabbage	25/09/2018	-	-	-	-
14	Fish Paste	25/09/2018	-	-	-	-
15	Khmer Fermented Rice Fresh Noodles	25/09/2018	3.9	4.6	4	9
16	Watercress*	04/10/2018	-	3.6	-	2
17	Coriander*	04/10/2018	-	5.4	-	10
18	Parsley*	04/10/2018	-	5.2	-	8
19	Green Cos*	04/10/2018	5.8	5.7	12	11
20	Green Botavia*	04/10/2018	4.7	5.4	11	8
		Total			92	110
					202	

(n) – number of isolates

* - samples packaged in modified atmosphere conditions

The three isolates (Gram-positive cocci) presenting antimicrobial activity by possible bacteriocin production were successfully identified by 16S rRNA gene sequencing (similarity values higher than 99%) as *Enterococcus faecium* RS7 (isolated from fermented shrimp, in M17 agar), *Enterococcus faecium* P12 (isolated from pork sausage, in M17 agar) and *Leuconostoc lactis* RK18 (isolated from khmer fermented rice fresh noodles, in MRS agar).

Table 3.2. Antimicrobial activity of all three LAB isolates against seven foodborne pathogens

Targets	LAB isolates				
	C	**CFS**	**CFSn**	**CFSnC**	**CFSnK**
Enterococcus faecalis ATCC 29212	+	-	-	-	-
Enterococcus faecium DSMZ 13590	+*	-			
Staphylococcus aureus ATCC 29213	-	n/a	n/a	n/a	n/a
Listeria monocytogenes NCTC 1194	+	+	+	+	-
Listeria monocytogenes CECT 911	+	+	+	+	-
Listeria monocytogenes CECT 936	+	+	+	+	-
Listeria monocytogenes CEP 104794	+	+	+	+	-

(C) live-cell; (CFS) cell-free supernatant; (CFSn) cell-free supernatant neutralized; (CFSnC) cell-free supernatant neutralized treated with 500 IU/ml catalase; (CFSnK) cell-free supernatant treated with proteinase K; (+) inhibition zone; (-) no inhibition zone; * positive result only for one isolate; n/a – not applicable.

Enterococcus faecium have been isolated from fermented food products, such as fermented Asian foods (fermented rice noodles and fermented fish) (Peng *et al*., 2017; Techo *et al*., 2019), from *Parkia biglobosa* seeds (Bello *et al*., 2018) and from Portuguese fermented foods (Barbosa *et al*., 2010). Also, some of these *E. faecium* strains had anti-listerial activity (Peng *et al*., 2017; Bello *et al*., 2018). *Leuconostoc* strains can also be found in a variety of fermented food products. Anti-listerial *Leuconostoc* isolated from fermented rice noodles (Hwang *et al*., 2018), *Parkia biglobosa* seeds (Bello *et al*., 2018) and from fermented mare milk (Arakawa *et al*., 2015). The majority of these isolates belong to *Leuconostoc pseudomesenteroides*, *Leuconostoc citreum* and *Leuconostoc lactis* species. Besides anti-listerial activity, activity against *Helicobacter pylori* by *Leuconostoc* strains isolated from fermented rice noodles was also reported (Techo *et al*., 2019).

3.3. Study of several criteria required for potential probiotics

Several tests were performed on selected LAB, *E. faecium* (RS7 and P12) and *Ln. lactis* (RK18), in order to evaluate their potential as probiotic organisms.

3.3.1. Safety criteria of potential probiotic

During the selection and evaluation of probiotics it is vital to analyse their safety. Consequently, evaluation of the safety criteria of the putative probiotic strains is required in order to prevent their potential negative side effects (Harzallah and Belhadj, 2013; Ray and Joshi, 2014; Flahaut and de Vos, 2015).

3.3.1.1. Antibiotic susceptibility

Antibiotic susceptibility is one important safety criteria. A bacterial strain is considered phenotypically resistant when is not inhibited at a concentration of a specific antimicrobial agent established by breakpoints (equal or higher) defined by EFSA (Flórez *et al*., 2016). Based on the lowest concentrations without visible growth (MICs), isolates were classified as resistant, intermediate or sensitive to each antibiotic investigated (EFSA, 2012; Table 3.3.).

Antibiotic susceptibility of enterococcal isolates (*E. faecium* RS7 and *E. faecium* P12) and *Ln. lactis* RK18 were evaluated for ampicillin, erythromycin, vancomycin, tetracycline, chloramphenicol, streptomycin, kanamycin and tetracycline, covering the main classes of antibiotics.

Overall, *Ln. lactis* RK18 was more susceptible to the majority of the antibiotics tested than *E. faecium* strains. Enterococcal isolates showed resistance to six of the eight antibiotics tested while *Ln. lactis* RK18 was sensitive to all antibiotics tested (Table 3.3.). Several authors reported similar results (Pan *et al*., 2011; Flórez *et al*., 2016).

Regarding to vancomycin, on the contrary of *Ln. lactis* RK18, which is intrinsically resistant (Swenson *et al*., 1990; Ogier *et al*., 2008; Flórez *et al*., 2016), *E. faecium* RS7 and *E. faecium* P12 were susceptible to this glycopeptide antibiotic (MIC ≤ 0.5 µg/ml). This was in agreement with previous studies (Busani *et al*., 2004; Gaglio *et al*., 2016; Sanlibaba and Senturk, 2018). The occurrence of vancomycin-resistant *Enterococcus* (VRE) decreased significantly due to banning

the use of avoparcin, an analogue of the glycopeptide vancomycin used as a growth promoter in livestock, consequently reaching humans less frequently through the consumption of animal food products (O'Driscoll and Crank, 2015).

Table 3.3. Antibiotic susceptibility and MICs (µg/ml) determined by the agar microdilution method, according to CLSI (2012) for *E. faecium* isolates and by broth microdilution method according to Klare *et al*. (2005) for *Ln. lactis* RK18

Antibiotics	Organism	Breakpoints EFSA	MICs (µg/ml)	Antibiotic susceptibility
Ampicillin	*E. faecium* RS7 and P12	2	4	R
	Ln. lactis RK18	2	0.5	S
Chloramphenicol	*E. faecium* RS7 and P12	16	32	R
	Ln. lactis RK18	11	4	S
Gentamicin	*E. faecium* RS7 and P12	32	8	S
	Ln. lactis RK18	16	1	S
Kanamycin	*E. faecium* RS7 and P12	1024	>1024	R
	Ln. lactis RK18	16	4	S
Streptomycin	*E. faecium* RS7 and P12	128	>256	R
	Ln. lactis RK18	64	8	S
Tetracycline	*E. faecium* RS7 and P12	4	>64	R
	Ln. lactis RK18	8	1	S
Vancomycin	*E. faecium* RS7 and P12	4	≤0.5	S
	Ln. lactis RK18	-	n/a	n/a
Erythromycin	*E. faecium* RS7 and P12	4	>8	R
	Ln. lactis RK18	1	<0.125	S

S – sensitive to tested antibiotic; R – resistant to tested antibiotic; n/a – not applicable

Leuconostoc lactis RK18 was susceptible to ampicillin and gentamicin. Other authors (Ammor *et al*., 2007 and Morandi *et al*., 2013) have shown that *Leuconostoc* strains isolated from dairy and meat products are susceptible to most of these antibiotics and in particular to the β-lactams (e.g. ampicillin). *Enterococcus faecium* RS7 and P12 were also susceptible to gentamicin. Similar results were described by Sanlibaba and Senturk (2018). In contrast, both strains were resistant to ampicillin. Although enterococci have intrinsically low resistance to β-lactams (Garrido *et al*., 2014), they can be resistant by increasing levels of penicillin-binding protein 5 (PBP5) expression (Vrabec *et al*., 2015). In this study, both *E. faecium* RS7 and P12 displayed resistance

to streptomycin, kanamycin, chloramphenicol, tetracycline and erythromycin. This observation corroborates data reported in previous studies (Pan *et al*. 2011; Sanlibaba and Senturk, 2018). Naturally, enterococci are resistant to aminoglycosides, macrolides, among others (Hollenbeck *et al*., 2012). Resistance determinants in enterococcal strains are generally located in conjugative plasmids or transposons (Sanlibaba and Senturk, 2018), that can be exchange horizontally between *Enterococcus* and *Leuconostoc* species.

