Bacteriocinogenic activity of Lactic acid bacteria isolates against potential pathogenic microbiota and evaluation of EK13 enterocin on the survival of *Listeria innocua*

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“Learn from yesterday, live for today, hope for tomorrow. The important thing is not to stop questioning”

Albert Einstein

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Abstract

Bacterial foodborne diseases are a constant concern to human health. Lactic acid bacteria (LAB) are known by their bacteriocinogenic potential property and could be used to reduce and control the pathogenic bacteria colonization in foodstuff, reducing the foodborne illness risk to consumers. The study aims to evaluate the potential bacteriocinogenic ability of different LAB, collected at a poultry slaughterhouse (n=94) and from different portuguese fermented meat products (n=15), against pathogens frequently detected in meat and meat products. All *Lactobacillus* and *Enterococcus faecium* EK13 tested showed potential bacteriocinogenic capability against the pathogens tested. *L. sakei* CV3C8 exhibited the highest inhibitory activity against all *Campylobacter* tested. *L. plantarum* P3B7 and P05-15 showed a high inhibition of *Salmonella enteritidis,* while strain P3B8 presented best results against *Enterococcus avium* (EA5) and *Listeria monocytogenes*. *L. plantarum* P05-67 had the best inhibitory results against *S. aureus* followed by isolate L1B8. B6C1-3-3 LAB isolate, from slaughterhouse collection, had high inhibition of *L. monocytogenes*. *Enterococcus faecium* EK13 did not have the highest inhibitory activity against *L. monocytogenes* however it was bacteriocin producer being selected to *in vitro* tests. The antilisterial effect of bacteriocin-like inhibitory substances (BLIS) produced by *E. faecium* EK13 was evaluated in an *in vitro* meat model, mimicking different fermentative conditions (1st step at 7ºC and 2nd step at 20ºC) during 96 hours. Two different concentrations (0.1% and 0.5%) of BLIS on meat were tested. EK13 BLIS presented good protective capacity reducing *Listeria innocua* counts but without inhibitory action for total mesophilic bacteria present in meat.

Key Words: bacteriocinogenic potential; EK13 BLIS; *Lactobacillus*; *Listeria; Campylobacter;* food safety

Resumo

As doenças de origem alimentar são um problema para a saúde humana. As bactérias ácido lácticas (LAB), conhecidas pelo potential bacteriocinogénico, podem ser usadas para reduzir e controlar a colonização bacteriana nos alimentos, reduzindo o risco de doença.

O objectivo deste estudo foi avaliar o potential bacteriocinogénico de diferentes LAB, isoladas de amostras recolhidas num matadouro de aves (n=94) e de produtos cárneos portugueses (n=15), contra patogéneos frequentemente detectados na carne e em produtos derivados.

Todos os isolados *Lactobacillus* e *Enterococcus faecium* EK13 mostraram potential bacteriocinogénico contra os patogéneos testados. *L. sakei* CV3C8 teve a maior capacidade inibitória contra todos os *Campylobacter* testados. *L. plantarum* P3B7 e P05-15 mostraram maior inibição contra *Salmonella enteritidis,* enquanto a estirpe P3B8 apresentou melhores resultados contra *Enterococcus avium* (EA5) e *Listeria monocytogenes*. *L. plantarum* P05-67 teve melhores resultados inibitórios contra *S. aureus* seguido pelo isolado L1B8. O *Lactobacillus* B6C1-3-3, isolado do matadouro apresentou melhores resultados contra *L. monocytogenes*. *Enterococcus* EK13 não apresentou a maior capacidade inibitória contra *L. monocytogenes* no entanto, sendo produtor de bacteriocinas foi escolhido para os testes *in vitro*.

O efeito antilisterial das substâncias inibitórias semelhantes a bacteriocinas (BLIS) produzidas por *E. faecium* EK13 foi avaliado num modelo *in vitro*, considerando diferentes condições fermentativas (1ª fase a 7ºC e 2ª fase a 20ºC) durante 96 horas. Foram testadas duas concentrações diferentes (0.1% e 0.5%) de BLIS. EK13 BLIS apresentou uma boa capacidade protectora reduzindo as contagens de *Listeria innocua* sem ter efeito inibitório na microbiota aeróbia mesófila total da carne avaliada.

Palavras-chave: Potencial bacteriocinogénico; BLIS EK13; *Lactobacillus*; *Listeria; Campylobacter;* segurança dos alimentos

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List of Abbreviations

ALOA	Agar Listeria by Ottaviani & Agosti
ANOVA	Analysis of variance
ATCC	American Type Culture Collection
AU	Arbitrary units
BHI	Brain Heart infusion
BLIS	Bacteriocinogenic-Like Inhibitory Substances
bp	Base pair
°C	Celsius degree
CDC	Center for Disease Control and Prevention
CECT	Spanish Type Culture Collection
CFU	Colony forming units
COS	Columbia Blood agar
DNA	Deoxyribonucleic acid
DNase	Deoxyribonuclease
EA5	*Enterococcus avium 5*
EFSA	European Food Safety Authority
EK13	*Enterococcus faecium 13*
Ent. Faecium	*Enterococcus faecium*
et al.	et alia (and others)
EU	European Union
FAO	Food and Agriculture Organization
FDA	Food and Drug Administration
g	gram
GC	Guanine-cytosine content
GRAS	Generally recognized as safe
h	hour
ISO	International Organization for Standardisation
LAB	Lactic acid bacteria
Log	Logarithm
min	minute
mL	milliliter
MRS	Man, Rogosa and Sharpe
OD	optical density
PCR	Polymerase chain reaction
pH	potential of hydrogen
QPS	Qualified Presumption of Safety
S. aureus	Staphylococcus aureus
sp.	species

WHO	World Health Organization
TSA	Trypticase Soy agar
µg	microgram
µL	microliter
%	percentage

Chapter 1

Introduction and Literature Review

Introduction

Food safety remains a major public health concern and is a challenge to be accomplished by food producers in order to provide adequate protection to consumers [1]. Contamination of meat with foodborne pathogens remains an important issue, because it can lead to illness by the ingestion of meat or meat products [2].

Consumers expect that the foods they purchase and consume are safe, and there is a need for close verification of potential pathogens absence [3]. Simultaneously, over the past years, microbial spoilage has reached a growing importance in food quality and food security.

Meat and meat products are excellent food substrates for spoilage and pathogenic bacteria [4]. Meat is a source of proteins, fat, vitamins and minerals. With these intrinsic factors the pathogenic bacteria can easily survive and/or growth to hazardous levels for human health when meat or meat products are ingested [3].

In developed countries, foodborne illness causes human suffering and loss of productivity, and significantly enhances the cost of food production and healthcare [2]. To reduce the number of foodborne outbreaks and control microbial spoilage, some regulatory frameworks and proactive safety management systems, such as good hygienic practices (GHP), good manufacturing practices (GMP), good agricultural practices (GAP) and hazard analysis critical control points (HACCP) have been developed for the production of safe foods [5].

Meat preservation and safety is demanded by consumers without use of chemical preservation. In this respect, biopreservation has gained increased interest as a mean of natural control of meat products shelf life and safety [6].

Microbiologists around the world got interest in bacteriocin-producing bacteria to overcome this problem that fulfils the requirement of food preservation [7]. Special attention has been given to those bacteria producing bacteriocin-like inhibitory substances (BLIS) with capability to inhibit foodborne pathogens and spoilage bacteria improving the safety and quality of foods [8].

Literature Review

1.1. Microbiology of meat

1.1.1. Spoilage bacteria in meat and factors influencing their multiplication

Meat is recognized as one of the most perishable foods [9]. This is due to its chemical composition, favourable water activity (aw) and pH values that promotes microbial growth to unacceptable levels, contributing significantly to meat deterioration. Spoilage can be defined as any modification in a food product that makes it unacceptable to the consumer from a sensory point of view concerning the food colour, texture, odour and taste, and also regarding changes of microbial counts [10, 11].

Microbial growth, oxidation and enzymatic autolysis are the main three basic mechanisms responsible for the spoilage of meat [12]. The initial microbial load of meat depends from the physiological status of the animal at slaughter, and from the spread of contamination in slaughterhouses and during processing, while temperature and other storage conditions during distribution could also influence the rate of spoilage [13,14].

When spoilage microorganisms are present in raw, cooked or fermented meat products, they compete to have space and nutrients utilization and several food modifications are induced becoming unappealing and unsuitable for human consumption [15,16]. The development of organoleptic spoilage is related to microbial consumption of meat nutrients, such as sugars and free amino acids and the release of undesired volatile metabolites (volatile organic compounds, VOCs) responsible for off-odours [10].

Meat spoilage is caused by only a fraction of Specific Spoilage Organisms (SSO) called Ephemeral Spoilage Organisms (ESO) that become dominant through selection depending on conditions prevailing during processing, transportation and storage [5].

A number of selective factors (e.g., pH, aw, temperature, nutrients, availability of oxygen, etc.) affects microbial growth and metabolism and so, influence the multiplication of particular bacteria and, as a consequence, a characteristic and specific microbial association develops and is present at the time of spoilage, leading to its characteristic spoilage features [17, 18].

Temperature is considered the most important factor influencing spoilage and meat safety [18, 19]. Bacteria relevant to meat, meat products and other food are divided into three groups according to the temperature range within which they can grow: mesophiles 10-45°C, psychrophiles 0-28°C and psychrotrophs 10–25°C. Mesophiles will not grow below 10°C but psychrotrophs, of which *Pseudomonas* are usually referred as important, will grow down to 0°C [20, 21].

In general, bacteria prefer to adhere to meat surface through different stages involving an attachment by glycocalix formation [9]. The development of these phases depends as already stated on the intrinsic and extrinsic ecological factors of a particular meat ecosystem such as pH, nutrient availability, meat surface morphology, redox potential, O_2 availability, temperature, relative humidity and presence and development of other bacteria [9].

The microorganisms that can colonize the fresh meat depend highly on the characteristics of meat and how it is processed and stored [18]. In Figure 1 are represented the most common microorganisms present in raw meat.

Figure 1 - Main microorganisms contaminating raw meat, adapted from Meat products Handbook: Practical Science and technology [22].

Meat spoilage is usually caused by Gram negative bacteria (*Pseudomonads, Enterobacteriaceae, Shewanella putrefaciens*) and several Gram positive (lactic acid bacteria (LAB), *Brochothrix thermosphacta, Clostridia*) that dominate under different storage conditions [23]. Spoilage organisms in raw meat, especially Gram-negative bacteria can be difficult to control due to the lipopolysaccharide layer of the outer membrane that functions as an effective permeability barrier [24]. A large number of LAB genera and species have been found in spoiled meat products and some of them are represented in Figure 2. The main LAB species associated with spoilage belong to the genera *Lactobacillus*, *Leuconostoc* and *Carnobacterium* [25, 26]. Species belonging to the genus *Lactobacillus* (e.g., *Lactobacillus sakei, Lactobacillus curvatus, Lactobacillus algidus, Lactobacillus fuchuensis, Lactobacillus oligofermentans*) are associated with acidification, emission of off-odour compounds and slime in the case of poultry, marinated meat, minced beef and pork stored under vacuum or modified atmosphere package (MAP) [23]. Furthermore, species from the genera *Weissella* and *Lactococcus* are also reported to cause spoilage [25, 26].

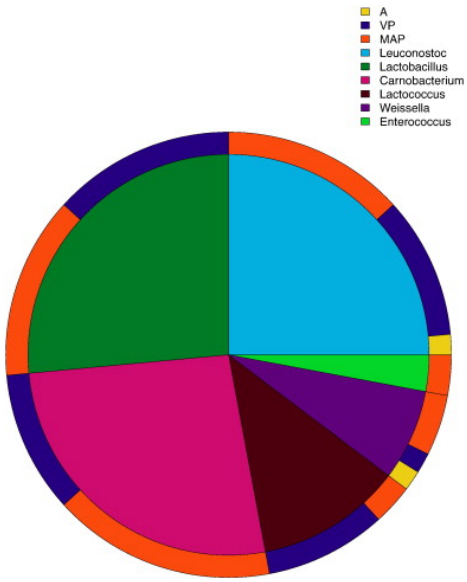

Figure 2 - Occurrence of different LAB genera as dominant members of the microbial community of spoiled meat (inner pie). Packaging technology implemented in every spoilage case (outer ring), A, air; VP, vacuum packaging; MAP, modified atmosphere packaging. Reports used for the construction of the present combination chart range from 1996 to 2014. The species of the major genera are sorted based on number of cases wherein their involvement was documented: *Leuconostoc (L. gelidum, L. mesenteroides, L. carnosum), Lactobacillus (Lb. sakei, Lb. algidus, Lb. curvatus, Lb. fuchuensis), Carnobacterium (C. divergens, C. maltaromaticum), Lactococcus (Lc. piscium, Lc. lactis)*, from Pothakos *et al*., 2015 [23].

1.1.2 Pathogenic microbiota present in meat and meat products

According to EFSA (European Food Safety Authority) foodborne diseases are caused by consuming food or drinking water contaminated by pathogenic microorganisms such as bacteria and their toxins, viruses and parasites [27, 28]. Numerically, the most important microorganisms contributing to foodborne illnesses are represented in Figure 3. *Campylobacter*, *Salmonella*, *Yersinia, E. coli* VETC and *Listeria monocytogenes* are the main pathogens link to the ingestion of food causing a picture of gastroenteritis or other symptoms. Also, *Staphylococcus aureus* are included in these frequent pathogens causing foodborne infections [28].

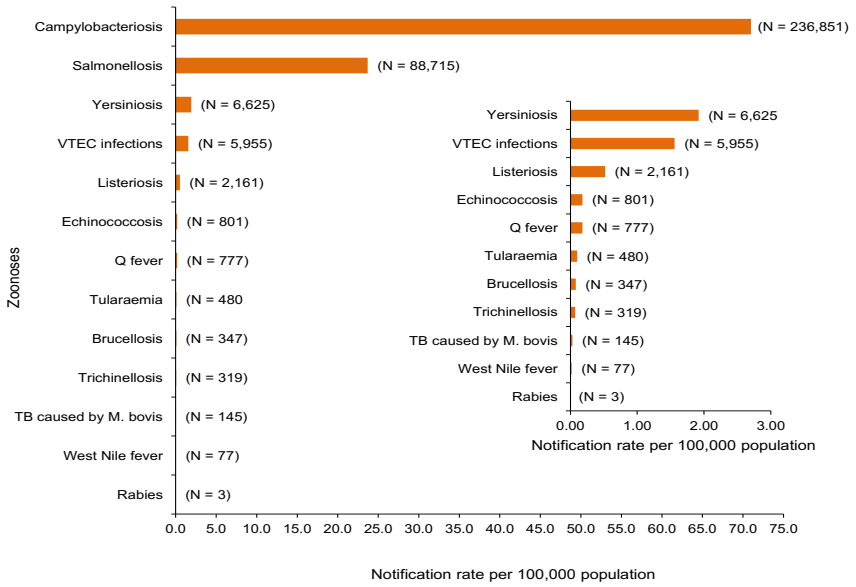

Figure 3 – Reported numbers and notification rates of confirmed human zoonoses cases in the EU, 2014. Data from: EFSA, CDC, 2015 [28].

1.1.2.1 *Campylobacter*

In 2014, *Campylobacter* continued to be the most commonly reported gastrointestinal bacterial pathogen in humans in the European Union (EU, Figure 3) and has been so since 2005 [28]. The European Food Safety Authority (EFSA) reported 214268 confirmed human cases (with 31 death occurring) due to campylobacteriosis compared to 91034 human cases of salmonellosis in 2012 in Europe [29].

The family *Campylobacteraceae* consists of four genera, comprising *Campylobacter, Arcobacter, Dehalospirilum,* and *Sulfurospirilum* [30].

According to the List of Prokaryotic Names with Standing in Nomenclature *Campylobacter* genus typically comprises 34 identified species [31]. They are Gram-negative, spiral-shaped in structure, anaerobic microorganisms [32] that under dramatic environmental conditions have the ability to enter the viable but non-culturable (VBNC) state and can revert back to a phase where the bacteria are able to regain culturability and pathogenicity when conditions are favourable [33].

The most common cause of human infection is *Campylobacter jejuni*, followed by *Campylobacter coli*. *Campylobacter lari, Campylobacter fetus* and *Campylobacter upsaliensis* have also been reported to cause human infections [34]. *C. jejuni* is involved in approximately 85% of infections [35] and can reside in the intestine of most warm-blooded animals, sometimes with distinct effects on the host such as severe disease symptoms, inflammation of gut mucosa and even penetration into deeper tissues by epithelial cell invasion. This major foodborne pathogen can also colonize the gut of animals without almost any symptoms of disease [29].

C. jejuni and *C. coli* are thermophilic; their optimum temperature for growth is 42ºC, close to the body temperature of poultry [36]. For that reason, poultry is an important reservoir and source of human campylobacteriosis [37]. However, cattle, pigs, sheep, and pet animals may also be a source of these microorganisms [38].

Human *Campylobacter* infection may be due to either consumption of undercooked meat and meat products or cross-contamination of ready-to-eat food during preparation or storage [38], [39]. Anyone may become ill from *Campylobacter* infection. However, infants and young children, pregnant women and older adults, are at a higher risk because they have weakened immune systems (such as those with HIV/ AIDS, cancer, diabetes, kidney disease, and transplant patients) [40]. The symptoms of human campylobacteriosis include diarrhea, abdominal pain, nausea and vomiting, which tend to last 5-7 days and can recur in 15-25% of cases with approximately 10% of these being hospitalized [35]. Campylobacteriosis is the most common infection preceding the onset of post-infectious Guillain–Barré syndrome, a severe demyelinating neuropathy, occurring in approximately 3/10 000 campylobacteriosis cases [37].

1.1.2.2. *Salmonella*

Salmonella is one of the most common causes of foodborne infection worldwide [41]. In 2014, a total of 88,715 confirmed salmonellosis cases were reported by 28 EU MS (Member State), resulting in an EU notification rate of 23.4 cases per 100,000 population. This represented a 15.3% increase in the EU notification rate compared with 2013 [28]. The highest notification rates in 2014 were reported by the Czech Republic (126.1 cases per 100,000 population) and Slovakia (75.3 per 100,000), while the lowest rates were reported by Portugal and Greece (≤ 4.0 per 100,000) [28].

Salmonella infection remains a major public health concern worldwide, contributing to the economic burden of both industrialized and underdeveloped countries through the costs associated with surveillance, prevention and treatment of disease [42].

The genus *Salmonella* is considered to have two species named *Salmonella enterica* and *Salmonella bongori* [43]. At present, over 2500 serotypes of *Salmonella* have been reported. The most common serotypes associated with human illness are *Salmonella enterica* serovar Typhimurium *(S. Typhimurium)* and *S. enterica* serovar *Enteritidis* (*S. Enteritidis*) in the United States and European countries [44]. *S. enterica* serovars are a diverse group of pathogens that have evolved to survive in a wide range of environments and across multiple hosts [41].

Salmonella is a rod-shaped, Gram-negative facultative anaerobe that belongs to the family *Enterobacteriaceae* and is about 2-3 x 0.4-0.6 μm in size [42]. *Salmonella* are non-fastidious as they can multiply under various environmental conditions outside the living hosts [45]. Most *Salmonella* serotypes grow at temperature range from 5 to 47°C with optimum temperature of 35 to 37°C but some can grow at temperature as low as 2 to 4°C or as high as 54°C [45]. They are sensitive to heat and often are destroyed at 70°C or above. *Salmonellae* grow in a pH range of 4 to 9 with the optimum between 6.5 and 7.5. They require high water activity (a_w) between 0.99 and 0.94, yet can survive at a_w <0.2 such as in dried foods. Complete inhibition of growth occurs at temperatures <7°C, pH <3.8 or water activity <0.94 [45].

They are widely distributed in the nature and their primary reservoirs are humans and animals [47]. In general, food animals such as swine, poultry and cattle are the prime sources of *Salmonella* infections [42]. It is also among the most commonly isolated foodborne pathogens associated with fresh fruits and vegetables [45].

Gastroenteritis is the most common manifestation of *Salmonella* infection worldwide, followed by bacteraemia and typhoid fever, also known as enteric fever disease [42, 46]. The incubation period is 12-36 hours [47]. Although most *Salmonella* infections result in mild to moderate self-limiting gastroenteritis requiring little or no intervention, serious extra intestinal complications, such as septicaemia, endocarditis, meningitis, and osteomyelitis, may occur [48].

1.1.2.3 *Listeria monocytogenes*

Listeria monocytogenes is one of the deadliest bacteria found in food related infections [49]. In 2014, 27 MS reported 2,161 confirmed human cases of listeriosis. The EU notification rate was 0.52 cases per 100,000 population which represented a 30% increase compared with 2013 [28].

Listeria monocytogenes is a Gram-positive, non-spore-forming, catalase positive, facultative intracellular pathogen that causes listeriosis, particularly in young, old, pregnant and immune-compromised individuals [50]. It is a facultative anaerobic bacterium, able to survive in the presence of oxygen [47].

The genus *Listeria* includes fifteen species and they are widely distributed in the environment particularly the closely related species *Listeria monocytogenes* and *L. innocua* and survive even under low temperatures, pH, high concentrations of salt or bile, oxidative stress, carbon starvation, and other adverse conditions [50, 51, 52].

Listeria has an optimum growth temperature of 30-37 ºC, can survive between 1 and 45 ºC, being considered a psychrotrophic bacteria, it is able to growth at refrigeration temperatures [53]. In ready-to-eat products, refrigeration is the principal method to control undesirable microorganisms and sometimes is the only method of preservation. However, *L. monocytogenes* can multiply with little or no change of products sensory characteristics. Therefore, the inhibition of *Listeria monocytogenes* is very relevant to food safety since this pathogen has been associated with several disease outbreaks [54].

Due to high rate of mortality of infected persons, foodborne diseases caused by *Listeria monocytogenes* present a public health risk [55]. Infections with this bacterium are currently associated with a fatality rate of approximately 17%, the highest rate observed among foodborne pathogens [50].

The symptoms of listeriosis usually last 7-10 days, with the most common symptoms being fever, muscle aches, and vomiting. Diarrhea is another, but less common symptom. If the infection spreads to the nervous system, it can cause meningitis [47].

