EFFECT OF *Leuconostoc mesenteroides* 11 BACTERIOCIN IN THE MULTIPLICATION CONTROL OF *Listeria monocytogenes* 4b[1]

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SUMMARY

The activity of a crude preparation of bacteriocin produced by the chicken meat isolate *Leuconostoc mesenteroides* 11, was evaluated at 8ºC and 15ºC against *Listeria monocytogenes*. The pathogen was inoculated in a crude preparation of the bacteriocin and its population was enumerated after 0.5 and 10 days. The title of the bacteriocin in the preparation was determined immediately before inoculation and after 10 days of incubation at both temperatures. As a negative control, a non-bacteriocin producing strain, *Leuconostoc mesenteroides* A13, was used. Bacteriocin of *L. mesenteroides* 11 partially inhibited *L. monocytogenes* at 8ºC, but at 15ºC it was unable to prevent growth of the pathogen. Our findings suggest that the use of the semi-purified bacteriocin of *L. mesenteroides* 11 probably will not be suitable as a single hurdle to prevent *L. monocytogenes* growth in foods.
Keywords: *Listeria monocytogenes*, *Leuconostoc mesenteroides*, bacteriocin, lactic acid bacteria.

RESUMO

EFEITO DA BACTERIOCINA DE *Leuconostoc mesenteroides* 11 NO CONTROLE DA MULTIPLICAÇÃO DE *Listeria monocytogenes* 4b. A atividade de uma preparação bruta de bacteriocina produzida por *Leuconostoc mesenteroides* 11, isolado de peito de frango, foi avaliada a 8ºC e 15ºC, contra *Listeria monocytogenes*. O patógeno foi inoculado em uma preparação bruta da bacteriocina e sua população foi enumerada depois de 0,5 e 10 dias de incubação. O título da preparação de bacteriocina foi determinado no tempo 0 e após 10 dias de incubação em ambas as temperaturas. Como controle negativo, foi utilizada uma cepa não produtora de bacteriocina, *Leuconostoc mesenteroides* A13. A bacteriocina de *L. mesenteroides* 11 inibiu parcialmente *L. monocytogenes* a 8ºC, mas a 15ºC a bacteriocina não foi capaz de impedir a multiplicação do patógeno. Nossos resultados sugerem que o uso da bacteriocina semi-purificada de *L. mesenteroides* 11 provavelmente não será adequada como único obstáculo para impedir a multiplicação de *L. monocytogenes* em alimentos.
Palavras-chave: *Listeria monocytogenes*, *Leuconostoc mesenteroides*, bacteriocina, bactérias láticas.

1 - INTRODUCTION

Nowadays, consumers are demanding natural and safe foods with no additives, reduced quantities of salt and no need to be cooked [9]. However, the emergence of psychrotrophic foodborne pathogens is an important cause of concern. To guarantee the safety of minimally processed foods, there is an intense research on natural antimicrobials derived from animals, plants and microorganisms. Bacteriocins are natural antimicrobials produced by bacteria and in general, present groups of twenty to forty amino acid residues, that are positively charged and are hydrophobic and/or amphiphylics [14]. These antimicrobial peptides present high potential to inhibit the psychrotrophic bacterium *Listeria monocytogenes*, that causes serious illnesses in imunocompromised individuals and expectant mothers. *L. monocytogenes* is able to survive at pH as low as 3.6 in foods and in salt concentration of up to 10%, in the presence of surfactants, sanitizers and after several cycles of freezing and thawing [1, 5, 15, 22]. *L. monocytogenes* has emerged in the last twenty years from relative microbial obscurity to become an important foodborne pathogen of humans [21].

Bacteriocins with activity against *L. monocytogenes*, have attracted great interest in the past ten years in food science area. Particularly, bacteriocins from lactic acid bacteria (LAB) are very important because they constitute a group of industrial microorganisms that may improve sensorial properties, shelf life and safety of foods [18].

