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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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 *L. plantarum* 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 *L. plantarum* L1B8. *Lactobacillus* B6C1-3-3, 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

1. 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 will be 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.

In fact, meat and meat products are excellent food substrates for spoilage and pathogenic bacteria [4]. Meat is a source of proteins, fat, vitamins and minerals, and 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].

2. MATERIALS AND METHODS

2.1 Sampling and isolation of LAB

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.

Once in laboratory, 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 catalase 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) [9]. Quantification of extracted DNA was performed spectrophotometrically (NANODROP

2000c Spectrophotometer, Thermo Scientific). The DNA was stored at -80ºC until use.

2.2.2.2 *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 according to Dubernet, Desmasures and Guéden, (2002) [10].

2.3 *Lactobacillus* from FMV collection

Lactobacillus from FMV collection isolated from fermented meat products and processing environment (n=15, Table 1) were used in this study. *Lactobacillus species* have been previously identified by PCR methods based on the methodologies described by Berthier and Ehrlich (1998) [11].

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

Table 1 - Lactobacillus strains from FMV collection tested

2.4 *Enterococcus faecium* (EK13)

The strain *Enterococcus faecium* EK13 (Enterocin A and P producer) was isolated from cattle dung water (Marekova *et al.,* 2003) [12].

2.5 Indicator bacteria collection

Indicator bacteria used in this study are presented in Table 2.

Codification	Genus	Specie
ATCC 11168	*Campylobacter*	*jejuni*
118.09	*Campylobacter*	*jejuni*
P101 VI CFAI	*Campylobacter*	*coli*
J8.5	*Campylobacter*	*jejuni*
C5.5	*Campylobacter*	*jejuni*
Zim 140	*Campylobacter*	*coli*
P93 VI CFAI	*Campylobacter*	*coli*
CECT 4300	*Salmonella*	*enteritidis*
EA5	*Enterococcus*	*avium*
ATCC 25923	*Staphylococcus*	*aureus*
CECT 934	*Listeria*	*monocytogenes*
CECT 910	*Listeria*	*innocua*

Table 2 - Indicator bacteria collection for testing

2.6 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 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.

2.7 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*. (Skalka, Pillich & Pospisil, 1983) [13].

In this study was used the strain *Enterococcus avium* (EA5) sensitive to bacteriocins and was used *Enterococcus faecium* EK13 as positive control.

All *Lactobacillus* isolated 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.*

2.7.1 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 1 describe the protocol use for testing bacteriocinogenic potential of isolates under study.

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

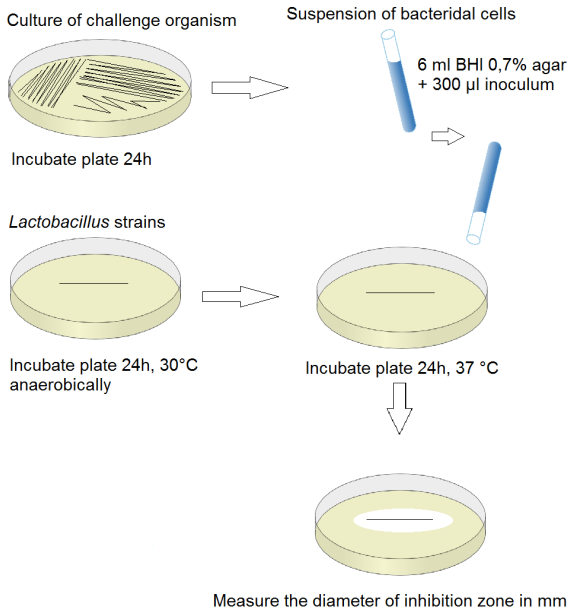

Figure 1 - Diagram procedure for lactic acid bacteria bacteriocinogenic potential activity evaluation (Original).

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.

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

2.8 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.

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 5mm 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. A proteinaceous nature of produced substances can be considered if they were sensitive to proteinase K enzyme.

Lactobacillus isolates were selected to this test 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.

2.9 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_{600nm} = 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 [12]).

The BLIS obtained was lyophilized in a ScanVac Freeze Dryers (CoolSafe model, Denmark) and stored at ambient temperature to subsequent work.

3 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 [14]. 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 a period of incubation, the areas of inhibition are observed. The results were 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.

4 *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 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. 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.

4.1 Preparation of the *Listeria innocua* CECT 910 inocula and growth conditions

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.

4.2 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).

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 BLIS; 4- raw meat with BLIS. The same design was performed to different concentrations of BLIS on meat (0.1% and 0.5%).

In condition 2 and 3 the bag with raw meat was 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 25g of raw meat was inoculated with a 1 ml of BLIS suspension with a concentration of 2.5% to obtain a final concentration of 0.1% in meat. The same procedure was done with a BLIS suspension with a concentration of 12.5% to obtain a final concentration of 0.5% in meat.