As mentioned before, *Ln. lactis* RK18 displayed a higher susceptibility to the majority of the antibiotics tested than *E. faecium* RS7 and *E. faecium* P12. These results indicate that *E. faecium* RS7 and *E. faecium* P12 are considered a potential source for the dissemination of antibiotic resistance since they were classified as a multidrug resistant (resistance to three or more antimicrobial agents of different classes) isolates.

3.3.1.2. Virulence factors

The presence of virulence factors is strongly associated to infections by bacteria as this can be used by microorganisms to invade the host, cause diseases and evade host defences (Peterson, 1996).

Pathogenicity was characterized by phenotypical tests such as biogenic amine-forming capacity, hydrolytic enzymes (gelatinase and DNase), hemolytic activity and the presence of virulence genes.

3.3.1.2.1. Determination of biogenic amine-forming capacity

Positive reactions in the screening medium were only observed for tyramine by *E. faecium* isolates (RS7 and P12). Similar results were previously demonstrated by others (Bover-Cid *et al*., 2001; Barbieri *et al*., 2019).

In the study of Liu (2016), also the inefficient ability of *Leuconostoc* spp. to produce biogenic amines from decarboxylation of amino acids was stated. In fact, *Ln. lactis* RK18 was not able to produce any of the biogenic amines tested. In addition, it has already been proven that some *Leuconostoc* species can produce undesirable compounds (BA) that can induce spoilage, but

species of this genus are considered as minor producers when compared to *Enterococcus* species (Ogier *et al*., 2008; Barbieri *et al*., 2019).

3.3.1.2.2. Production of hydrolytic enzymes: gelatinase and DNase

Regarding hydrolytic enzymes, gelatinase and DNase were not produced by none of the isolates. Gelatinase activity is strongly associated to enterococci and it has been found among isolates from dairy and fermented food products (Semedo *et al*., 2003a; Barbosa *et al*., 2010). Other reports have demonstrated that *E. faecalis* has higher capacity of hydrolyzing gelatin than *E. faecium* (Semedo *et al*., 2003a; Barbosa *et al*., 2010). Similarly, Kodama *et al*. (1956) and Antunes *et al*. (2002) also reported the absence of gelatinase production by *Leuconostoc* species.

According to Semedo *et al*. (2003a), the importance of DNase activity as a virulence factor in enterococci appears to be reduced. It has been reported the low incidence of DNase activity (Semedo *et al*., 2003a) or the absence of this enzyme activity by *E. faecium* (Barbosa *et al*., 2010) and other LAB (Haas *et al*., 2014).

3.3.1.2.3. Hemolytic activity

The absence of hemolytic activity is considered an important safety pre-requisite on the selection of a probiotic strain. In this study, both *E. faecium* (RS7 and P12) and *Ln. lactis* RK18 did not present hemolytic activity. *Enterococcus* and *Leuconostoc* species are known to be partial-hemolytic (α) or non-hemolytic (γ), although hemolytic activity in the *Leuconostoc* genus is yet to be clearly understood (Ogier *et al*., 2008).

3.3.1.2.4. Presence of virulence genes

The absence of biogenic amines production as well as hemolytic, gelatinase and DNase activities by *E. faecium* (RS7 and P12) and *Ln. lactis* RK18 does not necessarily mean that these microorganisms are not virulent. Thus, the presence of some virulence genes were studied by PCR (Table 1.2. and Table 2.4.): surface adhesin genes (*esp*, *ace*, *efaAfm* and *efaAfs*), aggregation protein gene (*agg*), extracellular metallo-endopeptidase gene (*gelE*), cytolysin genes (*cylA*, *cylB*, *cylM*, *cylL$_L$* and *cylL$_S$*), hyaluronidase gene (*hyl*) and aggregation substance precursor (*asa1*), since they could increase the capacity to cause infection, facilitating adhesion and colonization (Flórez *et al*., 2016). Also the presence of vancomycin-resistant genes (*vanA* and *vanB*) and genes

related to biogenic amines (*hdc1*, *tdc* and *odc*) were assessed, since, as well as the other genes studied, they can be expressed depending on external environmental factors (Eaton and Gasson, 2001). Some silent genes may become active not only depending on external factors, such as imbalance of gut microbiota (e.g. persistence of high cellular numbers of certain bacteria or synergisms activity) and gastrointestinal conditions, but also depending on the environment of the host (i.e. ingesting bacteria from contaminated foods or the contact with bacteria in clinical environments) (Eaton and Gasson, 2001).

In the present study, it was possible to observe the presence of *efaAfm* and *asa1* genes, and genes associated to the biogenic amines *hdc1*, *tdc* and *odc* for *E. faecium* RS7 and *E. faecium* P12. *Leuconostoc lactis* RK18 merely harboured the gene of aggregation substance precursor *asa1*.

The genes *efaAfs* and *efaAfm* are inherent to cell wall adhesins expressed in serum by *E. faecalis* and *E. faecium*, respectively (Eaton and Gasson, 2001). Therefore, the presence of *efaAfm* gene in *E. faecium* isolates was expected. Similar results were reported by Eaton and Gasson (2001) and Barbosa *et al*. (2010). Additionally, *E. faecium* isolates harboured *hdc1*, *tdc* and *odc* genes associated to biogenic amines histamine, tyramine and putrescine, respectively. Similar results were published by Marcobal *et al*. (2005) and Elsanhoty *et al*. (2016) for other LAB closely related to *E. faecium* strains.

Only correlation between the presence of tyrosine decarboxylase gene (*tdc*) and tyramine production was observed, since histamine and putrescine were not produced regardless the presence of *hdc1* and *odc* associated genes. The same correlation was observed by Muñoz-Atienza *et al*. (2011). This particular result emphasizes the importance of studying simultaneously both phenotypic and genotypic characteristics of an isolate.

The presence of the aggregation substance precursor encoded by the gene *asa1* was found for all isolates. This gene is known to be abundantly found in *E. faecium* strains and facilitates the aggregation of the bacteria to the host cells for transfer of transmissible conjugative plasmids, including virulent determinants (Franz *et al*., 2013; Abriouel *et al*., 2015; Biswas *et al*., 2016). The presence of this gene in other *Leuconostoc* species was already reported by Abriouel *et al*. (2015). The precursor for the aggregation substance is not directly associated to the presence and expression of the adherence potential of bacteria harbouring these genes, since infectivity happens when other factors are involved (Abriouel *et al*., 2015).

3.3.2. Technological Criteria

3.3.2.1. Lipase activity

The enzymatic activity has a potential biotechnological significance in fermentative processes and in human health as it promotes flavour development by releasing intracellular compounds, such as lipases and proteases, and replace strains with weaker enzymatic activities in human gut microbiota (Semedo *et al.*, 2003a; García-Cano *et al.*, 2019).

In this study, none of the isolates produced the enzyme lipase. Negative phenotypes were detected by Semedo *et al.* (2003a) for *E. faecium* and by Tiago *et al.* (2004) for other LAB isolates. Although not producing this enzyme, it does not mean that the isolates tested in this study do not have important technological properties. Further assays should be performed in the future in order to evaluate the eventual production of other important enzymes by these isolates.

Despite not producing lipase, but given the absence of virulence factors and genes, and the susceptibility to main classes of antibiotics, *Ln. lactis* RK18 was selected for further experiments.

3.3.3. Beneficial Criteria

A beneficial but non-mandatory criterion for the selection of probiotic strains may be the production of substances active against foodborne pathogens.

3.3.3.1. Characterization of the bacteriocin produced by *Ln. lactis* RK18

Since bacteriocins produced by LAB have large variations in size, inhibitory spectrum and physicochemical properties (Yang *et al.*, 2014), *Ln. lactis* RK18 bacteriocin (henceforward mentioned as RK18 bacteriocin) was characterized.