Listeria innocua is a specie closely related to *Listeria monocytogenes* but, in contrast, is non-pathogenic, and its presence in foods is not considered a hazard to human health [56]. This microorganism shares 2523 orthologous genes with *L. monocytogenes*, representing 88.4% of *L. monocytogenes* protein-coding genes [57].

1.1.2.4*. Staphylococcus aureus*

In 2014, 12 MS reported 393 foodborne outbreaks caused by staphylococcal toxins. This represents 7.5% of all outbreaks, a small increase compared with the reported 386 outbreaks in 2013 [28].

Staphylococcus aureus is a Gram-positive, catalase-positive, facultative anaerobic bacterium of *Staphylococcaceae* family [58]. This pathogen and their toxin has been identified as a potential food safety hazard for meat producers and food processors because it is widespread in the environment

and often detected in air, dust, sewage, water, raw milk, other foods, and on environmental surfaces. It survives to desiccation technology and tolerates high levels of salt [59, 60].

The success of *S. aureus* pathogenicity is due to the large number of virulence factors expressing microcapsules, toxins, and biocide resistance, its adaptability to various environments (e.g., host) and stressors [61].

Staphylococcus aureus is a common cause of hospital and community-acquired infections, and causes skin diseases, osteoarthritis and respiratory tract infections, as well as postoperative and catheter-related infections in hospitals [62].

Their classification thus distinguishes between *S. aureus* coagulase-producing strains, designated as coagulase-positive staphylococci (CPS) and noncoagulase-producing strains, called coagulase-negative staphylococci (CNS). Among CNS, some species are known to play an important role associated to the fermentation microbiota of meat and milk-based products and are therefore considered as food grade [60].

This microorganism produces heat-stable enterotoxins during their exponential phase when their level is at least 5-8 log CFU/g, on a variety of foods, including meat and poultry products, eggs, cream-filled pastries, potatoes, and some salads [59].

Staphylococcal food poisoning (SFP) is one of the most common foodborne diseases in the world and is caused by oral intake of enterotoxins of *Staphylococcus aureus* in food which are produced by enterotoxigenic strains of coagulase-positive staphylococci (CPS), mainly *Staphylococcus aureus* [64], [65]. Numerous staphylococcal enterotoxins have been described and it is the ingestion of these enterotoxins, and not of *S. aureus* cells that causes a rapid onset of nausea, vomiting, abdominal cramps, diarrhea and fever within 1–6 hours after consumption [63]. Less than 200 ng toxin is sufficient to cause disease symptoms [59].

Moreover, to induce SFP, conditions for staphylococci growth and enterotoxin production are needed [60].

1.2. Meat and meat products microbial spoilage prevention and safety

The safety of meat and meat products is of major concern to consumers, processors, retailers, food service industry, government agencies, educational institutions, public health professionals, researchers, and the general public [66].

The microflora composition in meat depends on various factors: (a) preslaughter husbandry practices (b) animal age at slaughtering time, (c) handling during slaughtering and processing, (d) temperature controls during slaughtering, processing and distribution (e) preservation methods, (f) type of packaging and (g) consumer handling and storage [12].

Application of good hygiene procedures and the HACCP principles in the meat industry is useful to prevent contamination of meat, foodborne pathogens and assure the safety of processed meat. The pre-requirements and the HACCP methods are fundamental to attain food safety [67].

Meat products results from the need to preserve meat in ancient times [68]. The essential of all food preserving methods, therefore, is the creation of conditions unfavourable to the growth or survival of

spoilage organisms by, for example, extreme heat or cold, deprivation of water and sometimes oxygen, use of salt or increased acidity [69].

Traditional methods that have been used for thousands of years to prevent meat spoilage and extend shelf life involve drying in wind and sun, salting, smoking and fermentation [70, 71]. Also, canning associated with thermic treatment dates from early in the 19th century allowing food to be stored for many years, since food is sterilised and protected from recontamination [71].

Curing means preserving food through variations and combinations of salting with drying and smoking or with cooking [72]. In the past, when refrigeration was not commonly available, curing was mainly applied to extend the storage life of entire pieces of muscle meat by using the preserving effects of common salt (in high concentrations) and to a lesser extent sodium nitrite. In modern meat processing, this aspect is less important as more efficient meat preservation methods, in particular cooling and freezing, are available. Curing is now mainly applied to achieve a pink-red colour as well as a typical flavour and taste in processed meat products [73].

The new technologies of food preservation include thermal treatment (pasteurization, heating sterilization), pH and water activity reduction (acidification, dehydration), addition of preservatives (organic compounds such as propionate, sorbate, benzoate, lactate, and acetate), refrigeration (chilling and freezing). Also the nonthermal inactivation is emerging with relevance to technologies such as ionization radiation, ultrasound, microfiltration, high hydrostatic pressure, and pulsed electric fields and packaging (vacuum packaging, modified atmosphere packaging, active packaging). The biopreservation and natural antimicrobial compounds such as essential oils, chitosan, lysozyme and bacteriocins has become attractive technologies to be applied on food because concerns of consumers related to the use of chemical additives such as nitrite and sulphite [74,75,76,77]. Among alternative food preservation technologies, particular attention has been paid to biopreservation to extent the shelf-life and to enhance the hygienic quality, minimizing the impact on the nutritional and organoleptic properties of perishable food products [78].

1.3. Biopreservation

Biopreservation can be defined as the extension of shelf-life and food safety by the use of natural or added microbiota and/or their antimicrobial compounds [79]. Natural antimicrobial compounds have been part of biopreservation practices since centuries [8]. Biopreservation raises as an interesting and profitable alternative to others technologies. However, this technology must be combined with different hurdles (refrigeration, vacuum-packing, salting, etc.) in order to be successful [80].

Lactic acid bacteria (LAB) are now commonly in use for the meat biopreservation, due to their ability to inhibit undesirable and spoilage microorganisms [81]. Biopreservation by LAB is a functional way of food preservation and, in addition their taste, smell, structure, nutritional adsorption and content may be improved [79].

The ability to reduce pathogens is due to the production of one or more active metabolites, such as organic acids (lactic, acetic, formic, propionic acids), that intensify their action by reducing the food pH, and also to the presence of other substances with antimicrobial properties, like fatty acids,

acetoin, hydrogen peroxide, diacetyl, bacteriocins (nisin, reuterin, reutericyclin, pediocin, lacticin, enterocin and others) and bacteriocin-like inhibitory substances – BLIS [8].
Biopreservation can be applied in food products by two basic methods: a) adding pure and viable microorganisms; b) adding crude, semi-purified or purified microbial metabolites (bacteriocins) [82].

1.4. Lactic acid bacteria (LAB)

Lactic acid bacteria are a Gram-positive group of bacteria, non-sporulating, nonmotile, catase-negative, with low proportions of G+C content in their DNA (<55%). They colonize a variety of habitats and this ability is a direct consequence of the wide metabolic versatility of this bacterial group [83].
LAB represents a controversial cohort of microbial species that either contribute to generation of offensive metabolites and the subsequent organoleptic downgrading of meat or serve as bioprotective agents with strains of certain species demonstrating reduced spoilage capacities and inhibitory activity against spoilage microbiota [23].
The general basis of lactic acid bacteria classification is the monograph published by Orla-Jensen in 1919 using the following criteria: cellular morphology (rods and cocci), mode of glucose fermentation (homofermentative or heterofermentative), range of growth temperature and sugar utilization patterns. The current taxonomic classification includes the LAB group in the phylum *Firmicutes*, class *Bacilli* and order *Lactobacillales* [84]. Currently, from a taxonomic point of view, in the List of Prokaryotic Names with Standing in Nomenclature, the order *Lactobacillales* is divided into 6 families and 44 genera [31]. Some genera of lactic acid bacteria associated with foods are represented in Figure 4.

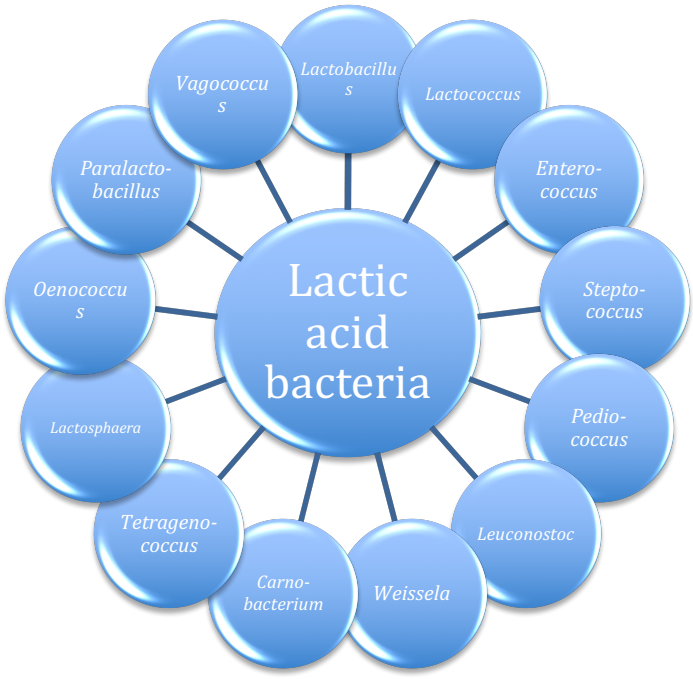

Figure 4 – Lactic acid bacteria genera associated with foods (Original).

LAB are able to grow in a wide range of temperatures, salt concentration, oxygen tension and pH [85]. This anaerobic or facultative aerobic cocci or rods produce lactic acid as one of the main fermentation products from carbohydrates metabolism either through homofermentative or heterofermentative pathway [79],[84],[86]. The metabolism of carbohydrate utilization depends on the kind of the sugar (e.g. hexoses, pentoses) and from the type of fermentation by the LAB [87].

Glucose is the first substrate used by most bacteria in raw meat during cold storage [23]. When glucose is consumed, other substrates such as lactate, gluconate, glucose-6-phosphate, pyruvate, propionate, formate, ethanol, acetate, aminoacids, nucleotides, urea and water-soluble proteins can be used by the majority of meat microbiota [23].

LAB are divided as obligate homofermentative, facultative heterofermentative and obligate heterofermentative [88].

The homofermentative LAB are able to convert hexoses almost exclusively (>85%) to lactic acid via the Embden-Meyerhof-Parnas (EMP) pathway or glycolysis. This type of fermentation includes some species of the genus *Lactobacillus*.

Facultative heterofermentative species ferment hexoses to lactic acid via EMP and are able to degrade pentoses and gluconate via an inducible phosphoketolase, an enzyme of the pentose phosphate (PP) pathway, with a resulting production of acetic acid, ethanol and formic acid under glucose limitation.

Finally, the obligate heterofermentative LAB metabolize pentoses and hexoses exclusively via phosphogluconate pathway (corresponding to the first part of the PP) and produce lactic acid, ethanol (or acetic acid) and CO_2. This type of fermentation includes organisms of the *genera Leuconostoc, Weissella* and *Oenococcus* as well as some species of the genus *Lactobacillus* [87, 88, 89].

The main fermentative microbiota of lactic acid bacteria (LAB) include *Lactobacillus, Pediococcus, Enterococcus, Leuconostoc, Lactococcus* and *Weissella* [90].

Heterofermentative LAB such as *Carnobacterium*, *Leuconostoc* and *Weissella* genera are usually more involved in meat spoilage than the homofermentative *Lactobacillus* and *Pediococcus* genera [91].

LAB produce bacteriocins that display a bactericidal mode of action, which can inhibit pathogenic and spoilage microorganisms usually of closely related species, extending the shelf life and contributing to food safety [92], [93].

Lactobacillus are the largest and heterogeneous genus of LAB with important implications in food fermentation [85]. Several *Lactobacillus* species are essential in fermented food production and are used as starter cultures or food preservatives [94]. The genus *Lactobacillus* was proposed by Beijerinck in 1901 based mainly on morphological and physiological properties [95]. They are Gram-positive, non-spore forming, rods or coccobacilli, catalase negative (even if some strains are able to produce pseudocatalase), microaerophylic and generally characterized by a low GC (guanine and cytosine) content [96]. Optimal growth temperature and pH are usually 20 - 40 ºC and 5.5 - 6.2, respectively [89].

To date (May 2016), the genus *Lactobacillus* is composed of 221 species and 29 subspecies [31]. Lactobacilli are almost ubiquitous growing in a variety of habitats, wherever high levels of soluble

carbohydrate, protein breakdown products, vitamins, and a low oxygen tension occur [97]. Different species having adapted themselves to grow under widely different environmental conditions and their production of high levels of lactic acid lowers the pH of the substrate and supresses the growth of many other bacteria [97, 98].

The majority of species were isolated from human and animal intestinal tracts and faeces but the second largest number of *Lactobacillus* species was isolated from vegetables and their associated fermentation products [94]. *Enterococci*, which belong to lactic acid bacteria (LAB) have become more recognized as emerging human pathogens [99]. Enterococcus species are Gram-positive, facultative anaerobic cocci, non-spore-forming and non-motile bacteria that occur singly, in pairs or in chains [100]. They show catalase negative and oxidase negative tests. The optimum temperature for the growth of enterococci is 35ºC while they can also grow in a wide range of temperatures, from 10 to 45ºC [101].

Enterococci are common inhabitants of the gastrointestinal tract of humans and other animals and are thus considered as indicator bacteria for faecal pollution of water and foods, but they have also emerged as significant cause of serious infection such as endocarditis, urinary and blood stream infections, intra-abdominal end intra-pelvic abscesses [102, 103].

Among *enterococci, Enterococcus faecalis* and *Enterococcus faecium* are responsible for the majority of infections in the most frequent causes of intrahospital infections particularly because of increasing resistance to a wide range of antibiotics [103]. *Enterococci* are intrinsically resistant to several antibiotics and also accumulate mutations and exogenous genes that confer additional resistance [102].

Certain species of genus *Enterococcus* (e.g., *Enterococcus viikkiensis, Enterococcus hermanniensis*) were found in spoiled meat [23]. The presence of *enterococci* in foods is highly controversial. While some authors consider them to be undesirable, indicators of fecal contamination, and responsible for the spoilage of meat products they are also producers of toxic substances such as biogenic amines and cause sausage modifications; others report their important role in flavour development and bioprotection [104]. Various studies have commented on the benefits of using *Enterococcus,* particularly *Enterococcus faecium* strains, as adjunct cultures in fermented foods, because of their ability to inhibit the growth of food-borne pathogens, commonly present in these kinds of products [105, 106,107].

1.4.1. Applications of Lactic acid bacteria

The beneficial role of lactic acid bacteria (LAB) and their safety in food fermentation have been well documented [108] as well their application by pharmaceutical and chemical industry (Figure 5). In food industry, Lactic acid bacteria are mostly used as starter cultures and antimicrobial agents where in pharmaceutical industry they are widely used as probiotic cultures.

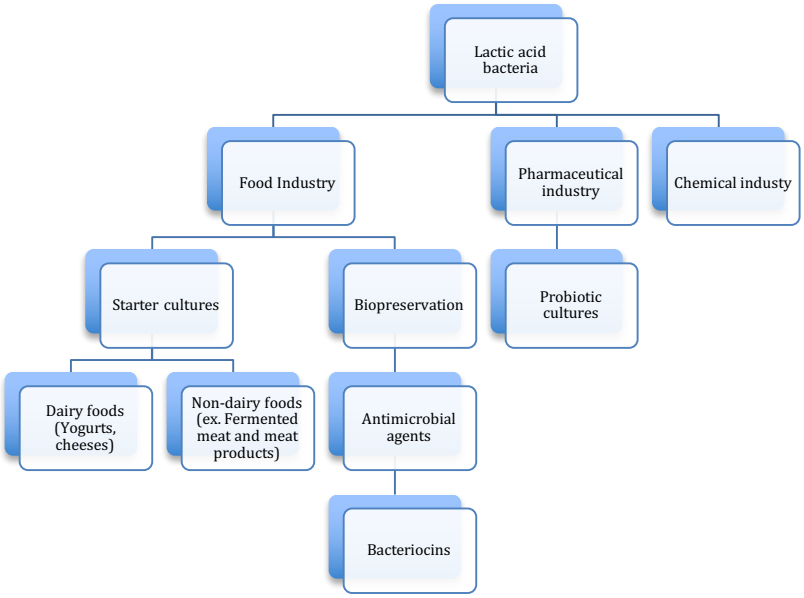

Figure 5 - Applications of lactic acid bacteria (Original)

1.4.1.1 Lactic acid bacteria as source of starter-cultures

A starter culture can be defined as a microbial preparation of a large number of one or more microorganisms, which are introduced to a raw material aiming to produce a fermented food by accelerating and steering its fermentation process [109].

LAB have a long history of safe use in fermented food production and consumption that support their GRAS (generally recognized as safe) and QPS (qualified presumption of safety) status provided by FDA (US Food and Drug administration) and EFSA (European Food Safety Authority), respectively [90]. They have been considered to be safe due to their occurrence as normal commensals of the mammalian microflora and their established safe use in a diversity of foods and health supplement products worldwide [91].

LAB are used as 'natural' or 'selected' starters in food fermentations, they improve nutritional, organoleptic, technological and shelf-life characteristics performing both acidification, due to the production of lactic and acetic acids, flavour-compound production, as well as protection of the food from spoilage and pathogenic microorganisms by producing organic acids, hydrogen peroxide, diacetyl, antifungal compounds such as fatty acids or phenyllactic acid and/or bacteriocins [109, 110].

In general, the choice of starter cultures is fundamental to guarantee the quality and safety of the final products. Ideally, each strain that is intended to be used as a starter culture should be tested individually before any use in food or medicine, should discard the presence of antibiotic resistance genes to avoid their transmission to commensal or pathogenic bacteria, must not have a single virulence factor and should be unable to produce biogenic amines (BA) [90, 111, 112].

1.4.1.1.1 Lactic acid bacteria used as starter cultures in fermented dairy foods

In the dairy industry, starters are used primarily to ferment lactose, but other LAB are deliberately added to milk to produce flavour components (such as diacetyl) or carbon dioxide [113]. Some of LAB used as starter cultures in fermented dairy foods are represented in Table 1. Certain LAB, such *Lb. delbrueckii subsp. bulgaricus* and *S. thermophilus*, are introduced in the milk to initiate lactose fermentation and give a lactate production with pH decline and casein gelation producing the known dairy product – Yogurt [115].These species are also able to produce vitamins such as folate. A controlled use of these bacteria may lead to dairy products with increased folate content [115].

Table 1 - Starter cultures in fermented dairy foods, adapted from Hati *et al*., 2013 [114].

Product	Microorganisms added
Yoghurt	*Lactobacillus delbrueckii ssp. bulgaricus, Streptococcus thermophilus*
Butter and buttermilk	*Lactococcus lactis ssp. lactis, Lactococcus lactis ssp. lactis var. diacetylactis, L. lactis ssp. cremoris, Leuconostoc mesenteroides ssp.cremoris*
Swiss and Italian type cheeses	*Lactobacillus delbrueckii ssp. lactis, L. helveticus, L. casei, L. delbrueckii ssp. bulgaricus, Streptococcus thermophilus*

1.4.1.1.2 Lactic acid bacteria used as starter cultures in fermented non-dairy foods

The use of starter cultures in meat products dates back to the 1940s in the United States, when they were first used to govern and accelerate fermentation. They are homofermentative lactic acid bacteria, i.e., the main end product of carbohydrate fermentation is lactate [116].

The predominant species in dry-fermented sausages are *Lactobacillus sakei*, *Lactobacillus curvatus*, *Lactobacillus plantarum*, *Leuconostoc mesenteroideus*, *Pediococcus* spp., *Enterococcus* spp., which growth is modulated and adapted to the existing stringent conditions of processing [90]. In Table 2 are represented some examples of microorganisms added as starter cultures to fermented food products. Generally, *Lactobacillus sakei* and *Lactobacillus curvatus* are used as main acidifiers for the production of European-style fermented sausage, whereas US-style fermented sausages usually rely on pediococci (Table 2).

Table 2 - Starter cultures in fermented products, adapted from Leroy & De Vuyst, 2004 [115].

Product	Microorganisms added
Fermented sausage	*Lactobacillus sakei, Lactobacillus curvatus*

(Europe)	
Fermented sausage (USA)	*Pediococcus acidilactici, Pediococcus pentosaceus*
Fermented vegetables	*Pediococcus acidilactici, Pediococcus pentosaceus, Lactobacillus plantarum, Lactobacillus fermentum*
Pickles	*Leuc. mesenteroides, P. cerevisiae, Lb. brevis, Lb. plantarum*

1.4.1.2 Bacteriocins produced by lactic acid bacteria

Bacteriocins defined as extracellularly produced primary or modified peptidic products of bacterial ribosomal synthesis, which can have a relatively narrow spectrum of bactericidal activity [117]. Antimicrobial substances that were not well characterized, are known as bacteriocin-like inhibitory substances (BLIS) [118].

Bacteriocins are antimicrobial peptides produced by bacteria that can kill or inhibit bacterial strains closely-related or non-related to produced bacteria [119]. They differ from most therapeutic antibiotics, being proteinaceous agents rapidly digested by proteases in the human digestive tract [120], which makes them safe for human consumption [76].

Based on the biochemical and genetic properties, bacteriocins were grouped into four classes [121]. Table 3 shows the various classes of bacteriocins produced by lactic acid bacteria.

Table 3 - Classification of bacteriocins from lactic acid bacteria, adapted from Rizzello *et al*., 2014 [121]

Class	**Subclass**	**Description**
I		Large group known as lantibiotics. They are small (3 to 10 kDa), heat-stable, cationic, amphilic and membrane-active peptides. Contain the unusual amino acids lanthionine and methyllanthionine
II	IIa IIb IIc	The non-lantibiotics. They are a large group of small (<10 kDa), heat stable peptides with anti-listerial effects defined by their non-modified nature. This class was divided into 3 subgroups based on structural and functional characteristics.
III		Unmodified bacteriocins with high molecular mass (>30 kDa) and heat sensitive.
IV		Complex peptides that contain lipid or carbohydrate moieties, which are essential for activity

Bacteriocins have been found in all major lineages of Bacteria and some members of the Archaea [86], being produced by different variety of living microorganisms, namely gram-positive and gram-

negative bacteria [122]. Bacteriocins are harmless against its own producers because the secretory bacterial strains have genetically pre-determined genes of immunity [123].