Different mechanisms of action have been proposed for bacteriocins: alteration of enzymatic activity, inhibition of spore germination and inactivation of anionic carriers through the formation of selective and non-selective pores. The formation of pores induces an efflux of intracellular substances of low molecular weight and causes the loss of citoplasmatic ATP, with consequent dissipation of proton-motive force, resulting in inactivation or cellular death [3, 16].

To implement the use of antimicrobial peptides for the biopreservation of foods stored under refrigeration, it is highly desirable to use bacterial strains that produce bacteriocin in the early phases of growth, have psychrotrophic nature and do not produce off-flavors. Moreover, bacteriocin should not interfere with other microorganisms used as starter cultures or be inactivated by food compounds [13].

In this work, the antilisterial activity of a semi-purified bacteriocin preparation of *L. mesenteroides* 11 was studied to estimate its potential of use in food systems.

2 - MATERIALS AND METHODS

2.1 - Bacterial Strains

L. monocytogenes ATCC 19151, serotype 4b was maintained at -70ºC in Brain Heart Infusion broth (BHI - Oxoid) supplemented with 20% (v/v) of glycerol (Merck).

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Bacteriocin-producing *L. mesenteroides* 11 was previously isolated by our research group from breast chicken [6]. The meat isolate *L. mesenteroides* A13 was used as a negative control for bacteriocin production. Both LAB strains were maintained at -70ºC in MRS broth supplemented with 20% (v/v) of glycerol.

2.2 - Evaluation of the antilisterial activity of the bacteriocin produced by *L. mesenteroides* against *L. monocytogenes*

L. mesenteroides 11 was inoculated at ca. 10^5 CFU/mL (1%, v/v) in 50 mL of BHI broth and incubated for 24 h at 25ºC. The broth culture was centrifuged at 4ºC at 6,720 g during 25 min (Sorvall RC Plus, Du Pont, USA). The supernatant fluid was neutralized to pH 7.0 and filter sterilized through membrane 0.22 µm with low capacity of binding proteins (GVWP, Millipore). The filtrate obtained was designated as "crude preparation of bacteriocin". The negative control *L. mesenteroides* A13 was treated following the same procedure.

An overnight culture of *L. monocytogenes* in BHI broth was inoculated (0.01%, v/v) into the crude preparation of bacteriocin, to yield ca. 10^5 CFU/mL and incubated at 8ºC and 15ºC to simulate refrigeration and abuse temperature for meat storage, respectively. The populations of *L. monocytogenes* were enumerated on triptone soy agar (TSA, Oxoid) plus 0.6% yeast extract (Oxoid) – TSAYE, after 0.5 and 10 days of incubation. The serial dilutions were surface plated, using "Spiral Plater", model D, K & R Technology, USA. The title of the bacteriocin in crude preparation was determined after 0 and 10 days, by the serial twofold dilution assay method, according to MAYR-HARTING, HEDGES and BERKELEY [12]. An overlay of BHI semi-solid agar (BHI broth plus 0.8% (w/v) of bacteriological agar - Oxoid) seeded with ca. 10^5 CFU/mL of *L. monocytogenes* was used as indicator layer. The titre was defined as the reciprocal of the highest dilution yielding an halo of inhibition of the indicator strain multiplied by 100 to express the results as arbitrary units per mL (AU/mL).

The same kind of test was performed using *L. monocytogenes* recovered from crude preparation on day 10, after bacteriocin exposition. For this, the inoculated crude preparation was centrifuged, washed twice with sodium chloride 0.85% (w/v) to remove traces of the inhibitory peptide and another critical dilution assay was done using those cells as indicator. This aimed to clarify if viable cells possibly present at the end of experiments were only survivors or resistants.

Three independent replicates of each experiment were done at each temperature.

Comparisons among different experimental groups were performed using three-way analysis of variance (ANOVA). Whenever appropriate, Post-Hoc Tuckey-HSD test for comparing group means [8] was also used. The level of significance was set at $p<0.05$ for all tests.