3.3.3.1.1. Maximum bacteriocin production (AU/ml) during growth of *Ln. lactis* RK18

The optimal growth temperature of *Leuconostoc* strains isolated from fermented foods range between 27 ℃ to 30 ℃ (Holzapfel *et al.*, 2015; Liu, 2016; Hwang *et al.*, 2018). Thus, a preliminary test was conducted to determinate the optimal growth temperature of *Ln. lactis* RK18 in order to perform all the succeeding tests. The growth of *Ln. lactis* RK18 at three different

temperatures for 21 h is presented in Figure 3.1.. It is possible to observe that *Ln. lactis* RK18 reached its highest growth at 30 ºC. Some authors that exploited the potential of bacteriocinogenic *Leuconostoc* strains to be used in industry also used MRS broth at 30 ºC for their growth (Héchard *et al.*, 1992; Chen *et al.*, 2018).
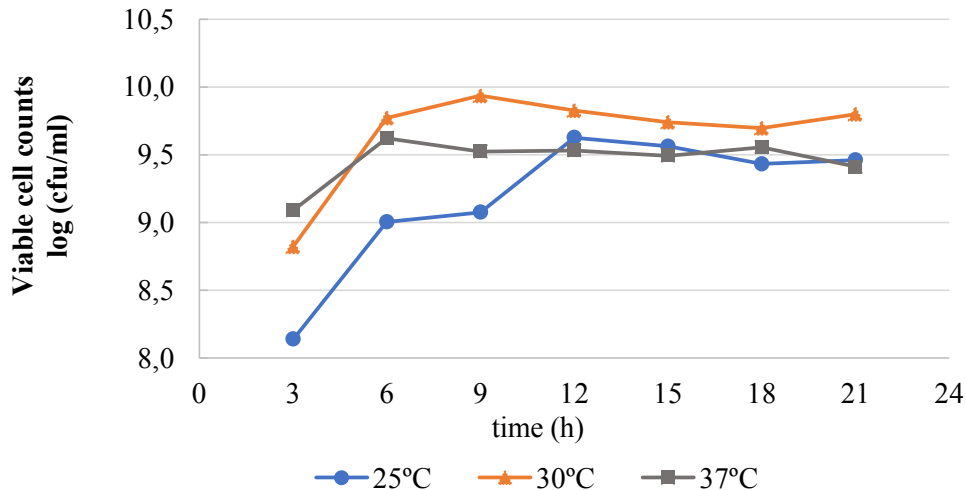

Figure 3.1.. Cell growth of *Ln. lactis* RK18 in MRS broth, at different temperatures: (●) 25ºC; (▲) 30ºC and (■) 37ºC. Colony forming units was enumerated and presented as log (cfu/ml).

The correlation between growth of *Ln. lactis* RK18 and its anti-listerial activity (AU/ml) was also determined. The antimicrobial activity (AU/ml) of the bacteriocin produced by *Ln. lactis* RK18 was evaluated against four serogroups of *L. monocytogenes* tested in the preliminary antimicrobial activity test: *L. monocytogenes* NCTC 1194 (L1; serogroup 1/4b), *L. monocytogenes* CECT 911 (L2; serogroup 1/2c), *L. monocytogenes* CECT 936 (L3; serogroup 1/2b) and *L. monocytogenes* CEP 104794 (L4; serogroup 1/2a) and the results are shown in Figure 3.2..
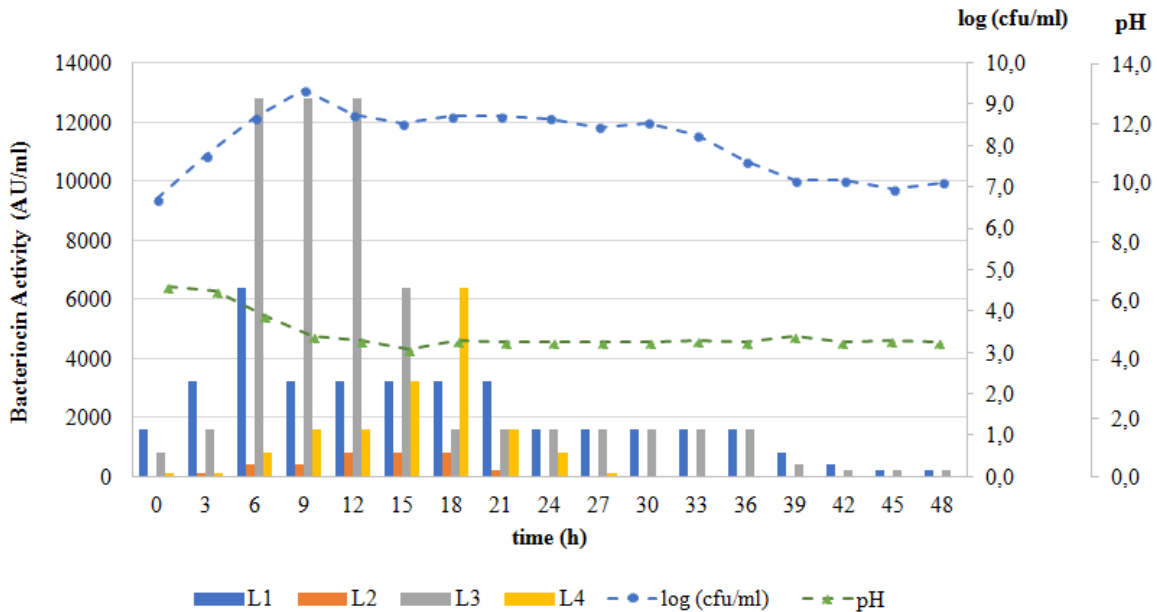
Figure 3.2. Production of bacteriocin by *Ln. lactis* RK18 in MRS broth at 30 ºC. Antimicrobial activity of treated cell-free supernatant is presented as AU/ml against four serogroups of *Listeria monocytogenes*: *Listeria monocytogenes* NCTC 1194 (**L1**); *Listeria monocytogenes* CECT 911 (**L2**); *Listeria monocytogenes* CECT 936 (**L3**) and *Listeria monocytogenes* CEP 104794 (**L4**). Viable cell counts of *Ln. lactis* RK18 are presented as log (cfu/ml) (-●-) and pH (-▲-) changes are indicated.

The higher activity of bacteriocin was observed against *L. monocytogenes* CECT 936 (L3) with 12800 AU/ml during growth of *Ln. lactis* RK18 for 6 h-12 h. For *L. monocytogenes* NCTC 1194 (L1) and *L. monocytogenes* CEP 104794 (L4) the maximum activity of the bacteriocin (6400 AU/ml) was reached after 6h and 18h, respectively. The lowest bacteriocin activity (800 AU/ml) was observed during the period of 12 h to 18 h against *L. monocytogenes* CECT 911 (L2). This shows that bacteriocin produced by *Ln. lactis* RK18 is strain-associated.

Also, the changes in pH values decreased from 6.5 (in the beginning of the assay) to 4.6 (at the end of the screening) reaching its minimum (pH 4.4) after 15 h of growth. The number of viable cells of *Ln. lactis* RK18 increased approximately, ca. 2.6 log (maximum cell growth at 9 h of growth). Afterwards, pH values reached a plateau and cell viability decreased 2 logs. Low levels

of bacteriocin activity were recorded after 24 h of growth decreasing gradually until 200 AU/ml against *L. monocytogenes* NCTC 1194 (L1) and *L. monocytogenes* CECT 936 (L3). The peptide lost activity against *L. monocytogenes* CECT 911 (L2) and *L. monocytogenes* CEP 104794 (L4) after 24 h and 30 h, respectively.