These peptides have a number of positive attributes that have made them especially attractive for various applications. LAB bacteriocins are inherently tolerant to high thermal stress and are known for their activity over a wide pH range. These antimicrobial peptides are also colourless, odourless, and tasteless, which further enhance their potential usefulness [124].

Several bacteriocins associated with lactic acid bacteria have been reported, and some have been extensively characterized by many researchers but the most widely known are nisin, plantaricin, lacticin and pediocin [76],[125],[126].

The history of bacteriocins extends to the early 1920s but they were not used in food products until 1951. In the 1960s, the first bacteriocin, called nisin, which is produced by *Lactococcus lactis* subsp. *lactis*, was purified and recognized as a food preservative by FAO/WHO in 1969. In 1988, the FDA approved the use of nisin as an additive in canned products in the United States to inhibit the growth of *Clostridium botulinum* [86]. Although many strains of lactic acid bacteria produce bacteriocins, only nisin, produced by *Lactococcus lactis* subsp. *lactis*, has GRAS (generally recognized as safe) status and remains the only commercially important bacteriocin being approved as a food preservative in over 40 countries [127]. The mode of action of nisin is through disruption of membrane function instigated by formation of pores in the bacterial cell membrane followed by leakage of the cellular material [128].

Nowadays, bacteriocins have been widely utilised especially in the field of food preservation [76]. Table 4 resumed previous studies where various LAB-produced bacteriocins have been applied as biopreservative agents and have been shown to be effective in the control of pathogenic and spoilage microorganisms.

These substances can be applied in food by several possible strategies: i) inoculation of the food with LAB as starter or protective cultures that produce the bacteriocin in the product (production in situ); ii) addition of the purified or semi purified bacteriocin as a food preservative, and iii) use of a product previously fermented with a bacteriocin-producing strain as an ingredient in food formulation [126].

Sant'Anna and co-author's [129] evaluated the antibacterial activity of bacteriocin-like substance P34 against *Listeria monocytogenes* in fresh chicken sausage and observed that BLIS P34 was a high inhibitor of *this pathogen*

In another study Acuña *et al.* [130], used recombinant PCR techniques integrating enterocin CRL35 and microcin V genes to obtain a bacteriocin called Ent35-MccV and evaluated the inhibitory effect of this hybrid bacteriocin on *Escherichia coli* and *Listeria monocytogenes* growth in a meat model. Ent35-MccV had a potent antimicrobial activity against various strains of *L. monocytogenes* and *E. coli*

Application of bacteriocins in food preservation may be beneficial in several aspects: (1) to decrease the risks of food poisoning, (2) to decrease cross-contamination in the food chain, (3) to improve the shelf life of food products, (4) to protect food during temperature-abuse episodes, (5) to decrease economic losses due to food spoilage, (6) to reduce the levels of added chemical preservatives, (7) to reduce the intensity of physical treatments, thereby achieving a better preservation of the food

nutritional value and possibly decrease processing cost (8), possibly to provide alternative preservation barriers for “novel food” and possibly satisfy the demands of consumers for foods with fresh-tasting, lightly preserved, and ready to eat [131].

Table 4 - Applications of some bacteriocins and bacteriocin-like inhibitory substances

Bacteriocin/BLIS	Producing strain	Potential use	References
Pediocin from *P. acidilactici*	*Pediococcus acidilactici*	Control of *Listeria monocytogenes* in fresh meat	Nielsen *et al.,* 1990 [132]
BLIS from *C. piscicola* L103	*Carnobacterium piscicola L103*	Control of *Listeria monocytogenes* in vacuum-packaged meat	Schöbitz *et al.,* 1999 [133]
Leucocins 4010	*Leuconostoc carnosum 4010*	Protective culture cold-stored, cooked, sliced, and vacuum-packed meat products	Budde *et al.,* 2003 [134]
Nisin	*Lactococcus lactis* subsp. *lactis* strain (*L. lactis* 69)	Preservation of salted meat products	*Biscola et al.,* 2013 [135]
Bacteriocin-like substance P34	*Bacillus* sp. strain P34	Control of *Listeria monocytogenes* in fresh chicken sausage	Sant'Anna *et al.,* 2013 [129]
Bacteriocin-like substance (CBLS)	*Bacillus cereus P9*	Meat and vegetable food biopreservation	Fangio & Fritz, 2014 [136]
Hybrid bacteriocin Ent35-MccV	*Enterococcus mundtii CRL35* *E. coli*	Meat model	Acuña *et al.,* 2015 [130]

The disadvantage of many LAB bacteriocins is that although they are active against Gram-positive microorganisms they are not effective against Gram-negative foodborne pathogens, such as *E. coli* or *Salmonella spp*., potential hazards in meat products [137]. Gram-negative bacteria are not affected due to the presence of outer barrier on their cells that prevent molecules of antibiotics, detergents and dyes to reach the membrane. However, some studies reported the effect of bacteriocin on gram-negative bacteria such as bacteriocin from *Lactobacillus plantarum* against *Salmonella typhimurium* [122].

Bacteriocins produced by *Enterococcus* strains were called enterocins [138]. Enterococcal bacteriocins were characterized as substances with strong antimicrobial activity against *L. monocytogenes* [128].

Enterocin AS-48, produced by *Enterococcus faecalis* A-48-32 and *Enterococcus faecium* S-32-81, has shown inhibitory effect against *Listeria monocytogenes* CECT 4032 in sausage indicating the possibility of application in sausages [139].Bacteriocins have also interest as therapeutics for medicine and veterinary [93]. In medicine since these peptides were produced by non-pathogen bacteria normally colonising the human body can easily be used with therapeutic uses [140]. They have shown cytotoxic effects against cancer cells, being suggested for cancer treatment in many studies [141], [142].

1.4.1.3 Bacteriocin regulation by quorum sensing

Bacterial communication via extracellular diffusible signalling molecules (Quorum sensing) allows populations of bacteria to synchronize group behaviour and facilitate the coordination of multicellular functionality [143].

Among lactic acid bacteria, several studies had established that most of bacteriocins synthesis is regulated by quorum sensing (QS) mechanism [144],[121]. Quorum sensing bacteriocin production has been identified in members of two of the biggest classes of LAB bacteriocins, class I (lantibiotics) and class II (pediocin-like) bacteriocins [145].

The regulation mechanism involved in the production of bacteriocins generally requires an inducer peptide and a two-component signal transduction system [146]. This consists of a membrane-located histidine protein kinase (HPK) monitoring one or more environmental factors and, a cytoplasmic response regulator (RR), which modulate the expression of specific genes [147].

Many LAB produced antimicrobial peptides (AMPs) frequently serving as signalling agents, often referred as auto inducers (AI) [148]. In the case of Lantibiotics, the bacteriocin itself acts as the inducing factor, whereas in the case of linear bacteriocin production (class II) other bacteriocin-like peptides were inducers of several genes expression [144]. These signals were produced while the bacterial population grows. When the concentration of the peptide (signals) reaches a certain level (quorum), it was sensed by the extracellular part of the histidine protein kinase becoming phosphorylated [149]. This phosphoryl group will be transferred to the response regulator causing the binding to specific promoters that will be activated [145].

Chapter 2

Objectives. Materials and methods

Justification and objectives

LAB are safe to consume and for that reason, have a major potential for use in biopreservation of foods. These microorganisms are commonly used in food fermentation and produce various metabolites like bacteriocins that can be applied as natural preservatives to improve food safety.

Cured and fermented meat products are commonly associated with human cases of listeriosis because are generally consumed without cooking. LAB are involved in the stability and safety of Portuguese fermented meat products consequent to fermentation. Their protective effect is influenced by several factors such as pH and the food matrix hence the importance to study the direct application of bacteriocins in meat since only thus it can be demonstrated the potential of bacteriocin producing strains.

This assay focus on the use of lactic acid bacteria as bioprotective cultures to inhibit pathogenic microbiota. This dissertation is divided in two parts. The first includes the isolation of lactic acid bacteria (LAB) from poultry caecum collected at a slaughterhouse and categorization as *Lactobacillus* genus. Then it was evaluated the bacteriocinogenic potential of the identified *Lactobacillus* collection and other from different origin (FMV collection) against different pathogens and deteriorative microorganisms that can be found in meat products.

In the second part it was made a partial purification of an enterocin (Bacteriocin Like Inhibitory Substance, BLIS) produced by *Enterococcus faecium* EK13 and was evaluated the effect on *L. innocua,* spoilage and fermentative microbiota growth in a meat model mimicking different fermentative conditions.

Materials and methods

2.1. Sampling and isolation of LAB

Sampling at a poultry slaughterhouse was carried out on two different days of work, 10^{th} february and 9^{th} march of 2015. A pool sample constituted by five poultry cecum collected randomly from different poultry flocks (n=12) were collected in a slaughterhouse in different working days. The sampling was performed after evisceration and before the rapid cooling of poultry carcasses.

Intestines were removed from the carcasses with aseptic requirements, collected in sterile bags and transported in isothermic box until delivery to the laboratory.

Once in laboratory, ceca were aseptically dissected and the faecal content with mucosa was scratched and roughly homogenized. The cecal content was used for bacterial isolation. Lactic acid bacteria isolates were obtained by a direct inoculation of cecal material onto MRS (Man, Rogosa and Sharpe) agar and incubated anaerobically (using GENbox anaer, bioMérieux, France) at 30ºC for 48h. The presumptive characteristic colonies were isolated by re-streaking onto MRS agar 3 times.

A total of 144 presumptive LAB isolates were collected from the two moments of sampling.

2.2. Identification of LAB isolates from the slaughterhouse

2.2.1. Phenotypic selection of LAB isolates

Representative colonies were presumed to be LAB by cell morphology on MRS (deMan, Rogosa, Sharpe) agar. The isolates obtained were characterized morphologically by microscopic observation after Gram staining and the catalase activity was also evaluated.

All isolates rods or cocci, Gram positive and catase negative were selected. Isolates were preserved in cryotubes with Brain Heart Infusion (BHI) broth medium containing 15% (v/v) glycerol. The vials were stored at -80°C (ThermoFisher Scientific, USA).

2.2.2. Molecular Identification of LAB isolates

2.2.2.1 DNA extraction

DNA extraction and the Total genomic DNA was extracted according to the Guanidine thiocyanate method described from Pitcher *et al*., (1989) [150], which was adapted for Gram-positive bacteria by a pre-treatment with lysozyme (10 mg/µl lysozyme in TE buffer) and incubation for 40 minutes at 37ºC.

Quantification of extracted DNA was performed spectrophotometrically (NANODROP 2000c Spectrophotometer, Thermo Scientific). The DNA was stored at -80ºC until use.

2.2.2. Lactobacili identification by amplification of 16S rDNA region by Polymerase Chain Reaction

To determine the genus of the LAB isolates was performed a PCR (Polymerase Chain Reaction) reaction with the use of primers described on Table 5, according to Dubernet, Desmasures and Guéden, (2002) [151].

Table 5 - Primer sets used for Lactobacillus

Primer	Sequence	Base pair (bp)
Lb MA1- rev	5'–CTCAAAACTAAACAAAGTTTC–3'	250
R16-1	5'–CTTGTACACACCGCCCGTCA–3'	

The reaction mixture for one 25 µl PCR reaction consisted of 1x Reaction Buffer, 2.0 mM MgCl2 (Nzytech, Portugal), 800 µM of dNTP's mix (dNTP set 100mM Nzytech, Portugal), 0.13 µM of each primer e 0.2 U/ µl de Taq Polimerase (NzyTaq DNA Polymerase).
Amplification was performed using a VWR Dopio thermal cycler (VWR, Belgium) with redenaturation at 95ºC for 5 min, followed by 30 cycles of denaturation at 95ºC for 30 s, annealing at 55ºC for 30 s and extension at 72ºC for 30s, with a 7 min final extension step at 72ºC, being obtained a products of reaction with approximately 250 bp.
The positive control used was the strain *L. sakei ATCC 15323*.
PCR products were analysed on 1.5% agarose gel electrophoresis stained with gel red 20x (Biotarget, Biotium). Gels were visualised under UV Light (ImageMaster VDS, Pharmacia Biotech). A molecular mass marker 1.5 kb (NZYDNA Ladder VI) was used to compare the size of PCR amplified fragments.

2.3. Lactobacillus from FMV collection

Lactobacillus from FMV collection isolated from fermented meat products and processing environment (n=15, Table 6) were used in this study. *Lactobacillus species* have been previously identified by PCR methods based on the methodologies described by Berthier and Ehrlich (1998) [152].

Table 6 - Lactobacillus strains from FMV collection tested

Codification	Genus	Specie	Origin
P05-15	*Lactobacillus*	*plantarum*	Chouriço

P3B7	*Lactobacillus*	*plantarum*	Paio
P2B2	*Lactobacillus*	*plantarum*	Paio
1L2-5	*Lactobacillus*	*plantarum*	Linguiça
P05-34	*Lactobacillus*	*plantarum*	Chouriço
P05-67	*Lactobacillus*	*plantarum*	Chouriço
P3B8	*Lactobacillus*	*plantarum*	Paio
S3M3	*Lactobacillus*	*plantarum*	Wall of stuffing room
L1B8	*Lactobacillus*	*plantarum*	Linguiça
CV3C2	*Lactobacillus*	*sakei*	Chouriço de vinho
L3B8	*Lactobacillus*	*sakei*	Linguiça
CV2C6	*Lactobacillus*	*sakei*	Chouriço de vinho
CV3C7	*Lactobacillus*	*sakei*	Chouriço de vinho
CV3C8	*Lactobacillus*	*sakei*	Chouriço de vinho
CV2C2	*Lactobacillus*	*sakei*	Chouriço de vinho

2.3.1. *Enterococcus faecium* (EK13)

The strain *Enterococcus faecium* EK13 (Enterocin A and P producer) was isolated from cattle dung water (Marekova *et al.,* 2003) [153] and kindly provided by Dra. Andrea Laukova of the Institute of Animal Physiology, Slovak Academy of Sciences, Laboratory of Animal Microbiology, Slovakia.

2.4. Indicator bacteria collection

Salmonella enteritidis CECT 4300, Staphylococcus aureus subsp. aureus ATCC 25923, Listeria monocytogenes CECT 934, Listeria innocua CECT 910 and *Campylobacter* spp. isolates (n = 7) (Table 7) used in the present study belong to the collection of the Laboratório de Tecnologia e Segurança dos Alimentos at the Faculdade de Medicina Veterinária da Universidade Técnica de Lisboa.

The sensitive to bacteriocins strain *Enterococcus avium* (EA5) was kindly provided by Dr. Andreia Laukova of the Institute of Animal Physiology, Slovak Academy of Sciences, Laboratory of Animal Microbiology, Slovakia.

The strain *C. coli* ZIM 140 was kindly provided by Sonja Možina from the University of Ljubljana, Ljubljana, Slovenia.

Table 7 - Indicator bacteria collection for testing and their growth conditions

Codification	Genus	Species	Origin	Atmosphere	Temperature of incubation	Incubation time
ATCC 11168	*Campylobacter*	*jejuni*	American Type Culture Collection (ATCC) isolated from human, faeces	microaerophilic	42°C	48h
118.09	*Campylobacter*	*jejuni*	Human faeces	microaerophilic	42°C	48h
P101 VI CFAI	*Campylobacter*	*coli*	Poultry breast	microaerophilic	42°C	48h
J8.5	*Campylobacter*	*jejuni*	Poultry legs marinated	microaerophilic	42°C	48h
C5.5	*Campylobacter*	*jejuni*	Poultry stogonoff	microaerophilic	42°C	48h
Zim 140	*Campylobacter*	*coli*	Poultry	microaerophilic	42°C	48h
P93 VI CFAI	*Campylobacter*	*coli*	Poultry breast	microaerophilic	42°C	48h
CECT 4300	*Salmonella*	*enteritidis*	Spanish Type Culture Collection	aerobic	37°C	24h
EA5	*Enterococcus*	*avium*	Isolated from faeces of piglet	anaerobic	30°C	24h
ATCC 25923	*Staphylococcus*	*aureus*	American Type Culture Collection (ATCC); Clinical isolate	aerobic	37°C	24h
CECT 934	*Listeria*	*monocytogenes*	Spanish Type Culture Collection; Isolated from brain of sheep with circling disease	aerobic	37°C	24h
CECT 910	*Listeria*	*innocua*	Spanish Type Culture Collection; Isolated from cow brain	aerobic	37°C	24h

2.5. Storage and culture of bacterial strains

All isolates used in this study were maintained as stock cultures at -80°C (ThermoFisher Scientific, USA) in Brain Heart Infusion broth (BHI Broth, Scharlau, Spain) containing 15% (v/v) glycerol.
LAB isolates and the indicator strains were streaked and re-streaked on an appropriate culture medium at frequent intervals of time.
When the culture was required, the vial was removed from the freezer and an inoculation loop was used to transfer approximately 10 µl of the content of the vial to BHI broth or MRS (Man, Rogosa and Sharpe, Scharlau, Spain) broth at 30 °C for 24 to 48 hours anaerobically.
LAB isolates were cultured in MRS agar at 30 °C for 24h.
Salmonella enteritidis CECT 4300, Staphylococcus aureus subsp. aureus ATCC 25923, Listeria monocytogenes CECT 934, Listeria innocua CECT 910 were cultured in Trypticase soy agar (TSA, Scharlau, Spain) at 37°C for 24h.
Camplylobacter spp. isolates were cultured in Columbia Blood agar (COS, Scharlau, Spain) with 5% of horse blood (BioMérieux, Inc., France) and incubated in microaerophilic atmosphere at 42 °C for 48h.

2.6. Screening Lactic Acid bacteria bacteriocinogenic potential activity: qualitative method

All isolates were screened for bacteriocinogenic potential activity (potential bacteriocin-like substance production) by the qualitative agar-diffusion technique according to Skalka *et al* [154] against *Enteroccocus avium* EA5, *Listeria monocytogenes CECT 934, Staphylococcus aureus ATCC 25923, Salmonella enteritidis CECT 430* and seven isolates of *Campylobacter*.
In this study it was used the strain *Enterococcus avium* (EA5) sensitive to bacteriocins and was used *Enterococcus faecium* EK13 as positive control.
All *Lactobacillus* isolated from samples colected in the slaughterhouse were evaluated regarding their bacteriocinogenic potential against *Enterococcus avium* (EA5) and *Listeria monocytogenes* CECT 934.
The LAB strains (*Lactobacillus* and *Enterococcus*) of the Faculty of Veterinary Medicine collection were studied against indicators: *Listeria monocytogenes* CECT 934, *Staphylococcus aureus* ATCC 25923, *Salmonella enteritidis* CECT 430 and seven isolates of *Campylobacter sp.*

2.6.1. Skalka modified method

LAB strains were inoculated in MRS 1.5% agar (MRS agar, Scharlau Chemie S.A., Spain) and incubated anaerobically at 30ºC for 24h. Figure 6 describe the protocol used for testing bacteriocinogenic potential of isolates under study.

Microorganisms indicators were cultured in a specific medium and incubated at a suitable temperature described previously in Table 7.

Thereafter, several LAB colonies were collected aseptically with a loop and inoculated in duplicate into row in MRS agar plates (MRS agar, Scharlau Chemie SA, Spain) and incubated at 30°C for 24h in anaerobiosis. After this period, the plates with LAB strains were overlaid with 6 ml of BHI soft agar (0.7% agar) inoculated with 300 µl of a culture of the indicator microorganism. The indicator microorganims cultures were at an optical density (OD_{625nm}) with values between 0.8 – 1 which corresponds approximately to 10^7- 10^8 CFU/ml.

The cultured plates were aerobically incubated at 37ºC for 24h. The presence of inhibitory zones was observed and their diameters (surrounding the spotted isolates) were measured. Each assay was performed in duplicate.

Isolates with an inhibition zone bigger than 5 mm were considered to have antimicrobial activity.

The agar diffusion technique was performed on MRS agar plates. For *Campylobacter* sp. was used Columbia Blood agar with 5% of horse blood.

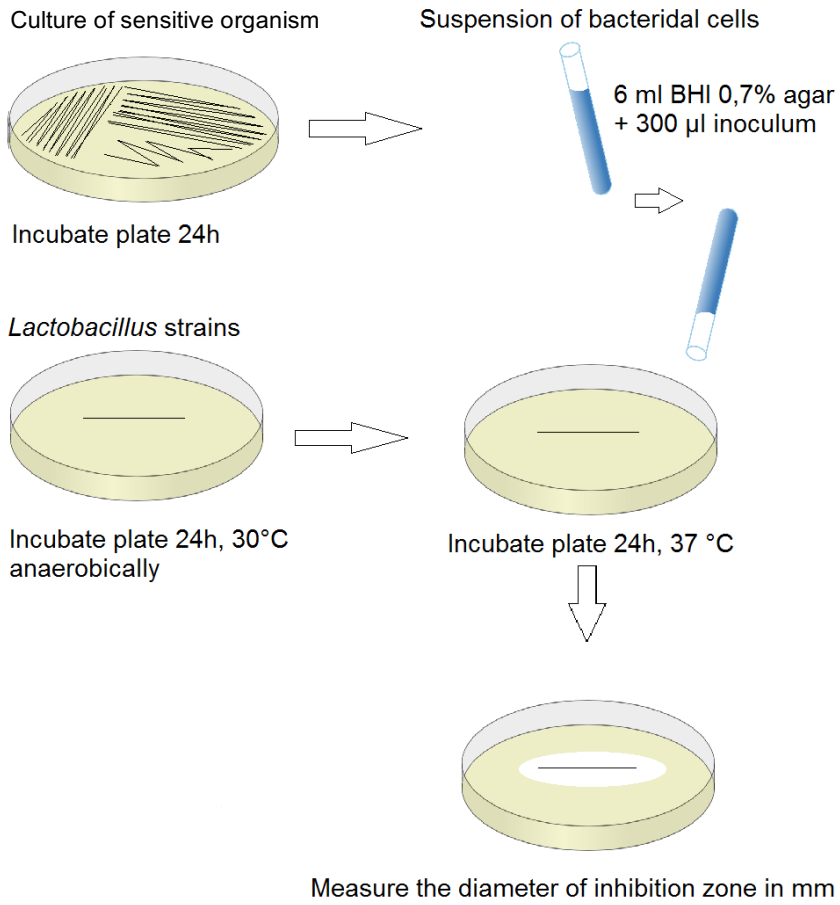

Figure 6 - Diagram procedure for lactic acid bacteria bacteriocinogenic potential activity evaluation (Original).