3 - RESULTS AND DISCUSSION

3.1 - Results

At 8ºC, for *L. monocytogenes* enumeration right after its inoculation 4.5 (±0.2) and 5.2 (±0.0) log CFU/mL were present in crude preparations from *L. mesenteroides* 11 and *L. mesenteroides* A13, respectively. Bacteriocin activity was 42.00±12.000 AU/mL in the broth prepared with *L. mesenteroides* 11 (*Figure 1*). Nonetheless, spots of growth of the indicator microorganism inside the halos of inhibition were observed.

On the fifth day of incubation at 8ºC, *L. monocytogenes* population decreased to 3.6 (±0.2) log CFU/mL in the presence of the bacteriocin, compared to 7.4 (±0.2) log CFU/mL for the negative control.

After ten days of maintenance at 8ºC, the population of *L. monocytogenes* was of 6.4 (±0.2) log CFU/mL in the crude preparation of bacteriocin of *Ln. mesenteroides* 11 and 8.4 (±0.1) log CFU/mL with *L. mesenteroides* A13. The title of *L. mesenteroides* 11 bacteriocin remained unchanged (*Figure 1*).

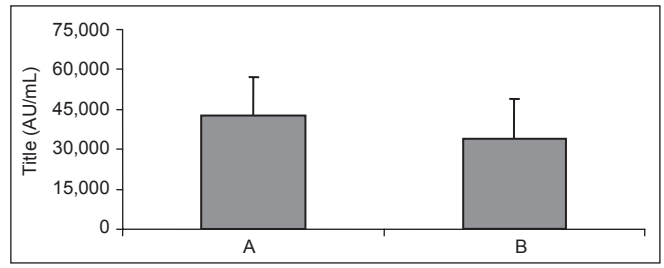

FIGURE 1 – Activity of *L. mesenteroides bacteriocin* (AU/mL) in crude preparation, using *L. monocytogenes* 4b as indicator. A: Immediately after inoculation; B: After ten days at 8ºC

In experiments performed at 15ºC, counts of *L. monocytogenes* right after its inoculation were 4.6 (±0.2) log CFU/mL in the presence of *L. mesenteroides* 11 bacteriocin and 4.9 (±0.3) log CFU/mL for negative control. The average title (AU/mL) of bacteriocin preparation is shown in *Figure 2*. The presence of growth points inside the halos of inhibition was also observed under this condition.

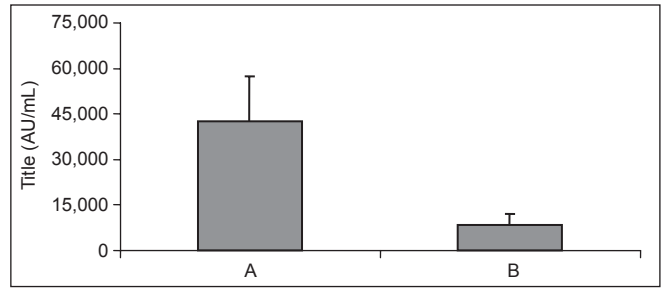

FIGURE 2 – Activity of *L. mesenteroides bacteriocin* (AU/mL) in crude preparation, using *L. monocytogenes* 4b as indicator. A: Immediately after inoculation; B: After ten days at 15ºC

On the fifth day at 15ºC, the population of *L. monocytogenes* quickly increased and reached the stationary phase regardless the presence of bacteriocin, with 8.8 (±0.4) log

CFU/mL in the broth containing the bacteriocin of *L. mesenteroides* 11 and 8.6 (±0.4) log CFU/mL in its absence.

On the tenth day at 15ºC, the number of cells remained stead and *L. monocytogenes* populations were of 8.7 (±0.5) log CFU/mL in the broth prepared from *L. mesenteroides* 11 and 8.6 (±0.6) log CFU/mL in the negative control. The title of the crude preparation of bacteriocin was lower than initial levels and it varied from 6.400 to 12.800 AU/mL (*Figure 2*). Growth spots of *L. monocytogenes* were also observed inside the halos of inhibition under these conditions.