A similar behavior was previously observed for the bacteriocinogenic *Pediococcus pentosaceus* K34, with bacteriocin production reaching maximum values of 12800 AU/ml against *L. monocytogenes* (Abrams *et al*., 2011). However, these results are somewhat different from the results reported by Hwang *et al*. (2018), since *Ln. lactis* SD501 demonstrated maximum activity (735 AU/ml) at 9 h of growth against *L. monocytogenes* ATCC 19114. This confirms that bacteriocin production of probiotic strains is strain-specific. Similarly, this bacteriocin activity decreased gradually after prolonged incubation. Nevertheless, similar results were reported to other bacteriocin-producing *Leuconostoc* strains and to other LAB closely related to this genus (Héchard *et al*., 1992; Arakawa *et al*., 2016; Chen *et al*., 2018; Lasik‑Kurdyś and Sip, 2019). The reduction of antimicrobial activity of bacteria may be due to various factors, including auto‑acidification after prolonged incubation, proteolytic degradation by extracellular proteases or protein aggregation (Papagianni and Papamichael, 2011; Papadimitriou *et al*., 2016).

Leuconostoc strains generally produce bacteriocins in early or in late stages of their growth cycle (Stiles, 1994). In this study, it was observed that *Ln. lactis* RK18 produced bacteriocin in early stages of its growth cycle, which brings an advantage in circumstances when competitive growth is desired or for pathogen inhibition purposes, since it can inhibit pathogenic bacteria before its exponential growth.

3.3.3.1.2. Effect of pH, enzymes, temperature, detergents, surfactants and protease inhibitors on bacteriocin activity

Different physicochemical treatments may affect antimicrobial activity of bacteriocins (Gharsallaoui *et al*., 2015). The effects of different conditions on the stability of the *Ln. lactis* RK18 bacteriocin are presented in Tables 3.4., 3.5. and 3.6..

Table 3.4. Reduction of antimicrobial activity of RK18 bacteriocin (expressed in percentage values) against four serogroups of *Listeria monocytogenes,* under the effect of pH, detergents, surfactants and protease inhibitors

		L1	L2	L3	L4
pH	2	50%	100%	13%	25%
	4	0%	0%	6%	3%
	6	0%	0%	6%	6%
	8	100%	25%	6%	25%
	10	100%	100%	25%	25%
	12	100%	13%	3%	3%
Detergents	Tween 20	0%	0%	100%	75%
	Tween 80	0%	0%	100%	50%
	Triton X-100	50%	75%	100%	50%
	SDS	50%	75%	100%	88%
	EDTA	50%	0%	100%	75%
	Ox-Bile	97%	88%	100%	97%
	Urea	50%	50%	50%	75%
	NaCl	0%	0%	25%	25%
	Sodium carbonate	50%	0%	94%	75%
	Sodium deoxycholate	50%	50%	75%	88%

(L1) *L. monocytogenes* NCTC 1194; (L2) *L. monocytogenes* CECT 911; (L3) *L. monocytogenes* CECT 936 and (L4) *L. monocytogenes* CEP 104794.

In table 3.4. it is posible to observe that the antimicrobial activity of RK18 bacteriocin was affected (25%-100% of reduction) at pH values below 2.0 and above 8.0, suggesting that peptide is sensitive to acidic and alkaline conditions. The bacteriocin remained stable after incubation for 1 h at pH 4.0 and 6.0 (0-6% of reduction). Even though the negative effect of pH on the antimicrobial activity bacteriocin still retained its high anti-listerial ability against strains of serogroups 1/4b and 1/2c. The acidification can cause solubilization of metals in some LAB, wich may be toxic, depending on their concentration, and low values of pH can result in the denaturation of proteins (Papadimitriou *et al*., 2016), corroborating the stated data. The stability of bacteriocin activity at different pH values was also reported for other *Leuconostoc* strains (Arakawa *et al*.,2016; Bello *et al*., 2018; Hwang *et al*., 2018) and to other LAB (Albano *et al*., 2007).

RK18 bacteriocin was sensitive to all the detergents tested but remained stable against *L. monocytogenes* CECT 911 after treatment with surfactants (Tween-20 and Tween-80), EDTA and salts (NaCl and sodium deoxycholate). Similar results were observed against *L. monocytogenes* NCTC 1194, since bacteriocin remained stable after treatment with surfactants and NaCl. However, almost a total loss of activity was observed against the other strains tested after treatment with all the detergents. This could indicate a high sensitivity of the bacteriocin to protease inhibitors, corroborating the reported data for other LAB species (Benmouna *et al.*, 2018).

Dissimilar results were reported for other *Leuconostoc* strains (Bello *et al.*, 2018) and other LAB (Albano *et al.*, 2007), showing that detergents, surfactants and salts had no effect on the activity of the antimicrobial susbtances produced.

Table 3.5. Reduction of antimicrobial activity of RK18 bacteriocin (expressed in percentage values) against four serogroups of *Listeria monocytogenes,* under the effect of temperature

		L1		L2		L3		L4	
		1h	2h	1h	2h	1h	2h	1h	2h
	4	0%	50%	0%	50%	94%	97%	88%	94%
	25	0%	50%	0%	75%	88%	94%	75%	88%
	30	0%	0%	0%	0%	0%	0%	0%	0%
T (ºC)	37	0%	0%	0%	0%	0%	0%	0%	0%
	60	0%	0%	0%	0%	0%	0%	0%	0%
	80	0%	0%	0%	0%	0%	0%	0%	0%
	100	50%	75%	50%	50%	88%	94%	88%	88%
	121	98%	98%	100%	100%	100%	100%	100%	100%

(L1) *Listeria monocytogenes* NCTC 1194; (L2) *Listeria monocytogenes* CECT 911; (L3) *Listeria monocytogenes* CECT 936 and (L4) *Listeria monocytogenes* CEP 104794.

In table 3.5., it is shown that RK18 bacteriocin is thermostable, since its activity remained stable at temperatures ranging from 30 ºC to 80 ºC. Residual activity was observed at lower temperatures (below 30 ºC) and at higher temperatures (at boiling and sterilization temperatures). Similar results were observed for other thermo-stable bacteriocins (Albano *et al.*, 2007a; Arakawa *et al.*, 2016; Bello *et al.*, 2018; Hwang *et al.*, 2018).

Complete inactivation of antimicrobial activity was observed after treatment with the proteolytic enzyme, proteinase K, confirming its proteinaceous nature (Table 3.6.). Overall, no reduction of activity was observed after treatment with other the proteases tested (papain and pepsin) and after the antioxidant enzyme activity (catalase). Resistance to catalase suggests that carbohydrates are not bound to the enzyme and its antimicrobial effect was not derived from hydrogen peroxide (H_2O_2). However, the concentration of enzymes seems to be an important factor, since there was a partial reduction (50% for catalase at 1 mg/ml) or almost total reduction of activity (ca. 88% and 75% of reduction for protease enzymes at 1 mg/ml and 0.1 mg/ml, respectivelly) against two serogroups of *L. monocytogenes* (L2 and L3). Similar results, were observed for other *Leuconostoc* strains (Arakawa *et al.*, 2016; Chen *et al.*, 2017; Bello *et al.*, 2018; Hwang *et al.*, 2018) and for other LAB (Albano *et al.*, 2007).

Table 3.6. Reduction of antimicrobial activity of RK18 bacteriocin (expressed in percentage values) against four serogroups of *Listeria monocytogenes,* under the effect of different enzymes, at two concentrations

Enzymes	L1		L2		L3		L4	
	1mg/ml	0.1mg/ml	1mg/ml	0.1mg/ml	1mg/ml	0.1mg/ml	1mg/ml	0.1mg/ml
Proteinase K	100%	100%	100%	100%	100%	100%	100%	100%
Papain	0%	0%	0%	0%	88%	75%	0%	0%
Pepsin	0%	0%	50%	0%	88%	75%	0%	0%
Catalase	0%	0%	50%	0%	50%	0%	0%	0%

(L1) *Listeria monocytogenes* NCTC 1194; (L2) *Listeria monocytogenes* CECT 911; (L3) *Listeria monocytogenes* CECT 936 and (L4) *Listeria monocytogenes* CEP 104794.