2.7. Nature evaluation of compounds with potential bacteriocinogenic

In order to verify if the substances produced by *LAB* isolates were of protein nature, a Skalka modified method was used with proteinase K to cleave the peptides produced by bacteria.
Proteinase K is classified as a serine protease and is widely used for proteins digestion. This protease cleaves peptide bonds at the carboxylic sides of aliphatic, aromatic, or hydrophobic amino acids [155]. *Lactobacillus* under study were scratched in line on MRS agar and incubated anaerobically at 30ºC over-night. After this period, the plates with *Lactobacillus* strains were overlaid with 6 ml of BHI soft agar (0.7% agar) inoculated with 300 µl culture of the indicator microorganism (*Enterococcus avium* EA5 and *Listeria monocytogenes CECT 934*). The plates were left to dry for thirty minutes. Then, wells of 5 mm in diameter were made on the agar layer with a sterile plastic straw and filled with 15 µL of proteinase K at a final concentration of 1 mg ml^{-1} in phosphate buffer (pH 7.0). After 24h of incubation in microaerophilic conditions at 30ºC, the inhibition zones were observed (Figure 7).

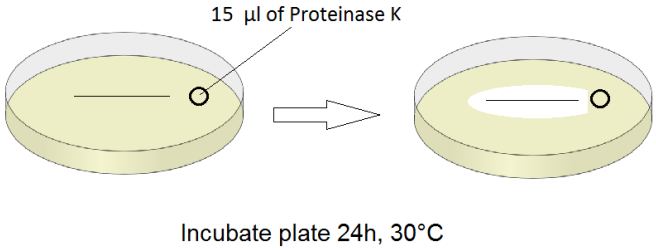

Figure 7 - Diagram of the procedure for the nature evaluation of compounds with bacteriocinogenic potential (Original)

Lactobacillus isolates were selected if they presented zones of inhibition against *E. avium* EA5 higher than 20 mm and against *Listeria monocytogenes CECT 934* higher than 25 mm in modified Skalka method. Thirty-seven strains were selected from the slaughterhouse and fifteen strains of FMV collection. A proteinaceous nature of produced substances can be considered if they were sensitive to Proteinase K enzyme.

2.8 BLIS EK13 production

The production of Bacteriocinogenic-like inhibitory substances (BLIS) from *Enterococcus faecium* EK13 was carried out by microbial culture for 48h at 37ºC in TSA medium. Then, 500 ml of MRS broth (Merck) were inoculated with 0-5ml of a freshly prepared *E. faecium* EK13 culture and incubated for 16h (overnight) at 37ºC until a OD 600 = 1,4. Then the culture of *E. faecium* EK13 in MRS broth (Merck) was centrifuged for 30 min at 10 000 x g in order to remove the cells. The pH of the supernatant was adjusted to 5 and ammonium sulphate was added to the supernatant to obtain 40% (w/v) saturation. This mixture was stirred at 4°C for 2-7h. After centrifugation at 10 000 x g for 30 min,

the resulting pellet (Bacteriocin Like Inhibitory Substances, BLIS) was resuspended in 10 mM phosphate buffer (pH 5.0) and frozen at -20°C (adapted from [153]).
The BLIS obtained was lyophilized (Figure 8) in a ScanVac Freeze Dryers (CoolSafe model, Denmark) and stored at ambient temperature to subsequent work.

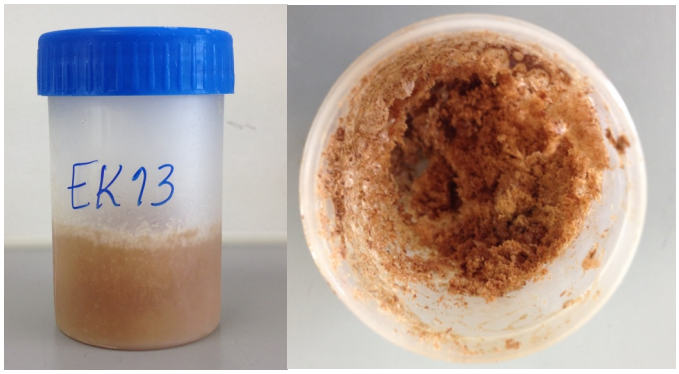

Figure 8 - BLIS from *Enterococcus faecium* EK13 lyophilized

2.9. Bacteriocinogenic activity quantification

The antimicrobial activity of BLIS produced by *Enterococcus faecium* EK13 was tested against seven isolates of *Campylobacter*, *Listeria monocytogenes* CECT 934, *Listeria innocua* CECT 910, *Enterococcus avium* (EA5) and *Salmonella enteritidis* CECT 4300.
The quantification of the bacteriocin activity was assessed using an adaptation of critical dilution method [156,157]. Dilutions of the BLIS were prepared with phosphate buffer (10 mM, pH 7.0), 1:1, 1:2, 1:4, 1:8, 1:16, 1:32, 1:64, 1:128, 1:256 and 10 µL of each dilution was spotted on to the surface of a layer of TSA agar plates previously overlaid with 6 mL of soft BHI agar inoculated with 300 µl of the indicator bacterium.
After incubation, the areas of inhibition were observed. The results are expressed in arbitrary units per mL (AU / ml), which represent the highest dilution of the bacteriocin capable of inhibiting the growth of the strains that are competing. The experiments were performed in duplicate.
For *Enterococcus avium* (EA5) instead of 0.7% BHI agar (BHI agar Scharlau Chemie SA, Spain) was used 0,7% agar MRS medium (MRS agar, Scharlau Chemie SA, Spain) while for *Campylobacter* culture was used Columbia Blood agar with 5% of horse blood.

3. In vitro evaluation of BLIS enterocin EK13 antimicrobial activity with a meat model

A meat fermentation model was prepared to simulate the ecological conditions in maturation and smoking with pork meat aseptically minced.
In this assay it was tested the effect of a partially purified enterocin produced by *Enterococcus faecium* EK13 on the growth of *L. innocua*, total mesophilic and fermentative microbiota in *a* meat model mimicking different fermentative conditions (1st step at 7ºC and 2nd step at 20ºC) during 96 hours.

Assays were performed in two temperatures reproducing the stages of production of sausages: maturation at 7°C and smoking at 20°C. Thus prepared samples were kept for 2 days at a temperature of 7°C, with tests carried out at 0h, 24h and 48 hours and then the temperature was changed to 20°C for another 2 days and the samples were analysed at 72 and 96 hours.

3.1. Preparation of the *Listeria innocua* CECT 910 inocula and growth conditons

Culture was prepared by growing the strain in TSA (Scharlau, Barcelona, Spain) for 24 hours at 37ºC and the optical density was adjusted in NaCl 0.9% to an OD_{625} of 0.5 which matches approximately to 7 $\log^{10}$ CFU/ml.

3.2. Study design

The study was conducted with a meat model under different conditions with and without bacteriocin: 1- control raw meat; 2- raw meat inoculated with *Listeria innocua* 910 CECT; 3- raw meat inoculated with *Listeria innocua* CECT 910 and free enterocin; 4- raw meat with free enterocin. *L. innocua* was tested against two different concentrations (0.1% and 0.5%) of BLIS in meat.
Assays were done in triplicate for each enterocin concentration contemplating the same procedures.

3.3. Meat samples preparation and inoculation procedure

The meat was minced (1x1cm) being twenty-five grams aseptically weighted in sterilized bags (n=5). Each one of the bags corresponds to an analysis time of the test (0, 24, 48, 72 and 96 hours).
In condition 2 and 3 the bag with raw meat was also inoculated with 1 ml of a suspension of *Listeria innocua* CECT 910 at approximately 7 $\log^{10}$ bacteria/ml.
In condition 3 and 4 the bag with raw meat was inoculated with a suspension of free BLIS. The preparation of this suspension in order to obtain a concentration of 0.1% of BLIS in meat was done by weighing 0,275g of BLIS added to 11ml of sterile distilled water (0.025g/ml). The suspension of BLIS to accomplish 0.5% in meat was prepared 0.125g/ml.

3.4. Microbiological analysis

The samples were subjected to microbiological analysis to monitor the dynamic changes in the main microbial groups responsible for ripening of fermented sausages and their hygienic quality. Microbiological analysis was performed 1 hour after inoculation (time 0), 24h, 48h, 72h and 96h, for total aerobic microorganisms at 30ºC, *Listeria spp.* counting, and lactic acid bacteria (LAB) counts according with the methods proposed by ISO (International Organization for Standardization).

3.4.1. Sample preparation for microbial analysis

Each sample (25g) was homogenized with 225 mL of sterile buffered peptone water solution (Scharlau, Barcelona, Spain) in a Stomacher blender (Stomacher Lab-Blender 400) for 2 min (10^{-1}

suspension). Further serial decimal dilutions were made with Tryptone Salt Broth for subsequent microbial enumeration.

3.4.1.1. Total aerobic microorganisms at 30ºC

For the enumeration of total aerobic microorganisms at 30ºC was used pour plate method technique and one mL from suitable dilutions were transferred aseptically into sterile petri dishes.
The total number of aerobic mesophilic microorganisms were determined on Triptone Glucose Agar (TGA agar, Scharlau Chemie S.A, Barcelone, Spain) and incubated at 30ºC for 48h according to ISO 4833 (ISO,2003). In the counting process were considered all colonies present, independently of their morphology. Results were presented as log CFU/g.

3.4.1.2. *Listeria* spp. count

Samples Decimal dilutions (0.1 ml) were plated on ALOA agar (ALOA, bioMérieux, France) surface being spread. Plates were incubated at 37ºC for 48h. All characteristic colonies of *Listeria spp.*, green-blue surrounded with or without opaque halo were counted. Confirmation for *Listeria* spp. was done according to ISO 11290-2: 2002. Results were presented as log CFU/g.

3.4.1.3. Lactic acid bacteria (LAB) count

For the Lactic acid bacteria (LAB) count was used pour plate method. Samples dilutions were cultured on MRS agar (Man Rogosa Sharpe Agar, Scharlau Chemie S.A, Barcelone, Spain) supplemented with (2-3-5 Triphenyl tetrazolium cloride 1% and thallium acetate 5%) under anaerobic conditions (Ref 96124, Genbox anaer, bioMérieux S.A, France) at 30°C for 48h. The presence of characteristic colonies of lactic acid bacteria were counted according to ISO 15214:1998. Results were presented as log CFU/g.

3.5. Statistical Analysis

Potential bacteriocinogenic activity of LAB against pathogenic bacteria were measured and the average of diameter of each inhibition zone (in mm) was calculated with Excel programme (Microsoft Corp., USA).
For data analysis of BLIS EK13 effect was used the Microsoft Excel 2011 program and Statistical Package for Social Sciences (SPSS) software, version 22. BLIS concentrations and time storage effects were evaluated using one-way analysis of variance (ANOVA) and Tukey test. The results were considered significantly different with $P < 0.05$.

Chapter 4

Results

4.1. Identification of *Lactobacillus* isolates

A total of 144 lactic acid bacteria were isolated from poultry ceca samples. All these isolates were Gram-positive, rods, catalase negative rods or cocci and were classified at the genus level based on biochemical tests and morphological properties. Using the 16S – 23S PCR approach all the isolates with a band with approximately 250bp were identified as *Lactobacillus* (94 isolates). On Figure 9 can be observed as example the electrophoretic gel (1.5% agarose) of a PCR amplification for 16S rRNA from isolated Lactobacillus.

Figure 9 - PCR amplification for 16S rRNA from isolated *lactobacillus* on 1.5% agarose gel. 1-12: presumptive isolates under test, M: 1.5 kb DNA marker, positive control: L. Sakei ATCC 15323, negative control

In figure 10 can be observed that from the initial 144 isolates collected from the slaughterhouse, only 65.30% (n=94) were identified as *Lactobacillus*.

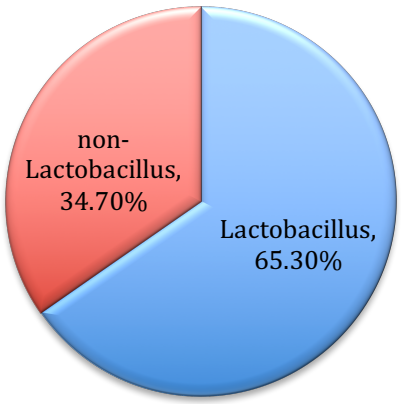

Figure 10 - Relative contribution of identified *Lactobacillus* for the total number of LAB isolates (n= 144)

4.2. Evaluation of *Lactobacillus* bacteriocinogenic potential

4.2.1. Lactobacillus isolates collection from the slaughterhouse

The antimicrobial activity of the isolates was evaluated by measuring the diameter of the inhibition zones (Figure 11). Isolates were selected based on the size of the zone of inhibition.

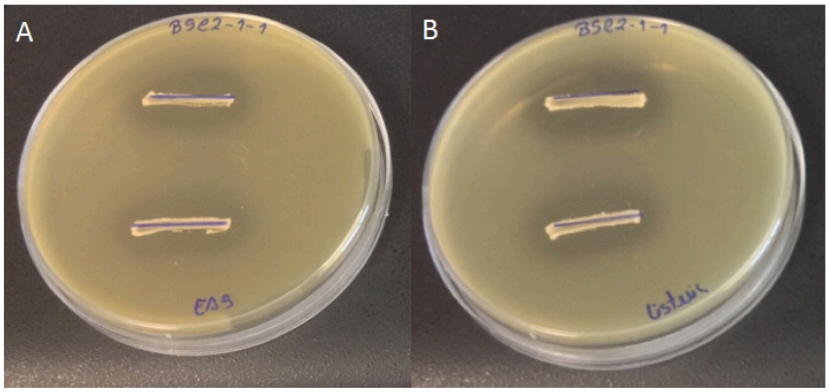

Figure 11 - Inhibition zones of a *Lactobacillus* isolate against *Enterococcus* EA5 (A) and *Listeria monocytogenes* (B)

The *Lactobacillus* isolates from the slaughterhouse were tested for their antagonism against two indicator strains (*Enterococcus avium* EA5 and *Listeria monocytogenes* CECT 934) by the Skalka method.

From the 94 *Lactobacillus* collected in a slaughterhouse was observed that all presented bacteriocinogenic activity against the microorganisms tested (*L. monocytogenes* (CECT 934) and *Enterococcus avium* (EA5).

Figure 12 shows the inhibition halos against *Enterococcus avium* (EA5). The majority of isolates, 5% of *Lactobacillus* presented an inhibition zone >10 and <15mm, 43% presented an inhibition zone >15 and <20mm and 41% presented an inhibition zone of >20 and <25mm and 11% presented an inhibition zone of >25mm,

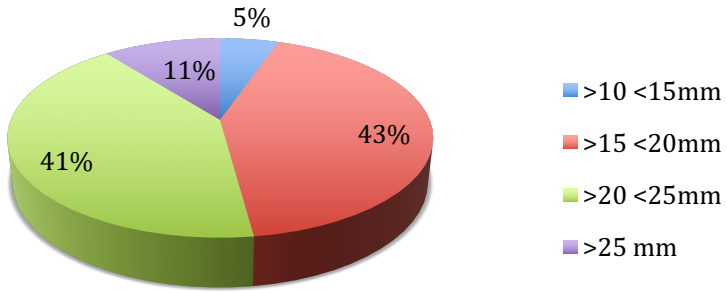

Figure 12 - Average diameters of inhibition zones from *Lactobacillus* against *Enterococcus avium* (EA5)

Lactobacillus isolates (n=94) were tested against *L. monocytogenes* (CECT 934) and 1% presented an inhibition zone of >10 and <15mm of diameter (Figure 13), 3% had an inhibition zone >15 and <20mm, 23% presented an inhibition zone of >20 and <25mm, 46% presented an inhibition zone >25 and <30 mm and 27% of the isolates presented an inhibition zone >30mm.

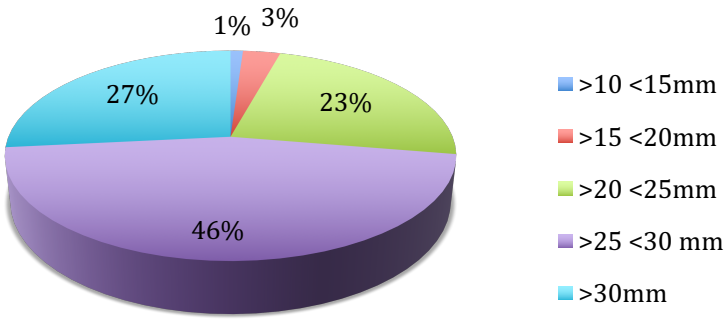

Figure 13 - Average diameters of inhibition zones from *Lactobacillus* against *L. monocytogenes* (CECT 934)

In the Figure 14 are presented only 7 isolates of the total *Lactobacillus* (n=94) evaluated which have shown inhibition zones higher than 30 mm against *L. monocytogenes* CECT 934 and higher than 25mm against *Enterococcus avium* (EA5) (n=34) in modified Skalka method. It can be observed that isolate B6C1-3-3 had the greater potential to inhibit *L. monocytogenes* CECT 934 while isolate B6C2-1-2 presented the higher inhibition zone against *Enterococcus avium* (EA5).

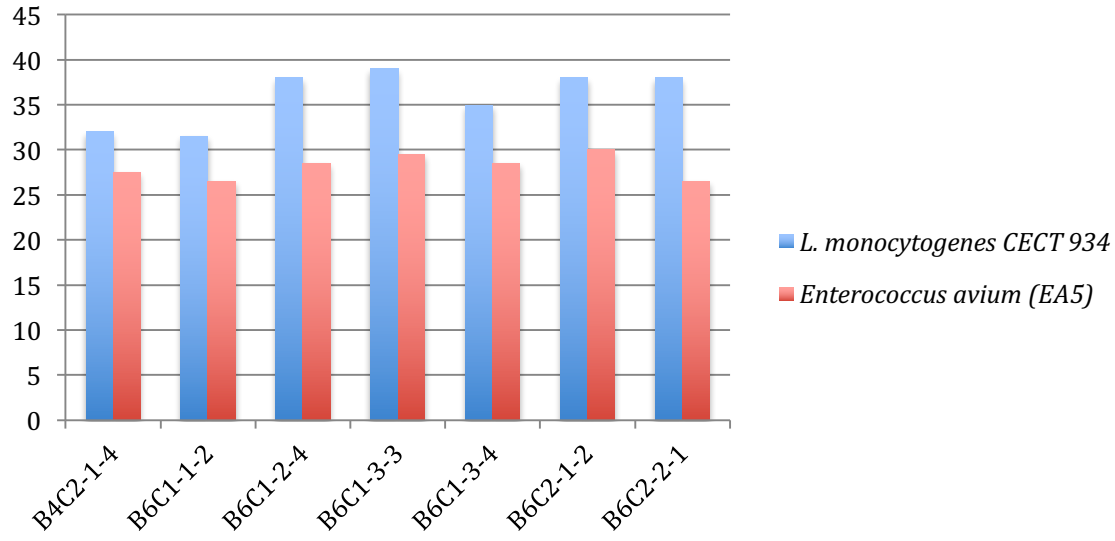

Figure 14 - Average of inhibition size (in mm) of inhibition zones formed by some *Lactobacillus* isolates from the slaughterhouse when cultured in the presence of strains of *Enterococcus avium* (EA5) and *L. monocytogenes* CECT 934

4.2.2. *Lactobacillus* strains from FMV collection and *Enterococcus faecium* EK13

Figure 15 presents the average of inhibition zones (mm) formed by *Lactobacillus* from the FMV collection when cultured in the presence of *Campylobacter* strains (n=7), *Salmonella enteritidis* CECT 4300, *Enterococcus avium* (EA5), *S. aureus* ATCC 25923 and *L. monocytogenes* CECT 934.

In view of the results for bacteriocinogenic potential activity, all strains presented bacteriocinogenic activity against all indicators tested of at least more than 5 mm.

L. sakei CV3C8 exhibited the highest inhibitory activity against all strains of *Campylobacter* tested, *L. plantarum* strains P3B7 and P05-15 showed higher inhibition against *Salmonella enteritidis CECT 4300* while the strain P3B8 presented best results against Enterococcus avium (EA5). P05-67 presented best results against *S. aureus ATCC* 25923 followed by L1B8. Isolate P3B8 also showed the highest halos against *Listeria* followed by P05-15.

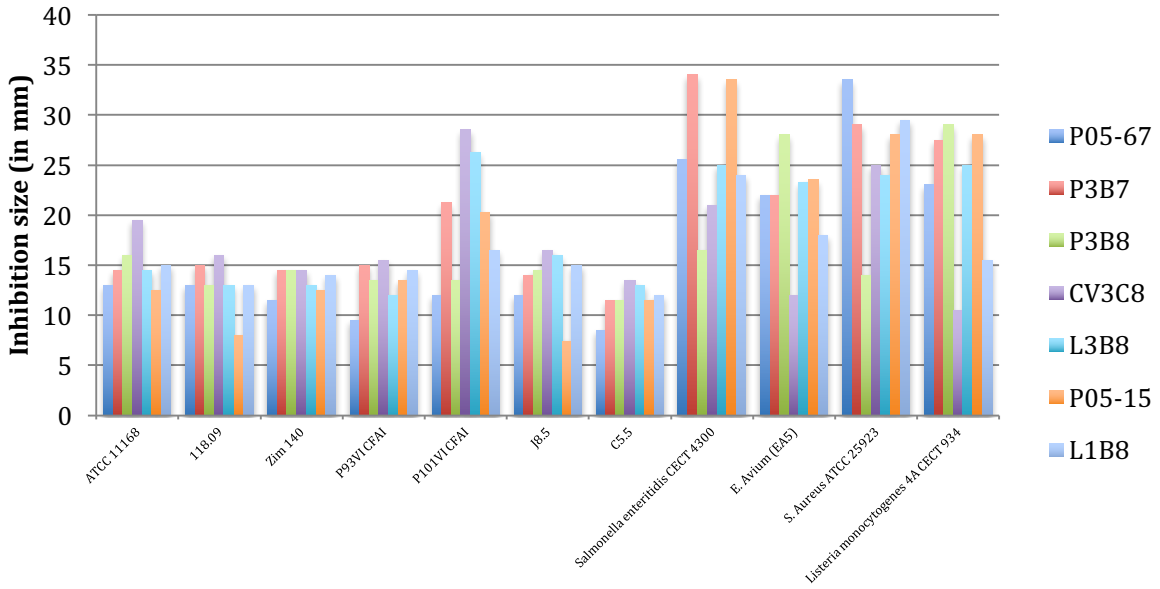

Figure 15 - Average of inhibition size (mm) of inhibition zones formed by *lactobacillus* from the FVM collection when cultured in the presence of strains of *Campylobacter* (n=7), *Salmonella enteritidis* CECT 4300, *Enterococcus avium* (EA5), *S. aureus* ATCC 25923 and *L. monocytogenes* CECT 934.

