Effect of *Enterococcus faecium EK13* bacteriocin-like inhibitory substances at differents concentrations on the survival of *Listeria innocua* in a meat model

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Abstract – The antilisterial effect of Bacteriocin-like inhibitory substances (BLIS) produced by *Enterococcus faecium* EK13 were evaluated in a meat model mimicking different fermentative conditions (1st step at 7ºC and 2nd step at 20ºC) during 96 hours. *Listeria innocua* was tested using two different concentrations (0.1% and 0.5%) of BLIS on meat. An inhibitory effect on *L. innocua* was observed using BLIS on meat at 0.5% with a significant difference of approximately 4 log cfu/g when compared with the meat model inoculated after 48h at 7ºC and after 96h at 20ºC.

When the temperature changed from 7ºC to 20ºC, the growth rate of *Listeria* after 96h under the effect of BLIS at 0.1% and 0.5% were the same but a difference of approximately 2 log CFU.g^{-1} was observed between these conditions.

Key Words – EK13 BLIS; *Listeria innocua*; meat safety.

I. INTRODUCTION

In recent years, bacteriocins have attracted increasing interest for their use as biopreservatives in food industry. They are used as food additives according to their GRAS (Generally Regarded as Safe) characteristics [1]. Lactic acid bacteria (LAB) can produce a high diversity of different bacteriocins, which comprise a huge family of ribosomally synthesized peptides that have antibacterial activity towards closely related strains [2].

Enterococcus species are producers of BLIS able to inhibit Gram-positives such as *L. monocytogenes* and some Gram-negatives in a lesser degree [1]. *Enterococcus faecium* strain *EK13* used in this study was isolated from cattle dung water and produces two bacteriocins, A and P [3]. Thus, the present study aimed to evaluate its mode of action against *L. monocytogenes*, an important foodborne pathogen, as a potential biopreservative used in dry fermented meat products with different temperature steps of fermentation.

II. MATERIALS AND METHODS

Enterococcus faecium EK13 BLIS production

E. faecium EK13 (CCM7419) strain was stored at -80ºC in Brain Heart Infusion broth (Scharlau, Spain) containing 15% (v/v) glycerol and propagated in MRS medium at 30ºC for 24h before use. The production of BLIS from *Enterococcus faecium* EK13 (EK13 BLIS) was carried out by ammonium sulphate precipitation. For that, the *E. faecium* EK13 culture was grown for 48h at 37ºC in TSA medium. Then, 500 ml of MRS broth (Merck) were inoculated with a freshly prepared *E. faecium* EK13 culture and incubated for 16h at 37ºC until an OD_{625} of 1.6. Then the EK13 culture was centrifuged for 30 min at 10 000 x g to remove the cells. The pH of the supernatant was adjusted to 5, ammonium sulphate was added to the supernatant (40% saturation, w/v and the mixture was stirred at 4°C for 2-7h. After centrifugation at 10 000 x g for 30 min, the resulting pellet was re-suspended in 10 mM phosphate buffer (pH 5.0) and frozen at -20°C [3]. The partially purified bacteriocin was then lyophilized in a ScanVac Freeze

Dryers (CoolSafe model, Denmark) and stored at ambient temperature to subsequent work.

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

Listeria innocua CECT 910 used in this study were maintained as stock culture at -80ºC in Brain Heart Infusion broth (Scharlau, Spain) containing 15% (v/v) glycerol.
Culture was prepared by growing the isolate in TSA 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} $CFU.ml^{-1}$.

Meat samples preparation and inoculation procedure

The meat was minced (1x1cm) and twenty five grams were 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 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 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 with different concentrations of BLIS in 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 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.

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* counting, and lactic acid bacteria (LAB) counts according with the methods proposed by ISO (International Organization of Standardization).
Assays were done in triplicate for each BLIS concentration contemplating the same procedures.

Statistical Analysis

For data analysis the Microsoft Excel 2011 program and Statistical Package for Social Sciences (SPSS) software - version 22, were used. 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 $P < 0.05$.

III. RESULTS AND DISCUSSION

The initial counts of total microorganisms at 30ºC in the meat model were approximately 4 log $cfu.g^{-1}$ on 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 5 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 2-3 log $cfu.g^{-1}$ at day 0. BLIS introduced in the meat model did not produce any inhibitory effect on LAB counts.

The evolution of *Listeria innocua* in the meat model with 0.1% and 0.5% EK13 BLIS stored over time (24h, 48h, 72h and 96h) is presented on figure 1.

The initial counts of *Listeria innocua* in 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 noticed 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 in meat with 0.5% and 0.1% of BLIS in the described storage conditions, presented a difference of approximately 2 log cfu.g^{-1}.

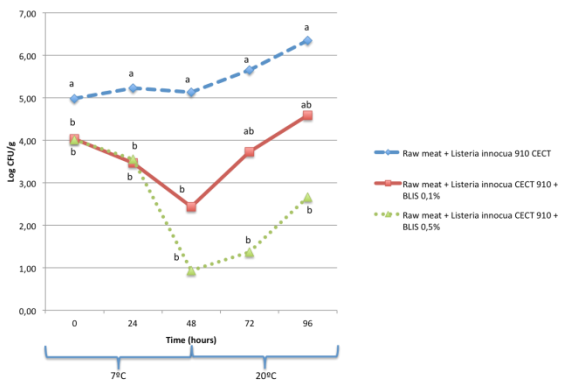

Figure 1 – Influence of EK13 BLIS on *Listeria innocua* growth innoculated in a meat model.

IV. CONCLUSION

EK13 BLIS shows a good capacity to inhibit *Listeria innocua* and does not have any inhibitory effects on total mesophilic microbiota and LAB.
The data obtained in this study provides useful insights on the influence of the BLIS produced by *Enterococcus faecium* EK13 on the survival and⁄or growth of *L. innocua* demonstrating that this peptide could be used as a natural preservative to reduce and control *Listeria spp.*, particularly *L. monocytogenes*, on fermented meat sausage processing.

ACKNOWLEDGEMENTS

This study was conducted with the financial support of ‘‘Centro de Investigação Interdisciplinar em Sanidade Animal’’ (CIISA/FMV) from Faculdade de Medicina Veterinária (Faculty of Veterinary Medicine, University of Lisbon), by project “Portuguese traditional meat products: strategies to improve safety and quality” (PTDC/AGR-ALI/119075/2010) and Project UID/CVT/00276/2013 financed by Fundação para a Ciência e a Tecnologia (FCT).

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