Overall, RK18 bacteriocin demonstrated high sensitivity to detergents, surfactants and protease inhibitors, but it remained stable when subjected to more acidic pH, keeping its antimicrobial activity against some *L. monocytogenes* strains. It was also demonstrated that the antimicrobial

activity of treated bacteriocin was different against the four serogroups of *L. monocytogenes*. This highlights the importance to perform these kind of studies for more than one target strain.

3.3.3.1.3. Cell lysis of target microorganisms

In figure 3.3. is presented the effect of RK18 bacteriocin, at its maximum activity on the growth of 4 serogroups of *L. monocytogenes*. When the bacteriocinogenic supernatant of *Ln. lactis* RK18 was added to a mid-log culture (4 h-old) of *L. monocytogenes* strains, a decrease of approximately 2 log cycles was observed for *L. monocytogenes* NCTC 1194 (L1) and *L. monocytogenes* CECT 911 (L2). Similarly, the addition of the proteinaceous substance (maximum activity of 12800AU/ml against L3 and maximum activity 6400AU/ml against L4, observed in section 3.3.3.1.1.) to a mid-log (5 h-old) culture of *L. monocytogenes* CECT 936 (L3) and *L. monocytogenes* CEP 104794 (L4) repressed cell growth in a similar way. However, after 7 hours it was observed a gradual growth of all *L. monocytogenes* until 11 h of incubation. The bacteriocin seems to have effect on the *L. monocytogenes* strain viability at the moment of addition, but this effect appears to be attenuated along the time. Therefore, *Ln. lactis* RK18 seemed to be bacteriostatic to exponential-phase cells of all four serogroups of *L. monocytogenes*. No changes in cell numbers of all *L. monocytogenes* were recorded for the untreated (control) samples.

Similar results were observed for other LAB strains closely related to the *Leuconostoc* genus where the addition of bacteriocin to a mid-log phase, resulted in a significant decline in the growth curve of the pathogenic strains (Albano *et al*., 2007; Abrams *et al*., 2011; Lasik‑Kurdyś and Sip, 2019).
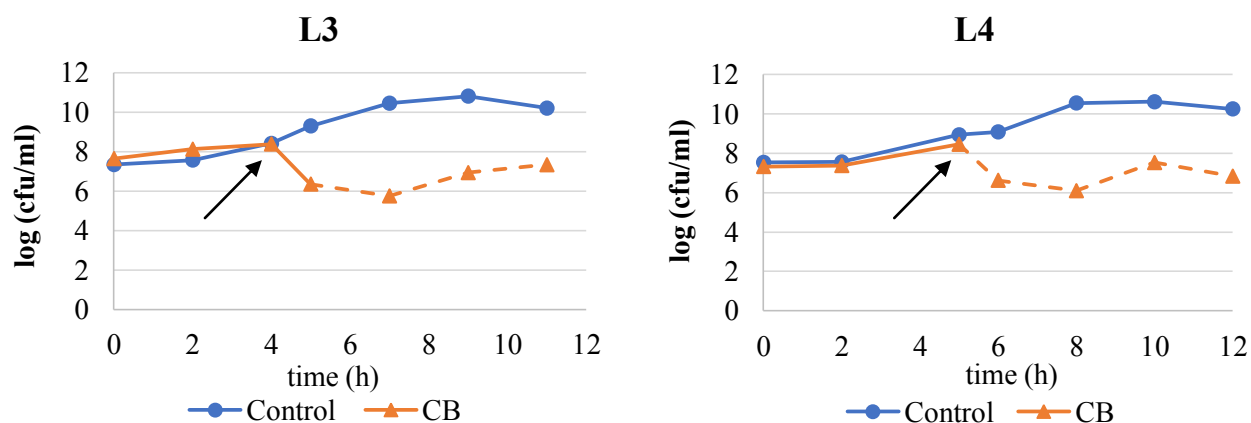
L3

L4
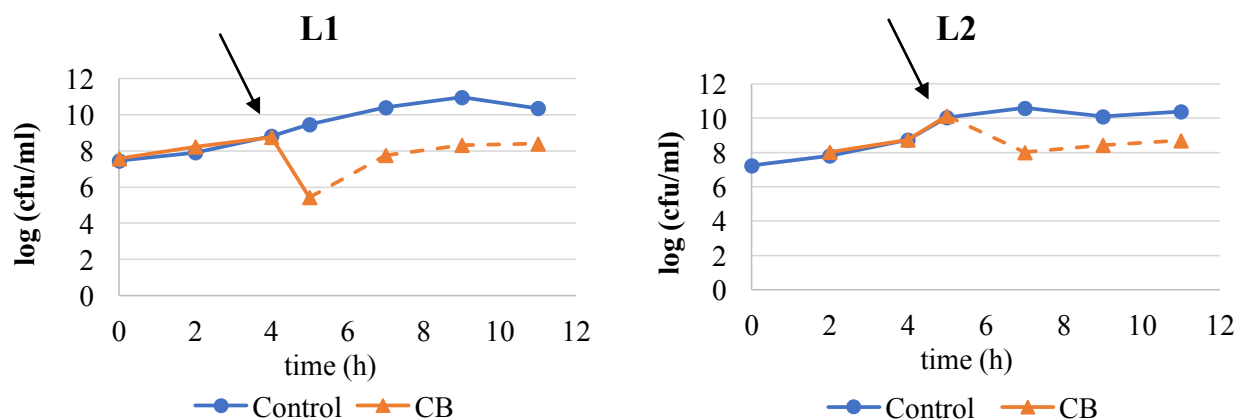
Figure 3.3. Effect of bacteriocin on growth of four serogroups of *Listeria monocytogenes*: *L. monocytogenes* NCTC 1194 (**L1**); *L. monocytogenes* CECT 911 (**L2**); *L. monocytogenes* CECT 936 (**L3**) and *L. monocytogenes* CEP 104794 (**L4**). Viable cell counts of each target are presented as log (cfu/ml). (**C**) represents target cultures without added bacteriocins (control); (**CB**) represents target cultures with added bacteriocins. (---) isolate was reduced to values below the detection limit of the enumeration technique.

2.4.3.1.2. Adsorption studies and partial purification of *Ln. lactis* RK18 bacteriocin

Bacteriocin activity was not detected after treatment of *Ln. lactis* RK18 with 100 mM NaCl at pH 2.0 (0 AU/ml) against all serogroups of *L. monocytogenes*, suggesting that the bacteriocin did not adhere to the surface of the producer cells. Similar results were reported for pediocin HA-6111-2 (Albano *et al*., 2007) and for the bacteriocin produced by bacPPK34 (Abrams *et al*., 2011).

3.3.3.1.4. Determination of the molecular size of *Ln. lactis* RK18 bacteriocin

Figure 3.4. shows an image of a Tricine-SDS PAGE gel of bacteriocin produced by *Ln. lactis* RK18. As observed, the RK18 bacteriocin was below 6.5 kDa in size. It is also possible to observe the position of the active bacteriocin by a zone of growth inhibition against *L.*