Figure 16 represents the inhibition zones formed by *Enterococcus faecium* EK13 against all indicators tested. *Enterococcus faecium* EK13 presented bacteriocinogenic activity against all indicators but showed higher inhibition against *Listeria monocytogenes* CECT 934.

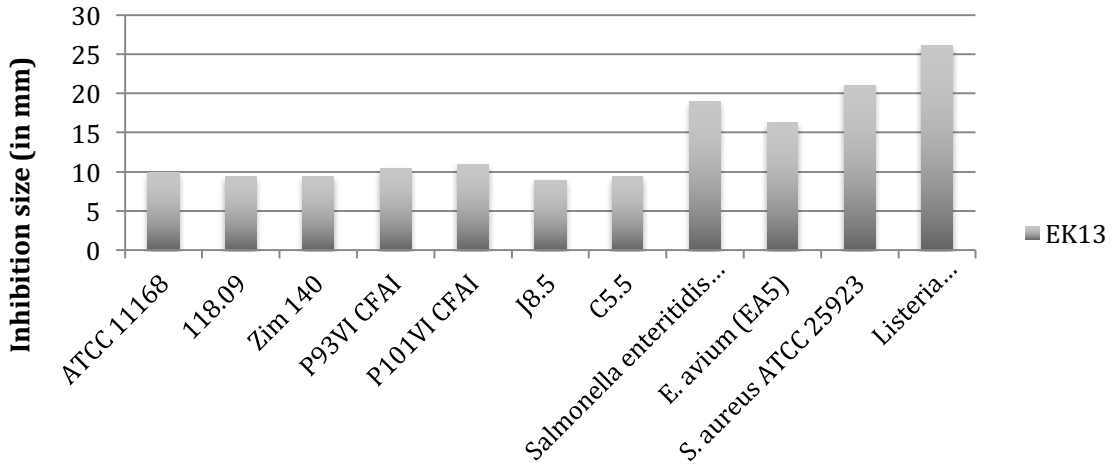

Figure 16 - Average of inhibition size (in mm) of inhibition zones formed by *Enterococcus faecium* EK13 when cultured in the presence of strains of *Campylobacter* (n=7), *Salmonella enteritidis* CECT 4300, *Enterococcus avium* (EA5), *S. aureus* ATCC 25923 and *L. monocytogenes* CECT 934.

Evaluation of compounds with bacteriocinogenic potential

LAB strains from the slaughterhouse that were selected according to their highest bacteriocinogenic potential (n=37) against EA5 (higher than 20 mm) and against Listeria innocua (higher than 25 mm) in modified Skalka method were tested to evaluate the nature of inhibitory compounds. Also, all strains of *Lactobacillus* from the FMV collection were tested (n=15). All LAB studied (both collection from FMV and the ones from slaughterhouse) showed inhibition caused by an organic acid or other non-protein compound.

The figures 17A and 17B shows the halo around the spot where it was placed proteinase K. In figure 17B was not shown a break in the halo around the spot where it was placed proteinase K which indicated that there was no production of bacteriocins and the antimicrobial activity was attributed to the production of organic acids. In the figure 17A it can be seen a break in the halo around the spot where it was placed proteinase K indicating the production of bacteriocins.

Since lactic acid bacteria isolated from both the cecum of chickens and from de FMV collection seems to be not producer of bacteriocin, the *Enterococcus faecium* EK13 was chosen for further study.

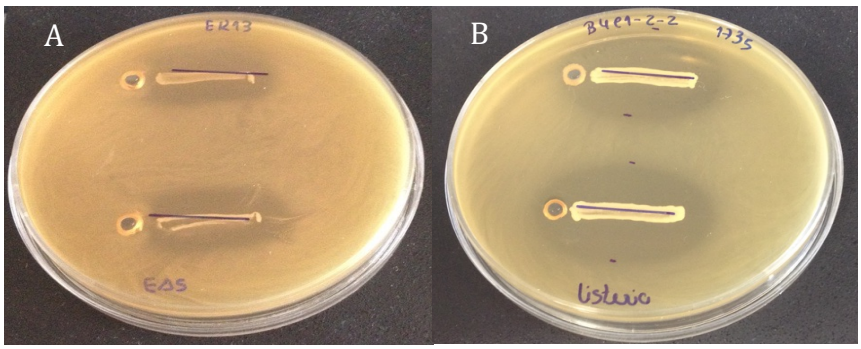

Figure 17 - Nature evaluation of compounds produced by EK13 (A) and *Lactobacillus* (B) isolated from poultry samples in a slaughterhouse

Bacteriocinogenic activity quantification of BLIS EK13

Using the quantitative dilution modified method was verified that BLIS produced by *Enterococcus* EK13 inhibited *Listeria innocua and L. monocytogenes* tested and *Enterococcus avium* EA5 (Table 7) forming a clear zone of inhibition in the place of the inoculation droplets (Figure 18).

Figure 18 - *Enterococcus faecium* EK13 showing inhibition zone for *L. monocytogenes* CECT 934.

From bacteriocinogenic activity quantification was observed that the greater the concentration of bacteriocin substance, the greater the inhibitory capacity.

BLIS EK13 showed no inhibitory capacity against the Gram-negative isolates of *Salmonella* and *Campylobacter* tested. In Table 8 we can see that the maximum activity of this bacteriocin (51200 UA ml^{-1}) was observed against *Listeria innocua* CECT 910.

Table 8 - Inhibitory activity of EK13 BLIS against indicator strains

Strain	**Inhibitory activity (AU/mL)**
Listeria monocytogenes CECT 934	25600 UA/ml
Listeria innocua CECT 910	51200 UA/ml
Enterococcus avium (EA5)	1600 UA/ml
Salmonella enteritidis CECT 4300	No inhibition halo
Campylobacter jejuni ATCC 11168	No inhibition halo
Campylobacter jejuni 118.09	No inhibition halo
Campylobacter coli Zim 140	No inhibition halo
Campylobacter coli P93 VI CFAI	No inhibition halo
Campylobacter jejuni P101 VICFAI	No inhibition halo
Campylobacter jejuni J8.5	No inhibition halo
Campylobacter jejuni C5.5	No inhibition halo

Meat fermentation model

Different temperature conditions and the addition of BLIS EK13 influenced the growth of studied microbiota and *Listeria innocua*.

The evolution of *Listeria innocua* in meat model with 0.1% and 0.5% EK13 BLIS under storage over time (24h, 48h, 72h and 96h) is presented on Figure 19.

The initial counts of *Listeria innocua* on meat at time 0 was reduced in 1 log cfu.g^{-1} under the effect of BLIS addition at 0.1% and 0.5%.

The growth of *Listeria innocua* at 7ºC after 48h of storage was significantly inhibited when 0.1% and 0.5% BLIS was in meat. The addition of free BLIS 0.1% in meat reduced *Listeria innocua* counts from 4 log cfu.g^{-1} to 2.4 log cfu.g^{-1} and, 0.5% BLIS in meat reduced *Listeria innocua* counts from 4 log cfu.g^{-1} to 0.93 log cfu.g^{-1}. The antilisterial activity was higher in meat samples with 0.5% BLIS compared to the 0.1% BLIS.

When the condition of temperature was changed to 20ºC it was notice an increase of *Listeria innocua* counts.

Listeria innocua growth rate in meat under the effect of BLIS 0.1% and 0.5% were the same. However, the final counts obtained on meat with 0.5% and 0.1% of BLIS in this storage conditions presented a difference of approximately 2 log cfu. g^{-1}.

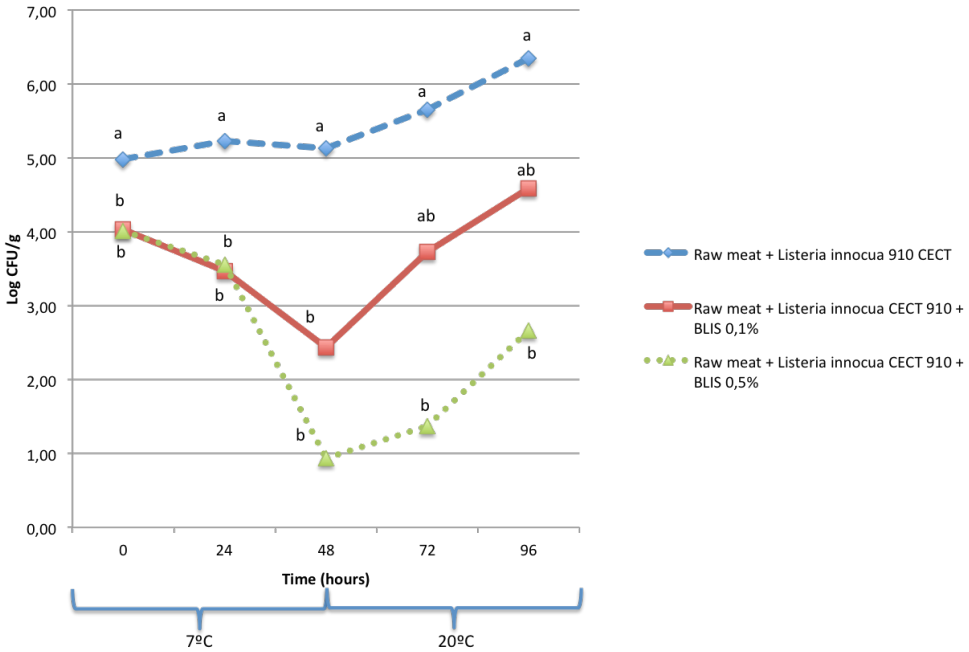

Figure 19 - Influence of EK13 BLIS on *Listeria innocua* counts. ab – means with different letters are significantly different with p<0.05.

Through the results obtained, it was built the graph shown in Figure 20, relative to total aerobic microorganisms at 30ºC counts in samples collected over time of storage. The initial counts of total microorganisms at 30ºC on meat model were between 4-5 log cfu.g^{-1} day 0 even under the effect of

BLIS at 0.1% and 0.5%. The total aerobic microorganisms at 30ºC counts slightly increased at 7ºC until 48h; when the temperature was changed to 20ºC it was noticed an exponential growth of this microbial group. Under the effect of all BLIS conditions tested the microbial counts were not inhibited with approximately 6 log cfu. g^{-1} after 48h of storage at 7ºC, and 9 log cfu. g^{-1} after 96h at 20ºC.

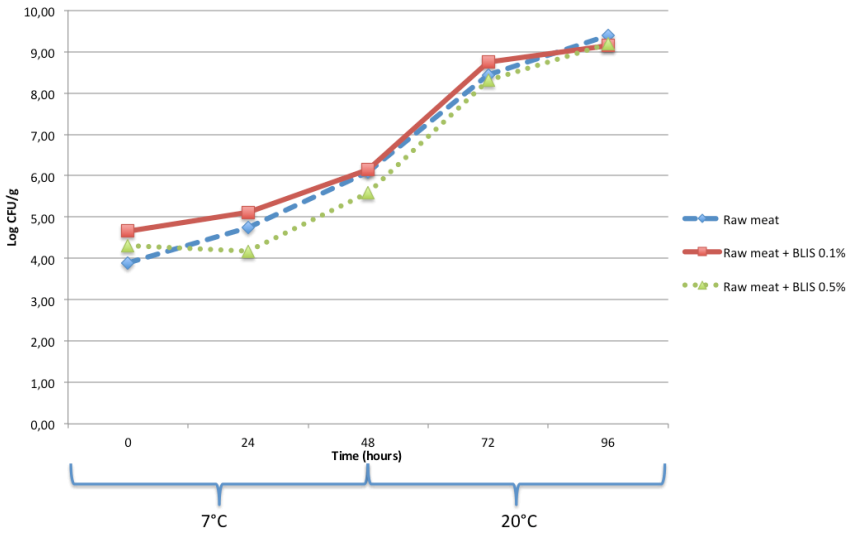

Figure 20 - Influence of EK13 BLIS at 0.1% and 0.5% on total aerobic microorganisms at 30ºC counts.

Figure 21 presents the evolution of Lactic acid bacteria in meat model with 0.1% and 0.5% BLIS EK13 and under storage over time (24h, 48h, 72h and 96h). The initial counts of lactic acid bacteria in meat model were 3-4 log cfu. g^{-1} at day 0. BLIS EK13 introduced in meat model did not produce any inhibitory effect on LAB counts.

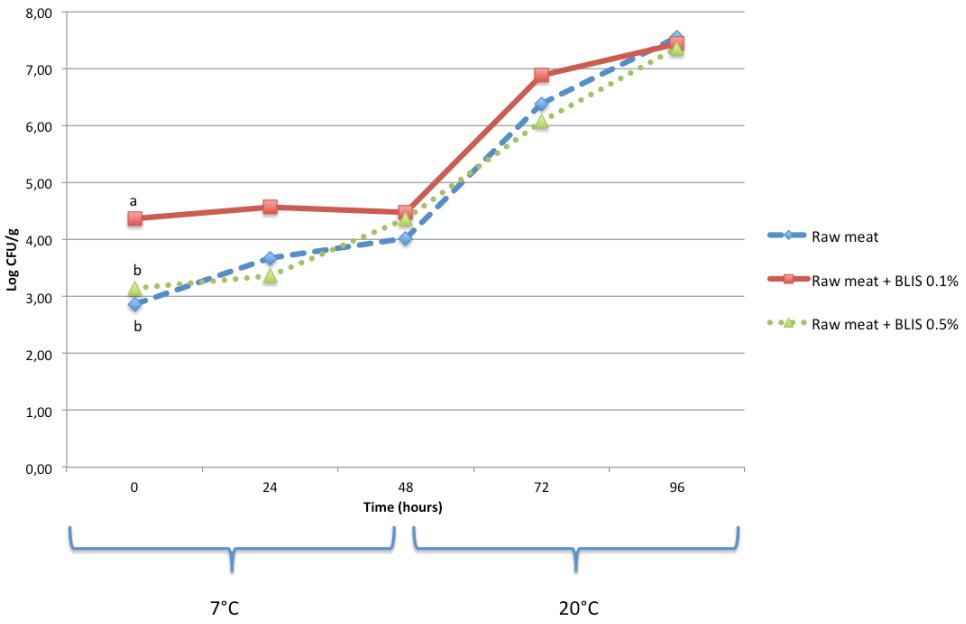

Figure 21 - Influence of BLIS EK13 at 0.1% and 0.5% on lactic acid bacteria counts (LAB). ab – means with different letters are significantly different with $p<0.05$.

Chapter 5

Discussion

Evaluation of bacteriocinogenic potential of LAB isolates

In this study, the isolation for LAB with biopreservation properties was initially performed with cecum isolates collected at a poultry slaughterhouse. Several authors also isolated from the same source LAB with bacteriocinogenic activity [158,159,160,161].

Phenotypic characteristics and specific PCR were used to identify 144 LAB isolates. They were Gram-positive, catalase negative and mostly rod-shaped. These results are in accordance with previous studies establishing that the major LAB in poultry intestinal tract are rod shaped [161], [162]. Only 94 (65.3%) isolates were confirmed by PCR as *Lactobacillus* genus. This percentage was higher than those reported by Noohi *et al.* [163] who showed that only 57.1% of their isolates from poultry intestinal tract belonged to *Lactobacillus*.

Most of the methods used for detecting antimicrobial activity were based on the production of inhibition halos on agar plates resulting from diffusion of inhibitory protein through the agar with growth inhibition of sensitives bacteria [164]. The antagonistic method used in this study (modified Skalka method) demonstrated the sensitivity of the indicators strains. Results obtained with modified Skalka method, suggest that certain LAB were producers of substances that inhibited indicator strains, such as organic acids (lactic ac., acetic ac.), or ethanol, carbon dioxide, hydrogen peroxide, diacetyl, bacteriocins (nisin, reuterin, reutericyclin, pediocin, lacticin, enterocin and others) and bacteriocin-like inhibitory substances (BLIS) [128]. Due to the production of organic acids and bacteriocins many LAB strains exhibited pronounced antagonistic activity against pathogenic microorganisms [165].

The antimicrobial activity assessment confirmed that all LAB from poultry origin and *Lactobacillus* from FMV collection had antagonistic activity against Gram-positive and Gram-negative microorganisms tested.

Among the poultry isolates, all presented antimicrobial activity against *L. monocytogenes* CECT 934 and *Enterococcus avium* (EA5). Both strains were selected as targets because previous studies demonstrated its susceptibility to antimicrobial substances produced by LAB. Tomé *et al.* [166] used *L. monocytogenes* CECT 934 as an indicator to study the inhibitory spectrum of bacteriocins produced by Lactic acid bacteria. *Enterococcus avium* (EA5) was used as indicator in a study made by Strompfová, Lauková & Mudroňová [167] to observe the effect of a bacteriocin like substance produced by *Enterococcus faecium* EF55.

Of all tested *Lactobacillus* with Skalka method stood out seven *Lactobacillus* with inhibition halos higher than 30mm against *Listeria monocytogenes* CECT 934, and higher than 25mm against *Enterococcus avium* EA5. *Lactobacillus* B6C1-3-3 were the isolate that had the highest antimicrobial property against *L. monocytogenes* (39mm). Carvalho *et al.* [168] studied the antilisterial activity of LAB isolated from Italian salami against *Listeria monocytogenes* and obtained an average of inhibition halos smaller than our results (20mm).

Lactobacillus B6C2-1-2 presented the best results against *Enterococcus avium* EA5 (30mm). Savino *et al.,* [169] also studied antagonistic effect of *Lactobacillus* strains against *Enterococcus faecalis* and

obtained an average of inhibition zones around 11mm for a strain of *L. delbrueckii* and 9mm for a strain of *L. plantarum*.

Lactobacillus, from FMV collection, were isolated from fermented meat products and processing environment. Several studies refer bacteriocin-producing *Lactobacillus* strains isolated from fermented meat products. Schillinger and Lücke [170] isolated various bacteriocin producing lactobacilli from fresh meat and different meat products. Also, Vignolo *et al.* [171] isolated lactobacilli from dry fermented sausages that were tested for the production of antimicrobial substances (bacteriocins). Among LAB, *L. sakei, L. curvatus* and *L. plantarum* are the species most frequently isolated in acid-fermented meat products [172]. Enterococci can also be isolated from these products.

Strains from FMV collection were tested against several microbial targets such as strains of *Campylobacter* (n=7), *Salmonella enteritidis* CECT 4300, *Enterococcus avium* (EA5), *S. aureus* ATCC 25923 and *L. monocytogenes* CECT 934. All the tested strains of *Lactobacillus (L. sakei* and *L. plantarum*) and *Enterococcus faecium* EK13 showed potential bacteriocinogenic capability against all the pathogenic microbiota tested. Results shown that the spectrum of inhibition was the same for isolates tested because they presented inhibitory activity against Gram-positive and Gram-negative bacteria.

It was assumed that the greater the diameter of the inhibition zone, greater was the antimicrobial activity of the isolate. Schved *et al.* [173] stated that zone of inhibitions can describe the degree of bacteria sensitivity and resistance.

L. sakei CV3C8 showed the highest inhibitory activity against all strains of *Campylobacter* tested. Jones *et al.* [174] also found two strains of *L. sakei* which demonstrated potential for *Campylobacter jejuni* control with inhibition zones between 1-10mm.

L. plantarum strain P3B7 showed higher inhibition against *Salmonella enteritidis CECT 4300,* while *L. plantarum* P3B8 presented best results against Enterococcus avium (EA5) and *L. plantarum* P05-67 presented best results against *S. aureus ATCC* 25923. Isolate *L. plantarum* P3B8 also showed the highest halos against *Listeria*. Arena *et al*., [175] also studied the antimicrobial activity of *L. plantarum* strains against various food pathogens (*L.monocytogenes, S. enteritidis and S. aureus*) and classified them as very strong inhibitors.

According to Al-Allaf, Al-Rawi, & Al-Mola, [176] LAB isolated from meat are probably the best candidates to improve the microbiological safety of these foods and act as a hurdle to inhibit spoilage and /or growth of pathogenic bacteria and the biopreservation techniques for meats.

Bacteriocins produced from LAB strains were reported to inhibit the growth of pathogenic bacteria in many studies. Bromberg *et al*., [117] studied the inhibitory activity of bacteriocin producing lactic acid bacteria isolated from meat and meat products against food spoilage and pathogenic bacteria. According to their results, the strains presented a broad inhibitory spectrum since they were able to inhibit many of the indicator strains tested such as *E. coli*, *S. aureus* and *L.monocytogenes*.

Also, Lü, Xin, *et al*. [177] tested the antimicrobial activity of a novel bacteriocin (lactocin MXJ 32A) produced by *Lactobacillus coryniformis* MXJ32, isolated from a traditional fermented vegetable against foodborne pathogens. They concluded that lactocin MXJ 32A had a broad antimicrobial

spectrum against Gram-positive (*Staphylococcus aureus*) and Gram-negative (*Escherichia coli*) foodborne pathogens.

However, the inhibitory activity against Gram-negative bacteria is an unusual phenomenon and has been reported only for a few LAB strains. This activity was described in a study performed by De Kwaadsteniet *et al.,* [178] with a strain of *Enterococcus mundtii* producing a 3,944-Da bacteriocin that was able to inhibit Gram-positive and Gram-negative bacteria. Also, Line *et al*., [179] reported the antimicrobial activity of the enterocin E-760 produced by *Enterococcus sp.* against Gram-negative organisms. This enterocin was able to reduce *Campylobacter* counts in 8 log^{10} in broiler chicken trials.

Liu *et al.,* [180] studied bifidocin A, a novel broad-spectrum bacteriocin produced by *Bifidobacterium animalis* BB04, isolated from the human faeces and observed antimicrobial activity against many Gram positive and Gram-negative foodborne spoilage and pathogenic bacteria.