4.3 Microbiological analysis

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).

Assays were done in triplicate for each BLIS concentration contemplating the same procedures.

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 Microsoft Excel 2011 program (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 $P < 0.05$.

6 RESULTS AND DISCUSSION

6.1 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). From the initial 144 isolates collected from the slaughterhouse, only 65.30% (n=94) were identified as *Lactobacillus*.

6.2 Evaluation of *Lactobacillus* bacteriocinogenic potential

6.2.1 *Lactobacillus* isolates collection from the slaughterhouse

The antimicrobial activity of the isolates was evaluated by measuring the diameter of the inhibition zones.

From the 94 *Lactobacillus* collected in a slaughterhouse was observed that all presented bacteriocinogenic activity against the microorganisms tested. *Lactobacillus* isolates (n=94) were tested against *Enterococcus avium* (EA5) and 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. *Lactobacillus* isolates (n=94) were also tested against *L. monocytogenes* (CECT 934) and 1% presented an inhibition zone of >10 and <15mm of diameter, 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.

Isolate *Lactobacillus* B6C1-3-3 had the great potential to inhibit *L. monocytogenes* CECT 934 while isolate *Lactobacillus* B6C2-1-2 presented

the higher inhibition zone against *Enterococcus avium* (EA5).

6.2.2 *Lactobacillus* strains from FMV collection and *Enterococcus faecium* EK13

In view of the results for bacteriocinogenic potential activity, all strains presented bacteriocinogenic activity against all indicators tested.

L. sakei CV3C8 exhibited the highest inhibitory activity against all strains of *Campylobacter* tested (n=7), *L. plantarum* strains P3B7 and P05-15 showed higher inhibition against *Salmonella enteritidis CECT 4300* while the strain *L. plantarum* P3B8 presented best results against Enterococcus avium (EA5). *L. plantarum* P05-67 presented best results against *S. aureus ATCC* 25923 followed by *L. plantarum* L1B8. Isolate *L. plantarum* P3B8 also showed the highest halos against *Listeria* followed by *L. plantarum* P05-15.

Enterococcus faecium EK13 presented bacteriocinogenic activity against all indicators but showed higher inhibition against *Listeria monocytogenes* CECT 934.

6.3 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 lactic acid bacteria studied (both collection from FMV and the ones from slaughterhouse) showed inhibition caused by an organic acid or other non-protein compound.

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

6.4 Bacteriocinogenic activity quantification of BLIS EK13

Using the quantitative dilution modified method was verified that BLIS produced by *Enterococcus* EK13 inhibited *Listeria monocytogenes* CECT 934, Listeria innocua CECT 910 and *Enterococcus avium* EA5 forming a clear zone of inhibition in the place of inoculation droplets.

BLIS EK13 showed no inhibitory capacity against the Gram-negative isolates of *Salmonella* and *Campylobacter* tested.

The maximum activity of this bacteriocin (51200 UA ml^{-1}) was observed against *Listeria innocua* CECT 910.

6.5 Meat fermentation model

The initial counts of total microorganisms at 30ºC on meat model were approximately 4 log cfu. g^{-1} day 0 even under the effect of BLIS introduced 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.

The initial counts of LAB in meat models were 3-4 log cfu. g^{-1} at day 0. BLIS introduced in meat model did not produce any inhibitory effect on LAB counts.

The evolution of *Listeria innocua* in meat model with 0.1% and 0.5% EK13 BLIS storage over time (24h, 48h, 72h and 96h) is presented on figure 3.

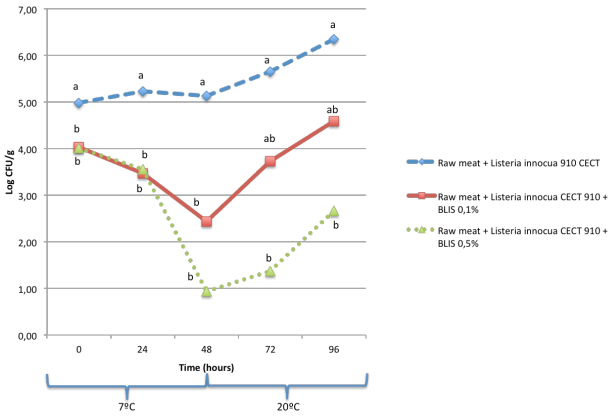

Figure 3 – Influence of EK13 BLIS on *Listeria innocua* growth inoculated in a meat model.

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 added to meat. The addition of free BLIS 0.1% reduced *Listeria innocua* counts from 4 log cfu. g^{-1} to 2.4 cfu. g^{-1} and 0.5% BLIS 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 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}.

7 CONCLUSION

Results showed great inhibitory effect of all analysed LAB against the food pathogens tested. Using the Skalka method were 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.

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