monocytogenes CEP 104794, which was firstly embedded in BHI soft agar. This zone of growth inhibition is coincident with the position of the active bacteriocin in the molecular mass marker. This small peptide is within the range of most bacteriocins reported for the genus *Leuconostoc* and for bacteriocins belonging to class II. Hwang *et al*. (2018) observed a single low molecular weight band of *Ln. lactis* SD501 (7kDa) and other authors have stated the classification of class IIa bacteriocins (known to be mainly anti-listerial proteinaceous substances) within *Leuconostoc* genus (Héchard *et al*., 1992; Arakawa *et al*., 2016; Chen *et al*., 2018; Hwang *et al*., 2018).
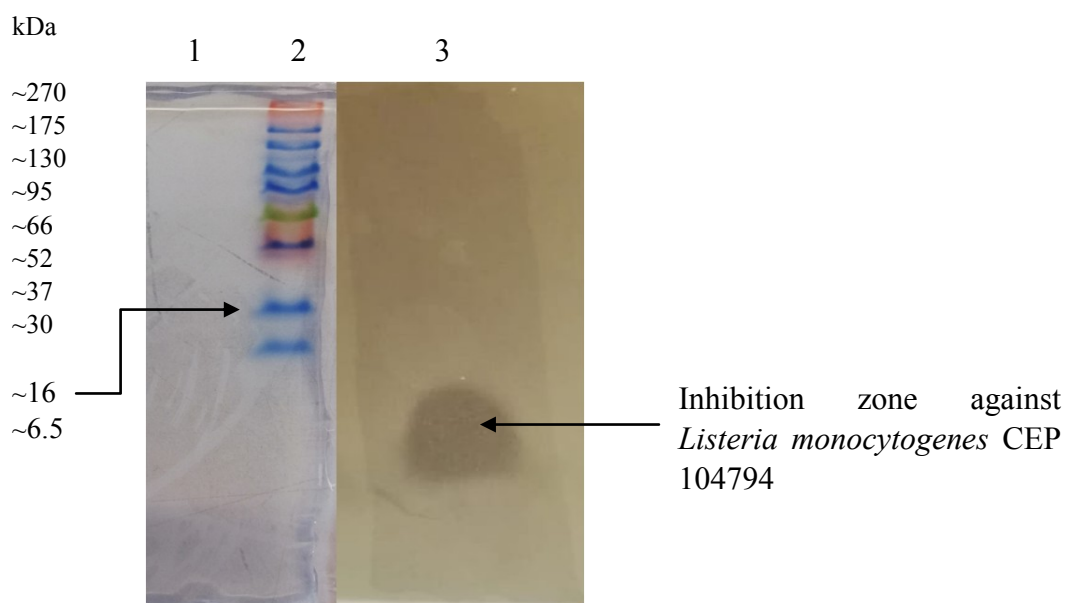

Figure 3.4. Tricine-SDS PAGE of RK18 bacteriocin. Lane 1: peptide bands stained with Coomassie Blue R250 (60% ammonium sulphate saturated)*;* lane 2: molecular mass marker; lane 3: zone of growth inhibition against *Listeria monocytogenes* CEP 104794.

The anti-listerial activity, wide pH resistance and thermostability suggest that bacteriocin produced by *Ln. lactis* RK18 may be classified as a class IIa bacteriocin (Yang *et al*., 2014). Genetic studies on the expression of the gene encoding bacteriocin production, amino acid sequence and mode of action will have to be done to confirm the hypothesis.

Other authors also found class IIa bacteriocins produced by *Leuconostoc* species, such as mesentericin Y105 produced by *Ln. mesenteroides* ssp. *mesenteroides* (Stiles, 1994).

3.3.3.1.5. Bacteriocin activity spectrum

The broad spectrum of antimicrobial activity against Gram-positive bacteria is characteristic of many class IIa bacteriocins and the activity against *L. monocytogenes* is particularly relevant (Peng *et al*., 2017; Bello *et al*., 2018). The inhibitory activity of bacteriocins may vary given that some only inhibit taxonomically related Gram-positive bacteria, and other are active against a broader range of Gram-positive and Gram-negative microorganisms (Harzallah and Belhadj, 2013).

The activity of RK18 bacteriocin was screened against 22 Gram-positive and 17 Gram-negative bacteria listed in Table 2.5.. The treated cell-free supernatant (neutralized and heated at 80 ºC; CFSnh) of *Ln. lactis* RK18 inhibited the growth of *L. monocytogenes* NCTC 1194, *L. monocytogenes* CECT 911, *L. monocytogenes* CECT 936, *L. monocytogenes* CEP 104794, *L. monocytogenes* L 7946, *L. monocytogenes* L 7947 and *L. monocytogenes* ScottA. Inhibitory ability against other Gram-positive microorganisms, such as *E. faecalis* ATCC 29212 and *L. innocua* 2030c, as well as one Gram-negative microorganism, *Yersinia enterocolitica* NCTC 10406 was also observed. Hwang *et al*. (2018) also reported antimicrobial activity of *Ln. lactis* SD501 against *L. monocytogenes* ATCC 19114 and *E. faecalis* (VRE) CCARM 0011, corroborating the data observed.

This bacteriocin activity spectrum showed that bacteriocin produced by *Ln. lactis* RK18 has an important anti-listerial activity, but also activity against other important microorganisms, such as *Enterococcus* spp.. This is an important advantage that contributes to turn the use of this microorganism even more appealing in the food industry.

3.3.4. Functional Criteria

Probiotics should resist to several conditions (tolerance to gastric substances and human bile) and remain viable through the gastrointestinal tract (adherence to epithelial cells and survival in the human GIT) to exert health benefits on the host (Hanchi *et al*., 2018).

3.3.4.1. Ability to resist to pH 2.5, pH 2.5 with pepsin and bile salts

A preliminary screening to simulate digestion fluids (pH 2.5, pH 2.5 with pepsin and 0.3% bile salts) was performed and the results are shown in figure 3.5..
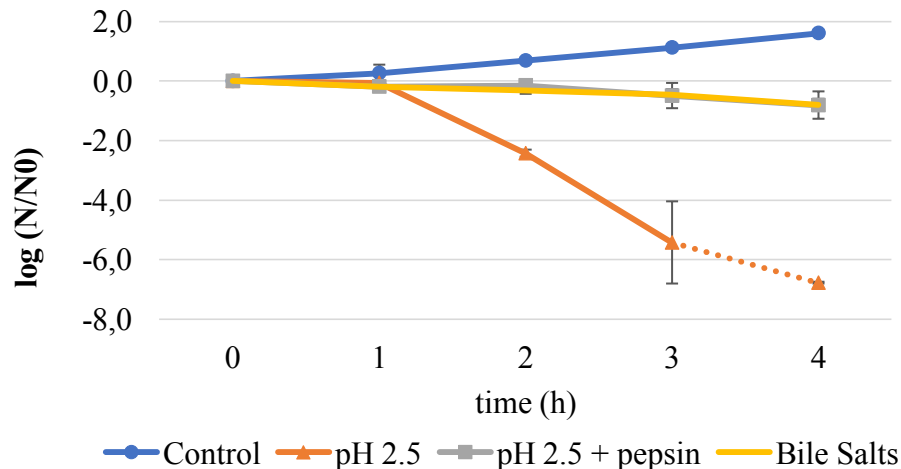

Figure 3.5. Logarithmic reduction of *Ln. lactis* RK18 observed under different conditions tested: growth in MRS broth at pH 7.0 (control) (●); MRS adjusted to pH 2.5 (▲); MRS adjusted to pH 2.5 with 1000 U/ml pepsin (■); MRS with 0.3% bile salts (—); (•••) isolate was reduced to values below the detection limit of the enumeration technique.

Unlike control, *Ln. lactis* RK18 demonstrated higher susceptibility to low values of pH, since after 3 hours of exposure, viable cell counts were reduced to values below the detection limit of the enumeration technique. Surprisingly, *Ln. lactis* RK18 cells exposed to pH 2.5 with pepsin were reduced in less than 1 log cycle. Despite the inability to explain this obtained result, variations on pepsin activity has been reported by several authors (DiPalma *et al*., 1991; Armand *et al*., 1995; Ulleberg *et al*., 2011).

Also the presence of bile salts had little effect on *Ln. lactis* RK18 cells (less than 1 log cycle reduction). Similar results were observed by Vinderola and Reinheimer (2003) for other LAB strains.

Although important, this experiment does not preclude the need to test for simulation of gastrointestinal conditions, since the sequential stresses which ingested microorganisms are exposed due to the continuous changing conditions during their passage through GIT *in vivo* (Marteau *et al*., 1997) are not simulated.

3.3.4.2. Survival through simulated gastrointestinal tract conditions

To guarantee that potential probiotic *Ln. lactis* RK18 was able to survive through the gastrointestinal tract (GIT), cells were exposed to simulated sequential GIT conditions in the presence of two food matrices: skimmed milk and *alheira*. These were considered as suitable matrices since probiotics are commonly added to dairy products and LAB isolates are frequently isolated from meat products (Barbosa *et al*., 2014). Results obtained are shown in table 3.7..