The resistance of Gram negative bacteria against the action of bacteriocins could be attributed to the lipopolysaccharide layer of the cell wall protecting the cell membrane, the site of action of bacteriocins. Bacteriocins from LAB only become active against Gram-negative bacteria when combined with other agents such as chelating agents, surfactants or osmotic shock (high salt) that compromise the integrity of the outer membrane [181], [182].

Nisin displays much lower activity against most Gram-negative bacteria, because the outer membrane can prevent the peptide to reach the periplasm and to exert activity binding lipid II in the inner membrane. When the outer membrane is destabilized using ethylenediaminetetraacetic acid (EDTA) or pyrophosphate, nisin can inhibit the Gram-negative bacteria more efficiently [183]. Other reports highlighted the specific benefits of combining nisin with EDTA, citrate, lysozyme and citric acid in improving the inhibitory activity of nisin towards Gram-negatives or even extending its inhibitory spectrum to cover Gram-negatives [184].

An understanding about the mode of action of bacteriocins against Gram-negative bacteria is important to ascertain their effective application as broad-spectrum biopreservatives in the food industry [185].

Protease sensitivity is a key criterion for the characterization of an inhibitory substance as bacteriocins [186]. Proteinase K test was used to confirm the proteinaceous nature of the inhibitory activity. Half-moon halos associated with proteases were indicative of the proteic nature of the antimicrobial substances produced by the tested isolates [187].

Among the LAB isolates obtained in the slaughterhouse and the ones from FVM collection, it was found that the antagonistic substances produced were not inactivated after treatment with Proteinase K. Only the inhibitory substances, produced by *Enterococcus faecium* EK13, were inactivated by the proteolytic enzymes, confirming its proteinaceous nature and indicating the presence of bacteriocins. This suggests that only *Enterococcus faecium* EK13 has a bacteriocinogenic inhibitory mechanism. The other isolates studied produced antimicrobial substances particularly lactic acid the main inhibitory substance against Gram-negative or Gram-positive bacteria [188].

Wilson, Sigee & Epton [189] investigate the mechanism of anti-listerial activity of a *Lactobacillus plantarum* strain and determined that it was due to lactic acid production alone.

It could be possible to induce bacteriocin production in *Lactobacillus* by several mechanisms. These include regulation by quorum sensing mechanisms, environmental factors such as pH, temperature, and other growth conditions [120]. Also, the presence of competing microorganisms has been reported as an environmental factor affecting production of bacteriocins by some LAB because bacteriocin production seems to be a defence mechanism directed to compete for nutrients against other bacteria in the same environment [190]. It is possible that inadequate pH or the presence of certain substances negatively influenced the binding of the induction factor to its receptor. One possible solution to induce bacteriocin production would be co-cultivation of strains with some inducing bacteria [191].

Numerous strains of enterococci associated with food systems, mainly *Enterococcus faecium* and *Enterococcus faecalis*, are capable of producing a variety of bacteriocin called enterocin with broad spectrum activity [192], [193], [194]. Enterocins usually belong to class II bacteriocins, which are small, heat stable and non-lantibiotics [100]. Many authors have demonstrated the bacteriocinogenic activity of *E. faecium* against food pathogens [195], [196], [197].

Vera Pingitore *et al.* [197] studied bacteriocins produced by two *Enterococcus* strains (*Enterococcus mundtii* CRL 35 and *Enterococcus faecium* ST88Ch), isolated from cheeses, and tested their capability to control *Listeria monocytogenes* 426 in experimentally contaminated fresh Minas cheese during refrigerated storage. The inhibition of *L. monocytogenes* was more significant in cheeses containing *Enterococcus mundtii* CRL 35 and these researchers underline the potential application of *E. mundtii* CRL 35 in the control of *L.monocytogenes* in Minas cheese.

Enterococcus faecium and *Enterococcus faecalis*, apart from l*actobacilli*, are commonly associated with anti-*Campylobacter* activity. In 2008, Svetoch *et al.* [198] identified and characterized an effective bacteriocin produced by *Enterococcus faecium* (NRRL B-30746). This peptide showed antimicrobial activity against *Campylobacter jejuni.* In the same year, Line *et al.* [179] isolated from chicken ceca a strain of *Enterococcus* (NRRL B-30745) antagonistic to *Campylobacter jejuni*, and 20 other *Campylobacter* species isolates. Both *Enterococcus faecium* (NRRL B-30746) and *Enterococcus* (NRRL B-30745) were also able to inhibit *Salmonellae*.

A recent *in vitro* study conducted by Robyn *et al.* [199] demonstrated that *E. faecalis* was inhibitory to *C. jejuni* MB 4185 infection under simulated broiler caecal condition.

In 2003, M. Mareková *et al.* [153] performed the partial characterization of bacteriocins produced by EK13 and concluded that this strain produced an anti-microbial substance which was determined to be enterocin A and found a second substance specified by PCR as enterocin P.

A partial purification of bacteriocins produced by *Enterococcus faecium* EK13 was performed by ammonium sulfate precipitation and antimicrobial activity of *Enterococcus faecium* EK13 was studied against *Listeria monocytogenes* CECT 934, *Listeria innocua* CECT 910*,* Enterococcus avium (EA5), *Salmonella enteritidis* CECT 4300 and seven strains of *Campylobacter* using an adaptation of the critical dilution method. *BLIS* EK13 inhibited *L. monocytogenes, L.innocua* and *Enterococcus avium* (EA5), but did not inhibit *Salmonella* and *Campylobacter* strains tested. These results are interesting because among the bacteria tested, *Salmonella* and *Campylobacter* were sensitive to *Enterococcus*

faecium EK13 with the Skalka method. However, they were resistant to the bacteriocin in the critical dilution method.

The lack of bacteriocinogenic activity in the critical dilution method may be due to the dilution with phosphate buffer in the partial purification of bacteriocin on which were removed the inhibitory compounds, particularly the lactic acid produced.

Application of BLIS EK13 in a meat model

Contamination of meat and meat products by *Listeria monocytogenes* is considered a major problem [28].

LAB are excellent candidates for pathogens control in meat or fermented meat products because they can inhibit growth of these bacteria through various mechanisms without causing unacceptable sensory changes [200]. Bacteriocins from *Enterococcus faecalis* and other *enterococci* species can be used as biopreservatives of food [122].

In this second part of the study, the BLIS (containing enterocins) produced by *Enterococcus faecalis* EK13 was inoculated onto fresh meat to investigate its effect *in vitro* against *Listeria innocua* CECT 910. *L. innocua* was frequently used as model of *L. monocytogenes* behaviour. In 2011, Křepelková & Sovják [55] studied the effect of pressure and time in fermented meat products of Czech origin inoculated with *Listeria innocua* using a non-pathogenic strain for experimental purposes, since *Listeria monocytogenes* and *Listeria innocua* are of very similar nature.

Dykes *et al.* [201] tested and compared both species of *Listeria* in cooked tiger prawns and conclude that *L. innocua* strain proved to be an effective model of *L. monocytogenes.*

Assays in this study were performed reproducing the temperatures of two stages of manufacture of sausages: maturation at 7°C and smoking at 20°C in order of the results could be linked to what happens in a Portuguese traditional fermented meat product.

The initial counts of *Listeria innocua* on meat at time 0 was reduced in 1 log cfu.g^{-1} under the effect of BLIS addition at 0.1% and 0.5% indicating that BLIS exerts its effect once introduced into the meat.

The growth of *Listeria innocua* at 7ºC after 48h of storage was significantly inhibited (0.93 log cfu.g^{-1}) when 0.1 and 0.5% BLIS was added to meat. The addition of free BLIS 0.1% in meat reduced *Listeria innocua* counts from 5 log cfu.g^{-1} to 2.4 cfu.g^{-1} and 0.5% BLIS in meat reduced *Listeria innocua* counts from 5 log cfu.g^{-1} to 0.93 log cfu.g^{-1}. The antilisterial activity was higher in meat samples with 0.5% BLIS compared to the 0.1% BLIS.

Listeria innocua growth rate in meat under the effect of BLIS 0.1% and 0.5% were the same. However, the final counts obtained on meat with 0.5% and 0.1% of BLIS in this storage conditions presented a difference of approximately 2 log cfu.g^{-1}. Similar results were reported by Nielsen, Dickson & Crouse [132] and Vignolo *et al*.,[202] when a bacteriocin was inoculated onto fresh meat, Listeria was remarkably reduced. The antilisterial effectiveness of pediocin-like bacteriocins has been demonstrated in meat products by Nielsen *et al*. [132]. Vignolo *et al.,* [202] reported that the combined effect of different bacteriocins against *L. monocytogenes* FBUNT in meat showed no viable counts after incubation for 3 h.

Although bacteriocins are effective in reducing *Listeria* populations and other microorganisms in model systems, bacteriocin activity is difficult to maintain in a range of foods particularly in meat products because the occurrence of relatively high numbers of survivors or regrowth in food systems is generally observed [137],[202]. This was in agreement with our results because even with 0.5% bacteriocin used it was not possible to avoid the regrowth of *Listeria* after 48h when the temperature changed to 20ºC.

Despite *Listeria* counts increased when the temperature changed to 20ºC, addition of 0.5 BLIS EK13 was able to inhibiting the growth of this pathogen in more than 3 log cfu.g^{-1} at 96h compared to control. This suggests that EK13 BLIS will be effective against *Listeria* in fermented meat products with fermentation conditions.

Currently, the role of LAB in fresh meat spoilage is quite controversial. While they are recognized as causative agents of meat spoilage [10], they have also a bioprotective function in meat as they can reduce pH and provide favourable antagonistic activity against other undesired microorganisms improving the safety and stability of the product [23].

Lactic acid bacteria and total aerobic microorganisms at 30ºC counts were significantly influenced by storage temperature and activity of EK13 BLIS on these microorganisms was not noticed. Similar results were reported by Wang [203] with nisin which had no effect on total aerobic mesophilic bacteria and LAB counts in Chinese style sausages.

LAB and total aerobic microorganisms at 30ºC counts showed a strong increase when stored at 20ºC (48h) in all conditions studied. The maximum growth of LAB reaches up to approximately 8 - 9 log cfu/g. At this point, their growth is eventually inhibited by the amount of acid produced or lack of nutrients. Due to the influence of abusive temperature mesophilic population was $\geq$ 6 log cfu g^{-1}, which is indicating meat putrefaction.

The mesophilic aerobic bacteria can be used as an indicator for the determination of shelf life and spoilage status of foods, and they are useful to evaluate the sanitary quality of foods because their presence implies contamination by pathogens in food [204].

Results from the present study suggest that BLIS EK13 shows a good capacity to reduce the levels of *Listeria innocua* and does not have effects on the microbiota under study.

The lack of effect of BLIS EK13 on mesophilic total populations in meat might be due to the fact that these microorganisms are mainly Gram-negative bacteria, including *Pseudomonas* spp. Bacteriocins produced by Gram-positive bacteria are not effective against Gram-negative bacteria [205]. Also, BLIS protects the bacteriocin producer from its own bacteriocin and this fact could explain why BLIS does not have effects on LAB.

Enterococcus faecium EK13 is a potential candidate for biopreservation of meat against *Listeria* monocytogenes in the food industry.

Conclusions and future work

Results showed great inhibitory effect of all analysed LAB against the food pathogens tested. Using the Skalka method was identified *Lactobacillus* isolates with stronger inhibition against *Enterococcus avium* (EA5), *Campylobacter*, *Salmonella enteritidis* CECT 4300, *S. aureus* ATCC 25923 and *L. monocytogenes* CECT 934. From all isolates tested the inhibitory action was not attributed to the production of bacteriocin like inhibitory substances

Among all the tested LAB with proteinase K, only *Enterococcus faecium* strain EK 13 was a bacteriocin producer.

Moreover, was investigated the ability of *Enterococcus faecium* strain for production of BLIS and the introduction of BLIS (at different concentrations) on meat inoculated with *Listeria innocua* to investigate the antilisterial activity of BLIS EK13 and the effect on microbiota of the meat. BLIS produced by *Enterococcus faecium* EK13 have potential uses to inactivate *Listeria monocytogenes* and is worth studying for its potential as natural food preservative to meat or meat products.

An interesting challenge for future work is to study the effect of BLIS in others concentrations.

Also, it would be appropriate to evaluate the antilisterial activity of encapsulated BLIS and compare the efficiency of free and encapsulated BLIS to control *L. monocytogenes.*

Further research on BLIS EK13 efficient production conditions, purification, mechanisms of action should be done to obtain the highest yield of enterocins, increase his activity and optimize the production cost to make these findings applicable to food safety.

References

[1] Deraz, S. F., Shehata, M. G., & Khalil, A. A. (2015). Significant Industrial Properties of Enteriococus faecium SFD as a Probiotic and Bacteriocin-Producing Strain. Life Science Journal, 12(3), 82-91.

[2] Mbata, T. I. (2005). Poultry meat pathogens and its control. International Journal of Food Safety, 7, 20-28.

[3] Hennekinne, J. A., Herbin, S., Firmesse, O., & Auvray, F. (2015). European Food Poisoning Outbreaks Involving Meat and Meat-based Products.Procedia Food Science, 5, 93-96.

[4] Sidira, M., Galanis, A., Nikolaou, A., Kanellaki, M., & Kourkoutas, Y. (2014). Evaluation of Lactobacillus casei ATCC 393 protective effect against spoilage of probiotic dry-fermented sausages. Food Control, 42, 315-320.

[5] Cheng, J. H., & Sun, D. W. (2015). Recent applications of spectroscopic and hyperspectral imaging techniques with chemometric analysis for rapid inspection of microbial spoilage in muscle foods. Comprehensive Reviews in Food Science and Food Safety, 14(4), 478-490.

[6] Sakaridis, I., Soultos, N., Batzios, C., Ambrosiadis, I., & Koidis, P. (2014). Lactic Acid Bacteria Isolated from Chicken Carcasses with Inhibitory Activity against *Salmonella* spp. and *Listeria monocytogenes.* Czech Journal of Food Sciences, 32(1), 61-68.

[7] Gautam, N., & Sharma, N. (2009). Bacteriocin: safest approach to preserve food products. Indian Journal of Microbiology, 49(3), 204-211.

[8] Favaro, L., Penna, A. L. B., & Todorov, S. D. (2015). Bacteriocinogenic LAB from cheeses–Application in biopreservation?. Trends in Food Science & Technology, 41(1), 37-48.

[9] Ercolini, D., Russo, F., Nasi, A., Ferranti, P., & Villani, F. (2009). Mesophilic and psychrotrophic bacteria from meat and their spoilage potential in vitro and in beef. Applied and Environmental Microbiology, 75(7), 1990-2001.

[10] Ercolini, D., Russo, F., Torrieri, E., Masi, P., & Villani, F. (2006). Changes in the spoilage-related microbiota of beef during refrigerated storage under different packaging conditions. Applied and Environmental Microbiology,72(7), 4663-4671.

[11] Brooks, J. C., Brashears, M. M., & Miller, M. F. (2007). Is there a link between food safety and food spoilage?. Journal of Dairy Science, 90, 137-137.

[12] Dave, D., & Ghaly, A. E. (2011). Meat spoilage mechanisms and preservation techniques: a critical review. American Journal of Agricultural and Biological Science, 6 (4), 486-510.

[13] Petrová, J. (2013). Microbiological quality of fresh chicken breast meat after rosemary essential oil treatment and vacuum packaging. Scientific Papers Animal Science and Biotechnologies, 46(1), 140-144.

[14] Kameník, J. (2012). The microbiology of meat spoilage: a review. Department of Meat Hygiene and Technology. University of Veterinary and Pharmaceutical Sciences, Brno. Czech Republic, 2, 3-10.

[15] Fung, D. Y. (2010). Microbial Hazards in food: food-borne infections and intoxications. In Handbook of meat processing. Toldrá F., Blackwell Publishing, 481-500.

[16] Doulgeraki, A. I., Ercolini, D., Villani, F., & Nychas, G. J. E. (2012). Spoilage microbiota associated to the storage of raw meat in different conditions. International Journal of Food Microbiology, 157(2), 130-141.

[17] Ercolini, D., Ferrocino, I., Nasi, A., Ndagijimana, M., Vernocchi, P., La Storia, A. & Villani, F. (2011). Monitoring of microbial metabolites and bacterial diversity in beef stored under different packaging conditions. Applied and Environmental Microbiology, 77(20), 7372-7381.

[18] Casaburi, A., Piombino, P., Nychas, G. J., Villani, F., & Ercolini, D. (2015). Bacterial populations and the volatilome associated to meat spoilage. Food Microbiology, 45, 83-102.

[19] Nychas, G. J. E. , Skandamis, P.N., Tassou, C. H. C. & Koutsoumanis, K. P., (2008): Meat spoilage during distribution. Meat Science, 78, 77-89.

[20] Salman, A. M., Hussien Abdella, H., & Mustafa, E. A. (2015). Some Quality Aspects of Fresh and Refrigerated Beef Cuts in Alkadaro Slaughterhouse, Khartoum- Sudan. Journal of Applied and Industrial Sciences, 3(2), 52-57.

[21] FAO.org. (2016). Guidelines for slaughtering, meat cutting and further processing. [online] Available at: http://www.fao.org/docrep/004/t0279e/t0279e03.htm [Accessed 25 May 2016].

[22] Feiner, G. (2006). Introduction to the microbiology of meat and meat products. In Meat Products Handbook: Practical Science and Technology. Elsevier. 574-584

[23] Pothakos, V., Devlieghere, F., Villani, F., Björkroth, J., & Ercolini, D. (2015). Lactic acid bacteria and their controversial role in fresh meat spoilage. Meat Science, 109, 66-74.

[24] Hyldgaard, M., Meyer, R. L., Peng, M., Hibberd, A. A., Fischer, J., Sigmundsson, A., & Mygind, T. (2015). Binary combination of epsilon-poly-l-lysine and isoeugenol affect progression of spoilage microbiota in fresh turkey meat, and delay onset of spoilage in *Pseudomonas putida* challenged meat. International Journal of Food Microbiology, 215, 131-142.

[25] Holzapfel, W.H. (1998). The Gram-positive bacteria associated with meat and meat products. In: The microbiology of meat and poultry. Davies, A. & Board, E. Blackie Academic & Professional, London, 35-84.

[26] Aymerich, M.T., Garriga, M., Costa, S., Monfort, J.M. & Hugas, M. (2002). Prevention of ropiness in cooked pork by bacteriocinogenic cultures. International Dairy Journal, 12, 239-246.

[27] EFSA (2016). Food-borne zoonotic diseases | European Food Safety Authority. [online] Available at: http://www.efsa.europa.eu/en/topics/topic/foodbornezoonoticdiseases [Accessed 25 May 2016].

[28] EFSA (2015). The European Union summary report on trends and sources of zoonoses, zoonotic agents and food-borne outbreaks in 2014. EFSA (European Food Safety Authority) and ECDC (European Centre for Disease Prevention and Control), EFSA Journal,13(12), 3 – 191.

[29] Indikova, I., Humphrey, T. J., & Hilbert, F. (2015). Survival with a Helping Hand: *Campylobacter* and Microbiota. Frontiers in Microbiology, 6 ,1-6.

[30] Modi, S., Brahmbhatt, M. N., Chatur, Y. A., & Nayak, J. B. (2015). Prevalence of *Campylobacter* species in milk and milk products, their virulence gene profile and antibiogram. Veterinary World, 8(1), 1-8.

[31] Whitman, W. B. (Ed.). (2015). Bergey's manual of systematics of Archaea and Bacteria.

[32] Silva, J., & Teixeira, P. (2015). Tackling *Campylobacter:* a Review. American Journal of Advanced Food Science and Technology, 3(2), 107-124.

[33] Smith, J., Corkran, S., McKee, S. R., Bilgili, S. F., & Singh, M. (2015). Evaluation of post-chill applications of antimicrobials against *Campylobacter jejuni* on poultry carcasses. The Journal of Applied Poultry Research, 24(4), 451-456.

[34] Whiley, H., van den Akker, B., Giglio, S., & Bentham, R. (2013). The role of environmental reservoirs in human campylobacteriosis. International Journal of Environmental Research and Public Health, 10(11), 5886-5907.

[35] Drinceanu, D., Stef, L., Julean, C., Cean, A., Lup, F. G., & Corcionivoschi, N. (2013). Poultry meat–as a source of *Campylobacter spp*., infection in humans. Scientific Papers Animal Science and Biotechnologies, 46(2), 17-21.

[36] Aroori, S. V., Cogan, T. A., & Humphrey, T. J. (2013). The effect of growth temperature on the pathogenicity of *Campylobacter*. Current microbiology,67(3), 333-340.

[37] Skarp, C. P. A., Hänninen, M. L., & Rautelin, H. I. K. (2016). Campylobacteriosis: the role of poultry meat. Clinical Microbiology and Infection, *22*(2), 103-109.

[38] Wieczorek, K., Szewczyk, R., & Osek, J. (2012). Prevalence, antimicrobial resistance, and molecular characterization of *Campylobacter jejuni* and *C. coli* isolated from retail raw meat in Poland. Veterinarni Medicina, 57(6), 293-299.

[39] *Campylobacter.* (2016). World Health Organization. Retrieved 25 May 2016, from http://www.who.int/mediacentre/factsheets/fs255/en/

[40] Käferstein, F. (2005). Food safety: a pressing public-health and economic issue. In Understanding the Global Dimensions of Health, Springer US., 199-212.

[41] McWhorter, Andrea R., Dianne Davos, and K. K. Chousalkar (2015). Pathogenicity of *Salmonella* strains isolated from egg shells and the layer farm environment in australia. Applied and Environmental Microbiology, 81 (1), 405-414.

[42] Eng, S. K., Pusparajah, P., Ab Mutalib, N. S., Ser, H. L., Chan, K. G., & Lee, L. H. (2015). *Salmonella:* A review on pathogenesis, epidemiology and antibiotic resistance. *Frontiers in Life Science*, *8*(3), 284-293.

[43] Park, S. Y., Pontes, M. H., & Groisman, E. A. (2015). Flagella-independent surface motility in *Salmonella enterica* serovar Typhimurium. Proceedings of the National Academy of Sciences, 112(6), 1850-1855.