When *L. monocytogenes* cells previously exposed to *L. mesenteroides* 11 bacteriocin for ten days, maintained at 8ºC and 15ºC, they were employed as indicators in critical dilution assays, no inhibition was observed.

Despite the development of resistant variants, statistically significant lower *L. monocytogenes* populations were obtained in the presence of bacteriocin at 8ºC, when compared to the results of experiments carried out simulating abuse temperature (15ºC).

3.2 - Discussion

In this work, the antilisterial activity of a crude preparation of *L. mesenteroides* 11 bacteriocin was evaluated. Bacteriocins produced by *Leuconostoc sp* with activity against *L. monocytogenes* were also studied by other authors [11, 17, 20]. SCHILLINGER, BECKER and HOLZAPFEL [20] tested the antilisterial activity of carnocin 54, a bacteriocin produced by *Leuconostoc carnosum* L454A isolated from vacuum-packaged meat product. In that work, all *Listeria* cells were inhibited in agar spot assays, but the bactericidal efficiency varied among different *L. mesenteroides* strains. Those authors also observed that log and stationary phase cells had the same susceptibility to the lethal action of carnocin 54 and that a certain percentage of the *Listeria* cells within the *Listeria* population appeared to be resistant, or less susceptible to the bacteriocin. Our results revealed an inhibition of listerial population with a decrease of ca. 0.9 log CFU/mL of *L. monocytogenes* after 5 days of incubation at 8ºC, followed by an increase of ca. 2.2 log CFU/mL on day 10. At 15ºC, there were no differences between both control groups (*L. mesenteroides* 11 and *L. mesenteroides* A13). This was likely due to the selection of resistant cells to *L. mesenteroides* 11 bactriocin, as indicated by scattered growth observed inside inhibition halos and corroborated by the additional critical dilution assays performed, where no inhibitions halos were observed.

Despite the great number of papers describing bacteriocin-producing strains or their purified bacteriocins, the use of this biological technology in foods is still restrict. It is believed that the application of biopreservation procedures using *Leuconostoc* sp. will be more successful if isolated and purified bacteriocins are used and not live cultures [7]. This may be attributed to intrinsic characteristics of the genus *Leuconostoc*, that presents heterolactic metabolism, causing the formation of CO_2 in packages, which may alter sensorial aspects of foods [2, 19].

However, JACOBSEN, BUDDE, and KOCH [10] found that the use of *L. carnosum* 4010 as a protective culture in a sliced gas packed meat product (saveloy) maintained at 10ºC was more effective in preventing *L. monocytogenes* growth when compared to the partially purified leucocin 4010, or the bacteriocin produced during fermentation before heat treatment of the meat. Besides this, BUDDE *et al.* [4] observed that *L. carnosum* 4010 presented strong antilisterial activity without producing any undesirable flavour components in meats. Results of their experiments revealed that the addition of 10^7 CFU/g of *L. carnosum* 4010 immediately reduced the number of viable *L. monocytogenes* cells to a level below the detection limit and no increase of listerial population was observed during storage of saveloy at 5ºC for 21 days.

L. gelidum has also been studied for use in meats. YOST & NATTRESS [23] observed the development of BAL community in vacuum packaged beef during six weeks, using molecular techniques. They noticed a transition of a mixed population of *Lactobacillus curvatus*, *L. sakei* and *Leuconostoc sp*. until a unique strain of *Leuconostoc* was preeminent.

In our study, despite the transitory inhibition of *L. monocytogenes* observed in the presence of *L. mesentereroides* 11 bacteriocin, higher *L. monocytogenes* populations were determined when abuse temperature (15ºC) was tested, in comparison with 8ºC (refrigeration temperature). These data strongly suggest the need for keeping foods biopreserved by the use of bacteriocins at low temperatures, to obtain a sinergistic inhibitory effect and maximize bacteriocin stability.

4 - CONCLUSION

We believe that the choice of using protective culture or purified bacteriocin for meat preservation appears to be case-specific especially with strains of the genus *Leuconostoc* and deserves to be carefully evaluated for each application.

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