Table 3.7. Logarithmic reduction of *Ln. lactis* RK18 through simulated gastrointestinal tract conditions in the presence and absence of food matrices

Conditions	time (min)		
	0	60[a]	120[b]
	Log N/N0		
Without matrix	0.00±0.00	-0.83±0.94	-6.30±1.20*
Skimmed Milk	0.00±0.00	0.21±0.38	-6.71±1.01*
Alheira	0.00±0.00	-0.09±0.07	-1.37±0.22

Survival is represented as the media of the logarithmic reduction – log (N/N0) ± the standard error of the mean; N is the cfu/ml at each sampling time; N0 is the cfu/ml at time zero;
[a]Survival after exposure to pH 3.0 in the presence of pepsin.
[b]Survival after exposure to pH 3.0 in the presence of pepsin and subsequent exposure to bile salts at pH 7.0.
*Isolate was reduced to values below the detection limit of the enumeration technique.

During simulation of the gastric transit (pH 2.5 with pepsin), there was a slight reduction (less than 1 log cycle) on the viability of *Ln. lactis* RK18 in the presence of *alheira* and without matrix protection. No reduction was observed for *Ln. lactis* RK18 cells in the presence of skimmed milk. This protection conferred by skimmed milk was already described by other authors for probiotic LAB (Chick *et al*., 2001; Guglielmotti *et al*., 2007).

When bile salts were added (simulated small intestine digestion), unlike for absence of matrix and in the presence of skimmed milk, for which *Ln. lactis* RK18 were reduced to values below the detection limit of the enumeration technique (6.30 and 6.71 log cycles, respectively), in the presence of *alheira* matrix there was a merely reduction of 1.37 log cycles. This shows that

survival of *Ln lactis* RK18 was influenced by the matrix of the food product and *alheira* conferred protection to the cells during digestion. Other authors have reported the ability of different strains of LAB closely related to the *Leuconostoc* genus to tolerate the GIT conditions (Nagata *et al*., 2009; Diana *et al*., 2015; Campana *et al*., 2017). The potential probiotic *Ln. lactis* RK18 was able to survive through simulated GIT conditions in the presence of *alheira* matrix, maintaining a number of cells about 10^9 cfu/ml. Although the need for further assays, especially to evaluate the behaviour of this strain in the presence of other food matrices, it seems quite possible the use of *Ln. lactis* RK18 to produce a probiotic food.

3.3.4.3. Human colon adenocarcinoma cell lines Caco-2 cells assays

3.3.4.3.1. Ability of *Ln. lactis* RK18 to adhere to Caco-2 cells

In vitro adhesion to Caco-2 cells was previously evaluated for fresh cells of *Ln. lactis* RK18, using *Lactobacillus rhamnosus* GG (ATCC) as control. The defined and optimized protocol for Caco-2 cells adhesion were then applied on *Ln. lactis* RK18 cells previously exposed to GIT conditions in the presence of *alheira* matrix (section 3.3.4.2.). Results obtained are presented in figure 3.6..
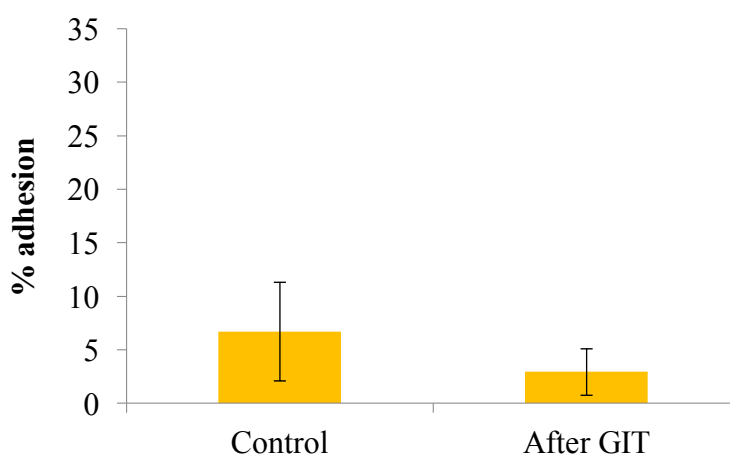

Figure 3.6. Percentage of adhesion of *Ln. lactis* RK18 cells before (control) and after gastrointestinal tract (GIT) simulation. Values represent the percentage of adhesion ± standard deviation of at least three independent experiments.

At optimal growth conditions *L. rhamnosus* GG cells (1.0 x 10^5 viable cells) were able to adhere to adenocarcinoma cells by 9.52 ± 0.73 %. These results were in accordance of reported data by Botes *et al*. (2008), therefore this control allowed the confirmation of the assay's effectiveness. Thenceforth all the *in vitro* assays were carried accordingly.

Growing in optimal conditions, 6.72 ± 4.61 % of *Ln. lactis* RK18 cells were able to adhere to Caco-2 cells. Similar results of adherence by other LAB were reported by other authors (Mishra and Prasad, 2005; Saxami *et al*., 2016).

The exposure to simulated GIT conditions seems to have had a negative impact on the cells, since the percentage of adhered cells decreased. This may be due to the second stress condition applied to the GIT exposed cells, i.e., the treatment with the proteolytic enzyme Triton. This enzyme is used to lysate Caco-2 cells in the last step of the protocol, but even used in low concentrations, increased sensitivity of the protein adhesins in the bacterial cell membrane to the Triton treatment could be a possible explanation (Botes *et al*., 2008). More tests are needed, including the response of this LAB after exposed to GIT under protection of other food matrices, but it is notable that *Ln. lactis* RK18 achieved the prerequisite of adhesion ability, which turns possible the ability of candidate probiotic to colonize and may modulate the host immune system.

3.3.4.3.2. Ability of *Ln. lactis* RK18 to invade Caco-2 cells

In vitro invasion ability of *Ln. lactis* RK18 to Caco-2 cells were evaluated for both fresh cells and cells exposed to simulated GIT conditions in the presence of *alheira* matrix and, in either case, Caco-2 cells were not invaded by *Ln. lactis* RK18 cells. This result reinforces the potential of *Ln. lactis* RK18 as probiotic strain, particularly in terms of safety.

3.3.4.3.3. Role of *Ln. lactis* RK18 on the inhibition of foodborne pathogens adherence and/or invasion to Caco-2 cells

A) Prevention of *L. monocytogenes* CEP 104794 adhesion to Caco-2 cells

In vitro ability of *L. monocytogenes* CEP 104794 to adhere to Caco-2 cells was evaluated. In parallel, the ability of *Ln. lactis* RK18 to prevent the adhesion by this foodborne pathogen was assessed.

In figure 3.7. are shown the results obtained for *L. monocytogenes* CEP 104794 adhesion.
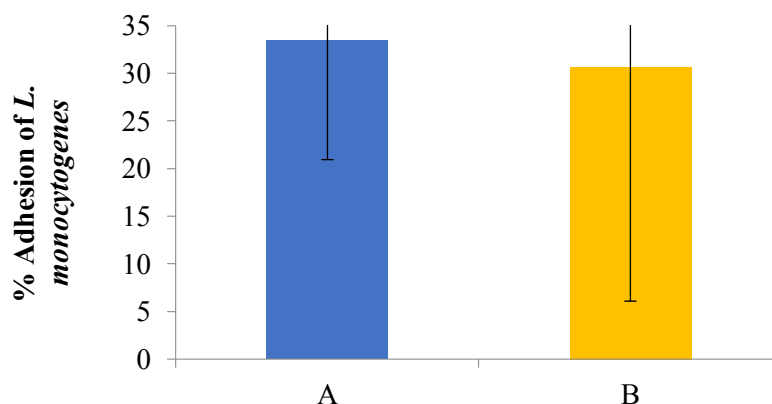

Figure 3.7. Percentage of *L. monocytogenes* CEP 104794 cells adhered to Caco-2 cells when tested alone (A) and in co-culture with *Ln. lactis* RK18 cells (B). Values represent the percentage of adhesion ± standard deviation of at least three independent experiments.