[44] Lee, K. M., Runyon, M., Herrman, T. J., Phillips, R., & Hsieh, J. (2015). Review of *Salmonella* detection and identification methods: aspects of rapid emergency response and food safety. Food Control, 47, 264-276.

[45] Wong, W. C., Pui, C. F., Chai, L. C., Tunung, R., Jeyaletchumi, P., Noor Hidayah, M. S. & Son, R. (2011). Review Article *Salmonella*: A foodborne pathogen International Food Research Journal, 18, 465-473.

[46] Ibrahim, H. M., Sabo, D. D. I. A., Mahmud, T., & Abubakar, N. M. (2015). Incidence of Typhoid Fever Among Patients Attending Aminu Kano Teaching Hospital Laboratory, Tarauni Local Government, Kano State, Nigeria.International Journal Of Scientific Research And Education, 3(09), 4409-4416.

[47] Addis, M., & Sisay, D. (2015). A Review on Major Food Borne Bacterial Illnesses. Journal of Tropical Diseases & Public Health, 3(4), 2-7.

[48] Maraki, S., & Papadakis, I. S. (2014). Serotypes and Antimicrobial Resistance of Human Nontyphoidal Isolates of *Salmonella enterica* from Crete, Greece. Interdisciplinary perspectives on infectious diseases. 1-5.

[49] Dussault, D., Vu, K. D., & Lacroix, M. (2016). Development of a model describing the inhibitory effect of selected preservatives on the growth of *Listeria monocytogenes* in a meat model system. Food Microbiology, 53, 115-121.

[50] Wu, S., Wu, Q., Zhang, J., Chen, M., & Hu, H. (2015). *Listeria monocytogenes* Prevalence and Characteristics in Retail Raw Foods in China. PloS one, 10(8), 1-16.

[51] Di Ciccio, P. , Meloni, D. & Ianieri, A. (2015) Morphological, physiological and epidemiological features of Listeria monocytogenes. In *Listeria monocytogenes:* Incidence, Growth Behavior and Control. Nova Science Publishers, 38-41.

[52] Rodríguez-Lázaro, D., Hernández, M., Scortti, M., Esteve, T., Vázquez-Boland, J. A., & Pla, M. (2004). Quantitative detection of *Listeria monocytogenes* and *Listeria innocua* by real-time PCR: assessment of *hly, iap*, and lin02483 targets and AmpliFluor technology. Applied and Environmental Microbiology, 70(3), 1366-1377.

[53] Johnson, J. L., Doyle, M. P., & Cassens, R. G. (1990). *Listeria monocytogenes* and other *Listeria spp*. in meat and meat products: a review. Journal of Food Protection, 53(1), 81-91.

[54] Pieniz, S., Andreazza, R., Okeke, B. C., Camargo, F. A. O., & Brandelli, A. (2015). Antimicrobial and antioxidant activities of Enterococcus species isolated from meat and dairy products. Brazilian Journal of Biology, 75(4), 923-931.

[55] Křepelková, V., & Sovják, R. (2011). Effect of high pressure treatment on *Listeria innocua* in dry cured fermented meat products. Agricultura Tropica et Subtropica, 44, 4.

[56] Kathariou, S. (2002). *Listeria monocytogenes* virulence and pathogenicity, a food safety perspective. Journal of Food Protection, 65(11), 1811-1829.

[57] Wurtzel, O., Sesto, N., Mellin, J. R., Karunker, I., Edelheit, S., Bécavin, C., & Sorek, R. (2012). Comparative transcriptomics of pathogenic and non-pathogenic *Listeria* species. Molecular Systems Biology, 8(1), 583.

[58] Jamali, H., Paydar, M., Radmehr, B., Ismail, S., & Dadrasnia, A. (2015). Prevalence and antimicrobial resistance of *Staphylococcus aureus* isolated from raw milk and dairy products. Food Control, 54, 383-388.

[59] Doyle, M. E., Hartmann, F. A., & Wong, A. C. L. (2011). White Paper on Sources of Methicillin-Resistant *Staphylococcus aureus* (MRSA) and other Methicillin-Resistant *Staphylococci:* Implications for our Food Supply. Food Research Institute, UW-Madison.

[60] Hennekinne, J. A., De Buyser, M. L., & Dragacci, S. (2012). *Staphylococcus aureus* and its food poisoning toxins: characterization and outbreak investigation. FEMS Microbiology reviews, 36(4), 815-836.

[61] Gopal, T., Nagarajan, V., & Elasri, M. O. (2015). SATRAT: *Staphylococcus aureus* transcript regulatory network analysis tool. PeerJ, 3, 2-8.

[62] Guillemot, D., Bonacorsi, S., Blanchard, J. S., Weber, P., Simon, S., Guesnon, B. & Carbon, C. (2004). Amoxicillin-clavulanate therapy increases childhood nasal colonization by methicillin-susceptible *Staphylococcus aureus* strains producing high levels of penicillinase. Antimicrobial Agents and Chemotherapy, 48(12), 4618-4623.

[63] FDA (2016). Foodborne Illnesses: What You Need to Know. Fda.gov. Retrieved 25 May 2016, from http://www.fda.gov/food/resourcesforyou/consumers/ucm103263.htm

[64] Danielsson-Tham, M. L. (2013). *Staphylococcal* Food Poisoning. Food Associated Pathogens, 16, 250-270.

[65] Johler, S., Giannini, P., Jermini, M., Hummerjohann, J., Baumgartner, A., & Stephan, R. (2015). Further evidence for *staphylococcal* food poisoning outbreaks caused by egc-encoded enterotoxins. Toxins, 7(3), 997-1004.

[66] Marshall, D. L., & Bal'a, M. F. (2001). Microbiology of meats. Meat science and applications, 149-169.

[67] Makwana, M., Rathod, P., Prajapati, B., Bariya, A., Chavada, P., & Roy, S. (2015). Review: Hazard analysis and critical control points in meat industry, 64, 109-120.

[68] Vandendriessche, F. (2008). Meat products in the past, today and in the future. Meat Science, 78, 104-113.

[69] Lee, R. (2015). Health and safety in meat processing. Gracey's Meat Hygiene, Eleventh Edition, 14, 291-317.

[70] Stiles, M. E. (1996). Biopreservation by lactic acid bacteria. Antonie van Leeuwenhoek, 70(2-4), 331-345.

[71] FAO (2016). Meat and meat products in human nutrition - Role of meat and meat products in human nutrition. Fao.org. Retrieved 25 May 2016, from http://www.fao.org/docrep/t0562e/t0562e03.htm.

[72] Honikel, K. O. (2010). Curing In Handbook of meat processing, Toldrá F., Wiley – Blackwell, 6, 125-141.

[73] FAO (2016)a. Cured meat cuts. Fao.org. Retrieved 25 May 2016, from http://www.fao.org/docrep/010/ai407e/ai407e14.htm

[74] Barbosa, L. N., Rall, V. L. M., Fernandes, A. A. H., Ushimaru, P. I., da Silva Probst, I., & Fernandes Jr, A. (2009). Essential oils against foodborne pathogens and spoilage bacteria in minced meat. Foodborne Pathogens and Disease, 6(6), 725-728.

[75] Zhou, G. H., Xu, X. L., & Liu, Y. (2010). Preservation technologies for fresh meat–A review. Meat Science, 86(1), 119-128.

[76] Zacharof, M. P., & Lovitt, R. W. (2012). Bacteriocins produced by lactic acid bacteria a review article. APCBEE Procedia, 2, 50-56.

[77] Deeth, H. C., Datta, N., & Versteeg, C. (2013). Nonthermal technologies in dairy processing. Advances in Dairy Ingredients, 161-215.

[78] Ghanbari, M. and Jami, M., (2013). Lactic acid bacteria and their bacteriocins: a promising approach to seafood biopreservation. INTECH Open Access Publisher, 16, 381-404.

[79] Saris, Per EJ (2014). Biopreservation by Lactic Acid Bacteria. Dairy Microbiology and Biochemistry: Recent Developments, 4, 86 - 105.

[80] Gómez-Sala, B., Herranz, C., Díaz-Freitas, B., Hernández, P. E., Sala, A., & Cintas, L. M. (2016). Strategies to increase the hygienic and economic value of fresh fish: Biopreservation using lactic acid bacteria of marine origin.International Journal of Food Microbiology, 223, 41 - 49.

[81] Akbar, A., & Anal, A. K. (2014). Occurrence of Staphylococcus aureus and evaluation of anti-staphylococcal activity of *Lactococcus lactis* subsp. *lactis* in ready-to-eat poultry meat. Annals of Microbiology, 64(1), 131-138.

[82] Hugas, M. (1998). Bacteriocinogenic lactic acid bacteria for the biopreservation of meat and meat products. Meat Science, 49 (S1), 139-150.

[83] Kalschne, D. L., Womer, R., Mattana, A., Sarmento, C. M. P., Colla, L. M., & Colla, E. (2015). Characterization of the spoilage lactic acid bacteria in “sliced vacuum-packed cooked ham”. Brazilian Journal of Microbiology, 46(1), 173-181.

[84] Quinto, E. J., Jiménez, P., Caro, I., Tejero, J., Mateo, J., & Girbés, T. (2014). Probiotic Lactic Acid Bacteria: A Review. Food and Nutrition Sciences, 5(18), 1765.

[85] Melgar-Lalanne, G., Rivera-Espinoza, Y., Farrera-Rebollo, R., & Hernández-Sánchez, H. (2014). Survival under stress of halotolerant lactobacilli with probiotic properties. Revista Mexicana de Ingeniería Química, 13(1), 323-335.

[86] Bharti, V., Mehta, A., Singh, S., Jain, N., Ahirwal, L., & Mehta, S. (2015). Bacteriocin: A novel approach for preservation of food. International Journal of Pharmacy and Pharmaceutical Sciences, 7(9), 20-29.

[87] Idler, C., Venus, J., & Kamm, B. (2015). Microorganisms for the Production of Lactic Acid and Organic Lactates. In Microorganisms in Biorefineries, Idler, C., Venus, J., & Kamm, B., Springer Berlin Heidelberg, 225-273.

[88] Gänzle, M. G. (2015). Lactic metabolism revisited: metabolism of lactic acid bacteria in food fermentations and food spoilage. Current Opinion in Food Science, 2, 106-117.

[89] Salvetti, E., Torriani, S., & Felis, G. E. (2012). The genus *Lactobacillus*: a taxonomic update. Probiotics and Antimicrobial Proteins, 4(4), 217-226.

[90] Fraqueza, M. J. (2015). Antibiotic resistance of lactic acid bacteria isolated from dry-fermented sausages. International Journal of Food Microbiology, 212, 76-88.

[91] Swetwiwathana, A., & Visessanguan, W. (2015). Potential of bacteriocin-producing lactic acid bacteria for safety improvements of traditional Thai fermented meat and human health. Meat Science, 109, 101-105.

[92] Todorov, S. D. (2009). Bacteriocins from *Lactobacillus plantarum* production, genetic organization and mode of action: produção, organização genética e modo de ação. Brazilian Journal of Microbiology, 40(2), 209-221.

[93] Dhewa, T. (2012). Screening, production purification and potential use of bacteriocins from lactic acid bacteria of meat and dairy food origin. InInternational Conference on Nutrition and Food Sciences 39, 35-41.

[94] Liu, W., Pang, H., Zhang, H., & Cai, Y. (2014). Biodiversity of lactic acid bacteria. In Lactic Acid Bacteria. Springer Netherlands, 2, 103-203.

[95] Schleifer, K. H., & Ludwig, W. (1995). Phylogeny of the genus *Lactobacillus* and related genera. Systematic and Applied Microbiology, 18(4), 461-467.

[96] Hammes, W. P., & Vogel, R. F. (1995). The genus *lactobacillus.* In The genera of lactic acid bacteria. Springer US. 19-54.

[97] Hammes, W. P., & Hertel, C. (2006). The genera *lactobacillus* and *carnobacterium*. Prokaryotes, 4, 320-403.

[98] Claesson, M. J., Van Sinderen, D., & O'Toole, P. W. (2007). The genus *Lactobacillus*—a genomic basis for understanding its diversity. FEMS Microbiology Letters, 269(1), 22-28.

[99] Gilmore, M. S., Clewell, D. B., Ike, Y., & Shankar, N. (2014). Enterococcal Bacteriocins and Antimicrobial Proteins that Contribute to Niche Control - Enterococci: from Commensals to Leading Causes of Drug Resistant Infection, 1-24.

[100] Banwo, K., Sanni, A., & Tan, H. (2013). Technological properties and probiotic potential of *Enterococcus faecium* strains isolated from cow milk. Journal of Applied Microbiology,114(1), 229-241.

[101] A. Javed, T. Masud, Q. ul Ain, M. Imran, I. Maqsood (2011). Enterocins of *Enterococcus faecium*, emerging natural food preservatives. Annals of Microbiology, 61 (4), 699–708.

[102] Arias, C. A., & Murray, B. E. (2012). The rise of the *Enterococcus:* beyond vancomycin resistance. Nature Reviews Microbiology, 10(4), 266-278.

[103] Mira, M. U., Deana, M., Zora, J., Vera, G., Biljana, M., & Biljana, R. (2014). Prevalence of different *enterococcal* species isolated from blood and their susceptibility to antimicrobial drugs in Vojvodina, Serbia, 2011-2013. African Journal of Microbiology Research, 8(8), 819-824.

[104] Garriga, M., & Aymerich, T. (2007). The microbiology of fermentation and ripening. Handbook of Fermented Meat and Poultry, 12, 107-115.

[105] Sabia, C., De Niederhäusern, S., Messi, P., Manicardi, G., & Bondi, M. (2003). Bacteriocin-producing *Enterococcus casseliflavus* IM 416K1, a natural antagonist for control of *Listeria monocytogenes* in Italian sausages ("cacciatore"). International Journal of Food Microbiology, 87(1), 173-179.

[106] Barbosa, J., Borges, S., & Teixeira, P. (2014). Selection of potential probiotic *Enterococcus faecium* isolated from Portuguese fermented food. International Journal of Food Microbiology, 191, 144-148.

[107] Oladipo, I. C., Sanni, A. I., Chakraborty, W., Chakravorty, S., Jana, S., Rudra, D. S., ... & Swarnakar, S. (2014). Bioprotective potential of bacteriocinogenic *Enterococcus gallinarum* strains isolated from some Nigerian fermented Foods, and of their bacteriocins. Polish Journal of Microbiology, 63(4), 415-422.

[108] El-Nagar, G. F., El-Alfy, M. B., Younis, M. F., & Atallah, A. A. (2007). Use of bacteriocins produced by some LAB as a natural preservative in yoghurt. Annals of Agriculture Science, 45, 1497-1510.

[109] Florou-Paneri, P., Christaki, E., & Bonos, E. (2013). Lactic acid bacteria as source of functional ingredients. INTECH Open Access Publisher, 25, 589-610.

[110] Corsetti, A., Settanni, L., & Van Sinderen, D. (2004). Characterization of bacteriocin-like inhibitory substances (BLIS) from sourdough lactic acid bacteria and evaluation of their in vitro and in situ activity. Journal of Applied Microbiology, 96(3), 521-534.

[111] Terzić-Vidojević, A., Veljović, K., Begović, J., Filipić, B., Popović, D., Tolinački, M. & Golić, N. (2015). Diversity and antibiotic susceptibility of autochthonous dairy enterococci isolates: are they safe candidates for autochthonous starter cultures? Frontiers in Microbiology, 6, 1-10.

[112] Spano, G., Russo, P., Lonvaud-Funel, A., Lucas, P., Alexandre, H., Grandvalet, C. & Rattray, F. (2010). Biogenic amines in fermented foods.European Journal of Clinical Nutrition, 64, 95-100.

[113] Johnson, M. E. (2014). Mesophilic and thermophilic cultures used in traditional cheesemaking. Cheese and Microbes American Society for Microbiology. New York, 4, 73-94.

[114] Hati, S., Mandal, S., & Prajapati, J. B. (2013). Novel starters for value added fermented dairy products. Current Research in Nutrition and Food Science Journal, 1(1), 83-91.

[115] Leroy, F., & De Vuyst, L. (2004). Lactic acid bacteria as functional starter cultures for the food fermentation industry. Trends in Food Science & Technology, 15(2), 67-78.

[116] Garriga, M. & Aymerich, T. (2007). The Microbiology of Fermentation and Ripening. In Handbook of Fermented Meat and Poultry. F. Toldrá (Ed.), (1a ed.) Oxford: Blackwell,125 – 135.

[117] Bromberg, R., Moreno, I., Zaganini, C. L., Delboni, R. R., & Oliveira, J. D. (2004). Isolation of bacteriocin-producing lactic acid bacteria from meat and meat products and its spectrum of inhibitory activity. Brazilian Journal of Microbiology, 35(1-2), 137-144.

[118] Abriouel, H., Franz, C. M., Omar, N. B., & Gálvez, A. (2011). Diversity and applications of Bacillus bacteriocins. FEMS Microbiology Reviews, 35(1), 201-232.

[119] Yang, S. C., Lin, C. H., Sung, C. T., & Fang, J. Y. (2014). Antibacterial activities of bacteriocins: application in foods and pharmaceuticals. Frontiers of Microbiology, 5(241), 1-10.

[120] Parada, J. L., Caron, C. R., Medeiros, A. B. P., & Soccol, C. R. (2007). Bacteriocins from lactic acid bacteria: purification, properties and use as biopreservatives. Brazilian Archives of Biology and Technology, 50(3), 512-542.

[121] Rizzello, C. G., Filannino, P., Di Cagno, R., Calasso, M., & Gobbetti, M. (2014). Quorum-sensing regulation of constitutive plantaricin by *Lactobacillus plantarum* strains under a model system for vegetables and fruits. Applied and Environmental Microbiology, 80(2), 777-787.

[122] Nagwa B. Elhag, El Rakha B. Babiker, Ahmed A. Mahdi (2015). Isolation and Characterization of Bacteriocin-Producing Lactic Acid Bacteria against Indicator Organisms. Journal of Agri-Food and Applied Sciences, 3 (1), 6-13.

[123] Zahid, M., Ashraf, M., Muhammad G., Yasmin A., Muhammad H & Hameed A. (2015). Antimicrobial Activity of Bacteriocins Isolated from Lactic Acid Bacteria Against Resistant Pathogenic Strains. International Journal of Nutrition and Food Sciences, 4 (3), 326-331.

[124] Perez, R. H., Zendo, T., & Sonomoto, K. (2014). Novel bacteriocins from lactic acid bacteria (LAB): various structures and applications. *Microbial cell factories*, *13*(1), 1-13.

[125] Wu, Y., Zheng, W., Zhang, T., & Song, H. (2012). Screening for bacteriocin-producing lactic acid bacteria from traditional fermented foods. International Conference on Bioinformatics and Biomedical Engineerin, 7(9), 1-6.

[126] Bemena, L. D., Mohamed, L. A., Fernandes, A. M., & Lee, B. H. (2014). Applications of bacteriocins in food, livestock health and medicine. International Journal of Microbiology Applied Sciences, 3(12), 924-949.

[127] Abrams, D., Barbosa, J., Albano, H., Silva, J., Gibbs, P. A., & Teixeira, P. (2011). Characterization of bacPPK34 a bacteriocin produced by *Pediococcus pentosaceus* strain K34 isolated from "Alheira". Food Control, 22 (6), 940-946.

[128] Reis, J. A., Paula, A. T., Casarotti, S. N., & Penna, A. L. B. (2012). Lactic acid bacteria antimicrobial compounds: characteristics and applications. Food Engineering Reviews, 4(2), 124-140.

[129] Sant'Anna, V., Quadros, D. A., Motta, A. S., & Brandelli, A. (2013). Antibacterial activity of bacteriocin-like substance P34 on *Listeria monocytogenes* in chicken sausage. Brazilian Journal of Microbiology, 44(4), 1163-1167.

[130] Acuña, L., Corbalan, N. S., Fernandez-No, I. C., Morero, R. D., Barros-Velazquez, J., & Bellomio, A. (2015). Inhibitory Effect of the Hybrid Bacteriocin Ent35-MccV on the Growth of *Escherichia coli* and *Listeria monocytogenes* in Model and Food Systems. Food and Bioprocess Technology, 8(5), 1063-1075.

[131] Gamal A. Mostafa, Sayed M. Mokhtar, Gehad S. Eldeeb, Refat A. Taha (2015). "Bacteriocins (From Bifidobacterium spp) Biopreservative Against Gram-Negative Pathogenic Bacteria in Minced Meat as a Critical Control Point". American Journal of Food Science and Nutrition 2 (4), 55-67.

[132] Nielsen, J. W., Dickson, J. S., & Crouse, J. D. (1990). Use of a bacteriocin produced by *Pediococcus acidilactici* to inhibit *Listeria monocytogenes* associated with fresh meat. Applied and Environmental Microbiology, 56(7), 2142-2145.

[133] Schöbitz, R., Zaror, T., León, O., & Costa, M. (1999). A bacteriocin from *Carnobacterium piscicola* for the control of *Listeria monocytogenes* in vacuum-packaged meat. Food Microbiology, 16(3), 249-255.

[134] Budde, B. B., Hornbæk, T., Jacobsen, T., Barkholt, V., & Koch, A. G. (2003). *Leuconostoc carnosum* 4010 has the potential for use as a protective culture for vacuum-packed meats: culture isolation, bacteriocin identification, and meat application experiments. International journal of food microbiology, 83(2), 171-184.

[135] Biscola, V., Todorov, S. D., Capuano, V. S. C., Abriouel, H., Gálvez, A., & Franco, B. D. G. M. (2013). Isolation and characterization of a nisin-like bacteriocin produced by a *Lactococcus lactis* strain isolated from charqui, a Brazilian fermented, salted and dried meat product. Meat Science, 93(3), 607-613.

[136] Fangio, M. F., & Fritz, R. (2014). Potential use of a bacteriocin-like substance in meat and vegetable food biopreservation. International Food Research Journal, 21(2), p677-683.

[137] Toldrá, F. (2008). Biotechnology of flavor generation in fermented meats. In *Meat Biotechnology*. Springer New York. 9, 199-215.

[138] Choyam, S., Lokesh, D., Kempaiah, B. B., & Kammara, R. (2015). Assessing the antimicrobial activities of Ocins. Frontiers in microbiology, 6: 1034.