B) Inhibition of *L. monocytogenes* CEP 104794 invasion to Caco-2 cells

In vitro invasion of Caco-2 cells by *L. monocytogenes* CEP 104794 was evaluated.

The ability of *Ln. lactis* RK18 to prevent invasion by this foodborne pathogen was measured by direct cell competition and by the use of its produced bacteriocin (10% (v/v) of cell-free supernatant neutralized and heated to 80 ºC for 10 min, CSFnh). Results obtained are presented in Figure 3.8..
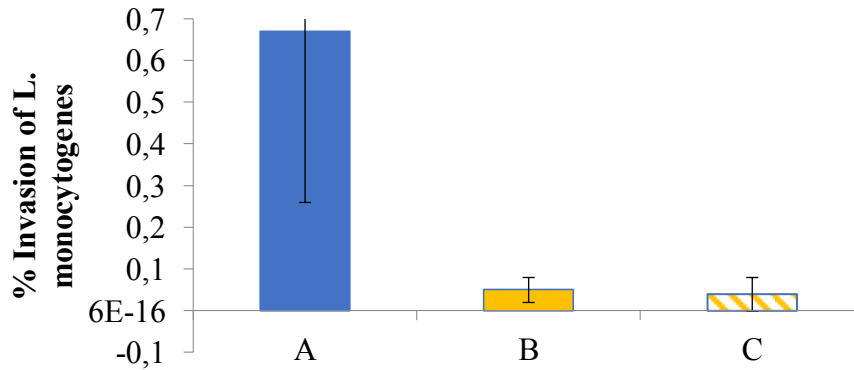
Figure 3.8. Percentage of *L. monocytogenes* CEP 104794 cells that invaded Caco-2 cells when tested alone (A), in co-culture with *Ln. lactis* RK18 cells (B) and in co-culture with 10% (v/v) treated cell-free supernatant of *Ln. lactis* RK18 (C). Values represent the percentage of invasion ± standard deviation of at least three independent experiments.

It is possible to observe that *L. monocytogenes* CEP 104794 cells were able to adhere (33.5 ± 12.6 %) to Caco-2 cells, but at the same time, the presence of *Ln. lactis* RK18 allowed a slight decrease in *L. monocytogenes* adhesion (Figure 3.7.).

In addition to adhere, *L. monocytogenes* CEP 104794 was also able to invade Caco-2 cells (Figure 3.8.), but although the low invasion percentage (0.67± 12.6 %), the presence of *Ln. lactis* RK18, cells and 10% (v/v) of its treated supernatant (CFSnh), inhibited *L. monocytogenes* CEP 104794 invasion capacity *in vitro*. Invasion capacity by different strains of *L. monocytogenes* had been reported for decades (Conte *et al*., 1994; Botes *et al*., 2008; Ferreira *et al*., 2011) Not neglecting the need to perform the same tests for other *L. monocytogenes* strains, this means that if *Ln. lactis* RK18 is used as a probiotic culture, when it cells are present and adhered to intestinal cells, they will be able to prevent the adhesion of foodborne pathogens, such as *L. monocytogenes*, which will contribute to the subsequent inhibition of the invasion by this pathogen and consequently inhibition of infection. Similar results were obtained for cells of *Lactobacillus plantarum* 423 in the prevention of *L. monocytogenes* ScottA when added prior to incubation (Botes *et al*., 2008), and also for *Lactobacillus casei* W56 and *Lactobacillus rhamnosus* W71 cells against various pathogenic bacteria, including *L. monocytogenes* ATCC 7644 (Campana *et al*., 2017).

The inhibition of *L. monocytogenes* CEP 104794 invasion capacity *in vitro* by the presence of treated cell-free supernatant (CFSnh) of *Ln. lactis* RK18 was an expected result due to its anti-listerial activity, already described in this study (section 2.2). Similar results were reported by Botes *et al*. (2008) with prevention of invasion by 5% CFS of *L. plantarum* 423. In the past, other authors stated that cell-free supernatants may interfere with the interaction between pathogens and epithelial cells, preventing the cell invasion (Bernet-Camard *et al*., 1997; Lammers *et al*., 2002). These results also allow inferring that after the passage of GIT, *Ln. lactis* RK18 does not lose the ability to produce an active bacteriocin against *L. monocytogenes*. At this point, it is not possible to know if inhibition of invasion occurs earlier, i.e., if it also prevents adhesion of this pathogen to intestinal epithelium, whereby it would be an important and interesting assay to perform.

IV.   Conclusions

In the present work, from 202 lactic acid bacteria isolated from several food products, only three isolates demonstrated antimicrobial ability against important human pathogenic microorganisms, such as *L. monocytogenes* and only one isolate from "khmer fermented rice fresh noodles" and identified as *Ln. lactis* RK18 presented important requirements to be considered as a potential probiotic candidate.

For commercial purposes, probiotic candidates should meet a number of requirements, including being safe, functional and with technological and physiological characteristics. A beneficial but non-mandatory criterion for the selection of probiotic strains may be the production of bioactive substances against foodborne pathogens. *Leuconostoc lactis* RK18 seems to meet the safety requirement, since, it did not show any of the virulence genes tested nor virulence factors, nor resistances to recommended antibiotics. As beneficial characteristics, *Ln. lactis* RK18 showed antimicrobial activity against *E. faecalis* and *L. monocytogenes* strains. Furthermore, it was found that its anti-listerial activity was due to the production of Class IIa bacteriocin (below 6.5 kDa in size). Bacteriocin produced by *Ln. lactis* RK18 remained stable at average temperatures (30 ºC to 80 ºC) and at pH values ranging from 4 to 6, and although susceptible to some detergents, it showed high resistance to several enzymes.

Production of lipase was the only enzymatic activity tested as technological criterion, but *Ln. lactis* RK18 did not produce this enzyme. On the contrary, *Ln. lactis* RK18 met the functional requirements tested. Despite being very sensitive to acidic environments, when incorporated into a complex food matrix such as *alheira*, *Ln. lactis* RK18 was able to survive through simulated gastrointestinal tract (GIT) conditions and also to adhere (but not invade) to human colon adenocarcinoma cell lines Caco-2 *in vitro*. Even though exposure to GIT conditions had influenced the adhesion ability of *Ln. lactis* RK18 cells, this potential probiotic was able to prevent the ability of *L. monocytogenes* CEP 104794 to adhere and invade Caco-2 cells. In addition, the presence of merely 10% of treated cell-free supernatant of *Ln. lactis* RK18 exposed to GIT conditions also allowed the inhibition of *L. monocytogenes* CEP 104794 to invade intestinal cells.

Overall, *Ln. lactis* RK18 appeared to be a safe strain, with no risk to human health, which harboured important features to be successfully considered as a potential biopreservative and probiotic culture. Nevertheless, further experiments should be performed for the validation of its application in food industry.

V. Proposals for future work

Despite the extensive study developed with *Ln. lactis* RK18 regarding its bacteriocinogenic and probiotic potential, there is still much interesting and necessary work to be done, such as:

(i)   To test for cholesterol assimilation and β-galactosidase production by *Ln. lactis* RK18;

(ii)   Biofilm formation ability and, in parallel, auto-aggregation and co-aggregation assays since they are important in the formation of biofilms to protect the host from colonization by pathogens;

(iii) Hydrophobicity assays since hydrophobicity plays a key role in the first contact between a bacterial cell and mucus or epithelial cells. Therefore there is a correlation between hydrophobicity potential and adhesion to Caco-2 cells;

(iv)   *In vitro* assays using other protocols or cell lines, to observe the behaviour of *Ln. lactis* RK18 in terms of adhesion and invasion;

(v)   Identification of genes encoding bacteriocin production, such as bacteriocin structural genes;

(vi)   Evaluation of antimicrobial activity by *Ln. lactis* RK18 against other important lactic acid bacteria belonging to the intestinal microbiota.

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