[139] Ananou, S., Garriga, M., Hugas, M., Maqueda, M., Martínez-Bueno, M., Gálvez, A., & Valdivia, E. (2005). Control of *Listeria monocytogenes* in model sausages by enterocin AS-48. International journal of food microbiology,103(2), 179-190.

[140] Pai, Aravinda, G. K. Sudhakar, and Venkatesh Kamath (2013). "Enzybiotics-A Review." International Journal of Pharmacological Research 3 (4), 69-71.

[141] Salar, R. K., Gahlawat, S. K., Siwach, P., & Duhan, J. S. (2014).Biotechnology: Prospects and Applications. Springer India. 197–215.

[142] Kamarajan, P., Hayami, T., Matte, B., Liu, Y., Danciu, T., Ramamoorthy, A., Worden, F., Kapila, S. and Kapila, Y., (2015). Nisin ZP, a bacteriocin and food preservative, inhibits head and neck cancer tumorigenesis and prolongs survival. PloS one, 10(7), p.e0131008.

[143] Dobson, A., Cotter, P. D., Ross, R. P., & Hill, C. (2012). Bacteriocin production: a probiotic trait?. Applied and environmental microbiology, 78(1), 1-6.

[144] Kuipers, O. P., de Ruyter, P. G., Kleerebezem, M., & de Vos, W. M. (1998). Quorum sensing-controlled gene expression in lactic acid bacteria. Journal of Biotechnology, 64(1), 15-21.

[145] Chanos, P., & Mygind, T. (2016). Co-culture-inducible bacteriocin production in Lactic Acid Bacteria. Applied microbiology and biotechnology, 100(10), 4297-4308.

[146] van der Ploeg, J. R. (2005). Regulation of bacteriocin production in Streptococcus mutans by the quorum-sensing system required for development of genetic competence. Journal of Bacteriology, 187(12), 3980-3989.

[147] Sturme, M. H., Francke, C., Siezen, R. J., de Vos, W. M., & Kleerebezem, M. (2007). Making sense of quorum sensing in *lactobacilli:* a special focus on *Lactobacillus plantarum* WCFS1. Microbiology, 153(12), 3939-3947.

[148] Quadri, L. E. (2002). Regulation of antimicrobial peptide production by autoinducer-mediated quorum sensing in Lactic Acid Bacteria. Antonie Van Leeuwenhoek, 82(1-4), 133-145.

[149] Nazzaro, F., Fratianni, F., & Coppola, R. (2013). Quorum sensing and phytochemicals. International Journal of Molecular Sciences, 14(6), 12607-12619.

[150] Pitcher, D.G., N.A. Saunders, and R.J. Owen, (1989). Rapid extraction of bacterial genomic DNA with guanidium thiocyanate. Lett. Applied Microbiology, 8, 151–156.

[151] Dubernet, S.; Desmasures, N.; Guéguen, M. (2002). A PCR-based method for identification of lactobacilli at the genus level. FEMS Microbiology, 214, 271–275.

[152] Berthier, F., Ehrlich, S. D., (1998). Rapid species identication within two groups of closely related Lactobacilli using PCR primers that target the 16S/23S rRNA spacer region. FEMS Microbiol. 161, 97-106.

[153] Marekova M., Laukova A., De Vuyst L., Skaugen M., Nes I.F. (2003) : Partial characterization of bacteriocins produced by environmental strain *Enterococcus faecium* EK13. Journal of Applied Microbiology, 94, 523-530.

[154] Skalka, B., Pillich, J. & Pospisil, L. (1983). Further observations on *Corynebacteriurn* renale as an indicator organism in the detection of exfoliationpositive strains of *Staphylococcus aureus*. Zbl. Bakt. Hygiene, 256 (a), 168-174.

[155] Thermo Fisher Scientific - Proteinase K, recombinant, PCR grade - Thermo Fisher Scientific. (2016). Thermofisher.com. Retrieved 26 May 2016, from https://www.thermofisher.com/order/catalog/product/EO0491

[156] Mayr-Harting, A., Hedgdes, A. J., Berkeley, R. (1972). Methods for studying bacteriocins. In: Norris, J. R.; Ribbons, D. W. (Eds.) Methods in Microbiology. New York: Academic Press, v. 7a, p. 313-342.

[157] De Vuyst, L., Callewaert, R. & Pot, B. (1996). Characterizatioan and antagonistic activity of *Lactobacillus amylovorus* DCE471 and large scale isolation of its bacteriocin amylovorin L471. Systematic and Applied Microbiology, 19, 9-20.

[158] Stern, N. J., Svetoch, E. A., Eruslanov, B. V., Perelygin, V. V., Mitsevich, E. V., Mitsevich, I. P., & Seal, B. S. (2006). Isolation of a *Lactobacillus salivarius* strain and purification of its bacteriocin, which is inhibitory to *Campylobacter jejuni* in the chicken gastrointestinal system. Antimicrobial agents and chemotherapy, 50(9), 3111-3116.

[159] Musikasang, H., Tani, A., H-kittikun, A., & Maneerat, S. (2009). Probiotic potential of lactic acid bacteria isolated from chicken gastrointestinal digestive tract. World Journal of Microbiology and Biotechnology, 25(8), 1337-1345.

[160] Messaoudi, S., Kergourlay, G., Rossero, A., Ferchichi, M., Prévost, H., Drider, D. & Dousset, X. (2011). Identification of *lactobacilli* residing in chicken ceca with antagonism against *Campylobacter*. International Microbiology, 14(2), 103-110.

[161] Musikasang, H., Sohsomboon, N., Tani, A., & Maneerat, S. (2012). Bacteriocin-producing lactic acid bacteria as a probiotic potential from Thai indigenous chickens. Czech Journal of Science, 57, 137-149.

[162] Sangsoponjit, S., & Soytong, K. (2013). Characterization of Lactic Acid Bacteria from gastrointestinal tracts of chickens for feed production. 563-570

[163] Noohi, N., Ebrahimipour, G., Rohani, M., Talebi, M., & Pourshafie, M. R. (2014). Phenotypic characteristics and probiotic potentials of *Lactobacillus spp.* isolated from poultry. Jundishapur journal of microbiology, 7(9).

[164] Alvarez-Cisneros, Y. M., Espunes, T. S., Wacher, C., Fernandez, F. J., & Alquicira, E. R. (2011). Enterocins: Bacteriocins with applications in the food industry. Science Against Microbial Pathogens: Communicating Current Research and Technological Advances. Mendez-Vilas, A.(ed.), 2, 1330-1341.

[165] Dzhakibaeva, G. T., Kebekbaeva, K. M., & Dzhobulayeva, A. K. (2015). Molecular genetic identification of lactic acid bacteria on the basis of the analysis of the nucleotide sequences of 16S RRNA GENE. Agriculture and Food. 3 (1), 1314-8591.

[166] Tomé, E., Todorov, S. D., Gibbs, P. A., & Teixeira, P. C. (2009). Partial characterization of nine bacteriocins produced by lactic acid bacteria isolated from cold-smoked salmon with activity against *Listeria monocytogenes*. Food Biotechnology, 23(1), 50-73.

[167] Strompfová, V., Lauková, A., & Mudroňová, D. (2003). Effect of bacteriocin-like substance produced by *Enterococcus faecium* EF55 on the composition of avian gastrointestinal microflora. Acta Veterinaria Brno, 72(4), 559-564.

[168] Carvalho, A. T., Paula, R. A., Mantovani, H. C., & de Moraes, C. A. (2006). Inhibition of *Listeria monocytogenes* by a lactic acid bacterium isolated from Italian salami. Food microbiology, 23(3), 213-219.

[169] Savino, F., Cordisco, L., Tarasco, V., Locatelli, E., Di Gioia, D., Oggero, R., & Matteuzzi, D. (2011). Antagonistic effect of *Lactobacillus* strains against gas-producing coliforms isolated from colicky infants. BMC microbiology, 11(1), 1.

[170] Schillinger, U. and F.K. Lucke, (1989). Antibacterial activity of *Lactobacillus sakei* isolated from meat. Applied Environmental Microbiology, 55, 1901-1906.

[171] Vignolo, G. M., Suriani, F., Holgado, A. P. D. R., & Oliver, G. (1993). Antibacterial activity of *Lactobacillus* strains isolated from dry fermented sausages. Journal of Applied Bacteriology, 75(4), 344-349.

[172] Rantsiou, K., Urso, R., Iacumin, L., Cantoni, C., Cattaneo, P., Comi, G., & Cocolin, L. (2005). Culture-dependent and-independent methods to investigate the microbial ecology of Italian fermented sausages. Applied and Environmental Microbiology, 71(4), 1977-1986.

[173] Schved F, Lalazar A, Henis Y, Junen J, (1993) Purification, partial characterization and plasmid–linkage of pediocin SJ-1, a bacteriocin produced by *Pediococcus acidilactici*. Journal Applied of Bacteriology, 74, 67-77.

[174] Jones, R. J., Hussein, H. M., Zagorec, M., Brightwell, G., & Tagg, J. R. (2008). Isolation of lactic acid bacteria with inhibitory activity against pathogens and spoilage organisms associated with fresh meat. Food Microbiology, 25(2), 228-234.

[175] Arena, M. P., Silvain, A., Normanno, G., Grieco, F., Drider, D., Spano, G., & Fiocco, D. (2016). Use of *Lactobacillus plantarum* Strains as a Bio-Control Strategy against Food-Borne Pathogenic Microorganisms. Frontiers in Microbiology, 7, 464.

[176] Al-Allaf, M. A. H., Al-Rawi, A. M. M., & Al-Mola, A. T. (2009). Antimicrobial activity of lactic acid bacteria isolated from minced beef meat against some pathogenic bacteria. Iraqi Journal of Veterinary Sciences, 23 (1), 115-117.

[177] Lü, X., Yi, L., Dang, J., Dang, Y., & Liu, B. (2014). Purification of novel bacteriocin produced by *Lactobacillus coryniformis* MXJ 32 for inhibiting bacterial foodborne pathogens including antibiotic-resistant microorganisms. Food Control, 46, 264-271.

[178] De Kwaadsteniet, M., Todorov, S.D., Knoetze, H., Dicks, L.M.T., (2005). Characterization of a 3944 Da bacteriocin, produced by *Enterococcus mundtii* ST15, with activity against Gram-positive and Gram-negative bacteria. International Journal of Food Microbiology 105, 433–444.

[179] Line, J. E., Svetoch, E. A., Eruslanov, B. V., Perelygin, V. V., Mitsevich, E. V., Mitsevich, I. P. & Stern, N. J. (2008). Isolation and purification of enterocin E-760 with broad antimicrobial activity against gram-positive and gram-negative bacteria. Antimicrobial Agents and Chemotherapy, 52(3), 1094-1100.

[180] Liu, G., Ren, L., Song, Z., Wang, C., & Sun, B. (2015). Purification and characteristics of bifidocin A, a novel bacteriocin produced by *Bifidobacterium animals* BB04 from centenarians' intestine. Food Control, 50, 889-895.

[181] Rahman, M. S. (2007). Handbook of food preservation. Rahman, M. S. CRC press. London, 11, 238-259.

[182] Perez, R. H., Perez, M. T. M., & Elegado, F. B. (2015). Bacteriocins from Lactic Acid Bacteria: A Review of Biosynthesis, mode of action, fermentative production, uses, and prospects. International Journal of Philippine Science and Technology, 8(2), 61-67.

[183] Zhou, L., van Heel, A. J., Montalban-Lopez, M., & Kuipers, O. P. (2016). Potentiating the Activity of Nisin against *Escherichia coli.* Frontiers in Cell and Developmental Biology, 4, 1 - 7.

[184] Smid, E. J., & Gorris, L. G. (1999). Natural antimicrobials for food preservation. Food Science and Technology, 2, 285-308.

[185] Y. Gao, D. Li, Y. Sheng, X. Liu (2011). Mode of action of sakacin C2 against *Escherichia coli*. Food Control, 22, 657-662.

[186] Holo, H., Nilssen, O., and Nes, I. F. (1991). Lactococcin A, a new bacteriocin from *Lactococcus lactis* subsp. *cremoris:* isolation and characterization of the protein and its gene. Bacteriology. 173, 3879-3887.

[187] Perin, L. M., & Nero, L. A. (2014). Antagonistic lactic acid bacteria isolated from goat milk and identification of a novel nisin variant *Lactococcus lactis*. BMC Microbiology, 14(1), 1-6.

[188] Djadouni, F., & Kihal, M. (2012). Antimicrobial activity of lactic acid bacteria and the spectrum of their biopeptides against spoiling germs in foods. Brazilian Archives of Biology and Technology, 55(3), 435-444.

[189] Wilson, A. R., Sigee, D., & Epton, H. A. S. (2005). Anti-bacterial activity of *Lactobacillus plantarum* strain SK1 against *Listeria monocytogenes* is due to lactic acid production. Journal of Applied Microbiology, 99(6), 1516-1522.

[190] Campos, C. A., Castro, M. P., Rivas, F. P., & Schelegueda, L. I. (2013). Bacteriocins in food: evaluation of the factors affecting their effectiveness, 2(1), 994 – 1004.

[191] Maldonado-Barragán, A., Caballero-Guerrero, B., Martín, V., Ruiz-Barba, J. L., & Rodríguez, J. M. (2016). Purification and genetic characterization of gassericin E, a novel co-culture inducible bacteriocin from *Lactobacillus gasseri* EV1461 isolated from the vagina of a healthy woman. BMC Microbiology, 16(1), 1.

[192] Franz, C.M.A.P., Schillinger, U. and Holzapfel, W.H.(1996) Production and characterization of enterocin 900, a bacteriocin produced by *Enterococcus faecium* BFE 900 from black olives. International Journal of Food Microbiology, 29, 255–270.

[193] Ennahar, S., Sashihara, T.,Sonomoto, K. andIsahizaki, A. (2001) Class IIa bacteriocins; biosynthesis, structure and activity. FEMS Microbiology Review, 24, 85–106.

[194] Giraffa, G. (2003). Functionality of *enterococci* in dairy products. International Jounal of Food Microbiology, 88, 215–222.

[195] A.L. Pinto, M. Fernandes, C. Pinto, H. Albano, F. Castilho, P. Teixeira, P.A. Gibbs. (2009) Characterization of anti-*Listeria* bacteriocins isolated from shellfish: Potential antimicrobials to control non-fermented seafood. International Jounal of Food Microbiology, 129, 50–58.

[196] C. Ibarguren, R.R. Raya, M.C. Apella, M.C. (2010). Audisio. *Enterococcus faecium* isolated from honey synthesized bacteriocin-like substances active against different *Listeria monocytogenes* strains. International Jounal of Food Microbiology, 48, 44–52.

[197] E. Vera Pingitore, S.D. Todorov, F. Sesma, B.D.G.M. Franco. (2012). Application of bacteriocinogenic *Enterococcus mundtii* CRL35 and *Enterococcus faecium* ST88Ch in the 67 control of *Listeria monocytogenes* in fresh Minas cheese. Food Microbiology, 32, 38–47.

[198] Svetoch, E. A., Eruslanov, B. V., Perelygin, V. V., Mitsevich, E. V., Mitsevich, I. P., Borzenkov, V. N. & Siragusa, G. R. (2008). Diverse antimicrobial killing by *Enterococcus faecium* E 50-52 bacteriocin. Journal of Agricultural and Food Chemistry, 56(6), 1942-1948.

[199] Robyn, J., Rasschaert, G., Messens, W., Pasmans, F., & Heyndrickx, M. (2012). Screening for Lactic Acid Bacteria capable of inhibiting *Campylobacter jejuni* in in vitro simulations of the broiler chicken caecal environment.Beneficial Microbes, 3(4), 299-308.

[200] Chen, Y., & Bulter, F. (2014). Technology to reduce pathogens in meat. Biosystems Engineering Research Review 19,25.

[201] Dykes, G. A., M. Vegar, P. B. Vanderlinde. (2003). Quantification of *Listeria spp.* contamination on shell and flesh of cooked black tiger prawns (*Penaeus monodon*). Letters of Applied Microbiology, 37(4), 309-313.

[202] Vignolo, G., Palacios, J., Farías, M. E., Sesma, F., Schillinger, U., Holzapfel, W., & Oliver, G. (2000). Combined effect of bacteriocins on the survival of various *Listeria* species in broth and meat system. Current Microbiology,41(6), 410-416.

[203] Wang, F. S. (2000). Effects of three preservative agents on the shelf life of vacuum packaged Chinese-style sausage stored at 20 C. Meat science,56(1), 67-71.

[204] Park, J., & Kim, M. (2013). Comparison of dry medium culture plates for mesophilic aerobic bacteria in milk, ice cream, ham, and codfish fillet products. Preventive Nutrition and Food Science, 18(4), 269.

[205] Lück, E., & Jager, M. (1996). Antimicrobial food additives: characteristics, uses, effects. Springer, 2, 167 - 174.

Supplementary data

Bacteriocinogenic activity of *Lactobacilli* isolates against potential pathogenic microbiota present in poultry meat products

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Bacterial foodborne diseases are a constant concern to human health. During the last decade, the consumption of poultry meat products increased rapidly all over the world being indicated as source of the most reported pathogens *Campylobacter jejuni, Salmonella enteritidis, Listeria monocytogenes and Staphylococcus aureus* causing foodborne diseases [1].
Lactic acid bacteria (LAB) group are known by their bacteriocinogenic potential property and could be used to reduce the pathogenic bacteria colonization in foodstuff, reducing the foodborne illness risk to consumers [2]. The aim of this study was to evaluate the inhibitory effect of different *Lactobacillus sakei* and *L. plantarum* strains isolated from portuguese fermented meat products against pathogenic microbiota present in ready-to-cook poultry meat products. Fifteen *Lactobacillus* strains where tested using the Skalka method (1986) against different wild strains of *Campylobacter jejuni and C. coli and C. jejuni ATCC 11168, Listeria monocytogenes CECT 934, Salmonella enteritidis CECT 4300, E. avium (EA5, as indicator strain)* and *S. aureus* ATCC 25923. Seven different *Campylobacter* wild strains were isolated from ready to cook poultry meat products and identified by PCR as *C. coli* and *C. jejuni*. The strains of *Lactobacillus sakei* and *Lactobacillus plantarum* showed potencial bacteriocinogenic capability against the pathogenic microbiota tested. *L. sakei* strain CV3C8 exhibited the highest inhibitory activity against all the strains of *Campylobacter* tested. *L. plantarum* strains P3B7 and P05-67 showed higher inhibition against *Salmonella enteritidis CECT 4300* while the strain P05-67 presented best results against *S. aureus ATCC* 25923. *L. plantarum* strain P3B8 had the best inhibition results against *Listeria monocytogenes CECT 934*.
Further research will be developed with selected strains to know if the inhibitory effect was due to the production of inhibitory metabolites, such as organic acids, peptides or a combination of metabolites.

Keywords: *Lactobacillus*; food safety; bacteriocinogenic potential; *Campylobacter; Listeria monocytogenes; Salmonella.*

References:

[1] Al-Nehlawi, A., Guri, S., Guamis, B., Saldo, J. “Synergistic effect of carbon dioxide atmospheres and high hydrostatic pressure to reduce spoilage bacteria on poultry sausages” LWT – Food Science and Technology. 2014. Volume 58, Issue 2, Pages 404-411.

[2] Neal – Mckinney JM, Lu X, Duong T, Larson CL, Call DR, Shah DH, et al. Production of organic acids by probiotic lactobacilli can be used to reduce pathogen load in poultry. PloS One. 2012; 7(9): e43928. Doi: 10.1371/journal.pone. 0043928.

Effect of *EK13* enterocin on the survival of *Listeria innocua* in a meat model under different temperature fermentative conditions

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Bacteriocins are antimicrobial peptides produced by particular bacteria strains that kill or inhibit the growth of other bacteria (Begley et al. 2010). Many lactic acid bacteria (LAB) produce a high diversity of different bacteriocins, from these *Enterococci* produce peptides known as enterocins that attract considerable interest for their potential use as natural and nontoxic food preservatives. The aim of this study was to evaluate the effect of a partially purified enterocin produced by *Enterococcus faecium* EK13 on the growth of *L. innocua* and total and fermentative microbiota *in a* meat model mimicking different fermentative conditions (1st step at 7ºC and 2nd step at 20ºC) during 96 hours. *Enterococcus faecium* strain *EK13* was isolated from cattle dung water and produces two bacteriocins, enterocin A and P (Laukova et al.,2003). The meat was minced (1x1cm) being 25g aseptically weighted and inoculated with 1 ml of a suspension of *Listeria innocua* CECT 910 at approximately 7 log^{10} bacteria/ml. The meat model was stored at 7°C for 2 days and then the temperature was changed to 20°C for another 2days. The study was conducted with meat model under different conditions with and without bacteriocin: 1-control raw meat. 2- raw meat inoculated with *Listeria innocua* CECT 910; 3- raw meat inoculated with *Listeria innocua* CECT 910 and free enterocin at 0,1%. 4- raw meat with free enterocin at 0,1%.

Microbiological analysis was performed 1 hour after inoculation, 24h, 48h, 72h and 96h, for total aerobic microorganisms at 30ºC, *Listeria* counting, and lactic acid bacteria (LAB) counts according to International Standards ISO. The addition of enterocin to meat inhibits the *Listeria innocua* with a decrease on counting of almost 2 log cfu/g during the condition 7ºC during 48h. When the condition of temperature was changed to 20ºC it is notice an increase of *Listeria* counts but with less 2 log cfu/g when compared to the meat model only inoculated with Listeria (condition 2). The effect of the enterocin on total aerobic counts and LAB were not noticed.

The data obtained in this study provides useful insights on the influence of the enterocin produced by EK13 on the survival and/or growth of *L. innocua* demonstrating that this peptide could be used as an natural preservative to reduce *Listeria* on meat.

Keywords: EK13 enterocin; *Listeria innocua*; meat model.

References:

[1] Marekova M., Laukova A., De Vuyst L., Skaugen M., Nes I.F. (2003) : Partial characterization of bacteriocins produced by environmental strain *Enterococcus faecium* EK13. J. Appl. Microbiol., 94, 523-530.

[2] Begley, M., Cotter, P.D., Hill, C. and Ross, R.P. (2010) Glutamate decarboxylase-mediated nisin resistance in *Listeria monocytogenes*. Appl Environ Microbiol 76, 6541-6546.