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Antimicrobial activity of the Nisin Z producer *Lactococcus lactis* subsp. lactis Lc08 against *Listeria monocytogenes* in skim milk

[*Atividade antimicrobiana de* Lactococcus lactis *subsp. lactics Lc08 produtor de Nisina Z contra* Listeria monocytogenes *em leite desnatado*]

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ABSTRACT

The presented study aimed to verify the effect of different pH values, enzyme solutions and heat treatments on the antimicrobial activity of the bacteriocinogenic strain *Lactococcus lactis* subsp. lactis Lc08 and to test their antimicrobial activity against *Listeria monocytogenes* in reconstituted skim milk at refrigeration temperatures. This strain was previously described as a nisin Z producer and capable of inhibiting *L. monocytogenes* growth in in vitro tests. The antimicrobial activity of the bacteriocin cell-free supernatant of Lc08 was sensitive to enzyme treatments (except papain). The pH values and heating (65°C for 30min, 75°C for 15s) had no apparent effect on the antimicrobial activity of the bacteriocin produced by Lc08. Only treatment at autoclave conditions result in loss of their antimicrobial activity. Lc08 presented antimicrobial activity against *L. monocytogenes* in the milk system after 12h at 25°C. No effect was found at 7°C. The results show the application viability of the Lc08 in food systems as a biopreservative against *L. monocytogenes*.

Keywords: nisin Z, milk, antagonistic activity, *Lactococcus lactis* subsp. *lactis, Listeria monocytogenes*

RESUMO

O presente estudo teve como objetivo verificar o efeito de diferentes valores de pH, soluções enzimáticas e tratamentos térmicos na atividade antimicrobiana da cepa bacteriocinogênica Lactococcus lactis *subsp.* lactis *Lc08 e testar sua atividade antagonista contra* Listeria monocytogenes *em leite desnatado reconstituído em diferentes temperaturas de estocagem. Essa cepa já foi descrita como produtora de nisina Z e capaz de inibir o desenvolvimento de* L. monocytogenes *em testes* in vitro. *A atividade antimicrobiana do sobrenadante de Lc08 contendo a bacteriocina produzida e livre de células foi sensível ao tratamento pelas enzimas testadas (exceto papaína). A aplicação de diferentes valores de pH e o tratamento térmico (65°C por 30 min, 75°C por 15s) não influenciaram na atividade antimicrobiana da bacteriocina produzida por Lc08. Apenas o tratamento em autoclave resultou em perda da sua capacidade em inibir o desenvolvimento de* L. monocytogenes. *A cepa Lc08 apresentou atividade antagonista contra* L. monocytogenes *em leite após período de estocagem de 12h a 25°C. Não foi observado efeito a 7°C. Os resultados mostram a viabilidade de aplicação da cultura Lc08 ou de sua bacteriocina em produtos lácteos como bioconservador contra* L. monocytogenes.

Palavras-chave: nisina Z, leite, atividade antagonista, Lactococcus lactis *subsp.* lactis, Listeria monocytogenes

INTRODUCTION

Novel lactic acid bacteria (LAB) strains possessing technological and antimicrobial properties are continuously required by the food industry for use as starters and biopreservatives. Isolated microorganisms with these characteristics are usually identified as members of the genus *Lactococcus*, of which *L. lactis* subsp. *lactis* is the main species capable of producing and expressing bacteriocins,

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particularly nisin, and their variants (Cotter *et al.*, 2005).

Nisin belongs to the lantibiotic class of bacteriocins; lantibiotics are proteins that contain the unusual amino acid lanthionine. Nisin has been applied in a number of food systems to inhibit the development and survival of spoilage microorganisms and foodborne pathogens, especially *Listeria monocytogenes* (Deegan *et al.*, 2006; Dal Bello *et al*., 2012). The control of this pathogen is a concern in the food industry because it can grow at low temperatures and pH usually adopted for food storage (Carpentier and Cerf, 2011).

Nisin (called nisin A) was the first well-characterised bacteriocin, and structural variants of this protein include nisin Q, U and Z (Piper *et al.*, 2011; Perin *et al.*, 2012). Nisin Z usually exhibits a higher inhibitory activity than nisin A, most likely due to its higher diffusion capacity in the solid culture media employed in tests to detect antagonistic activity (Piper *et al.*, 2011). The structure of nisin Z differs from that of nisin A by the substitution of an asparagine with a histidine at position 27, which improves its solubility and diffusion at pH 6.0 (de Arauz *et al.*, 2009). This characteristic may be relevant in some food systems, such as cheeses, where diffusion is limited.

In previous studies from our group (Ortolani *et al.*, 2010; Perin *et al.*, 2012), *L. lactis* subsp. *lactis* Lc08 was isolated from raw milk and characterised molecularly and phenotypically as a bacteriocinogenic strain capable of producing nisin Z and presenting a broad inhibitory activity against several foodborne pathogens. These characteristics justified additional tests to verify its applicability in food systems because the food matrix and industrial processes can interfere in the production of bacteriocins as well as antimicrobial activity (Deegan *et al.*, 2006). Therefore, when a bacteriocinogenic strain is isolated, it is necessary to evaluate the effect of distinct factors on the production and stability of the bacteriocins, such as pH, enzymes, and thermal processes (Gálvez *et al.*, 2010).

The aim of this work was to evaluate the effect of different pH values, enzyme solutions and heat treatments on the antimicrobial activity of the bacteriocins, including nisin Z, produced by the Lc08 strain and characterise the inhibitory activity of Lc08 against *L. monocytogenes* in reconstituted skim milk at refrigeration temperatures to simulate the usual storage conditions of this milk product.

MATERIALS AND METHODS

LAB strains Lc08 and Lc25 were isolated from raw milk (Ortolani *et al.*, 2010) and identified as *L. lactis* subsp. *lactis* based on 16s rDNA sequencing and species-specific PCR (Perin *et al.*, 2012). Lc08 was previously characterised as a bacteriocinogenic strain capable of producing nisin Z. Lc25 was identified as a non-bacteriocinogenic strain and was used in the present study as a negative control for bacteriocin production. Based on the antimicrobial activity of Lc08 against several foodborne pathogens and spoilage microorganisms (Perin *et al.*, 2012), *L. monocytogenes* ATCC 7644 was selected as the target. LAB strains were stored in de Mann, Rogosa and Sharpe broth (MRS, Oxoid Ltd., Basingstoke, England), and *L. monocytogenes* was stored in Trypticase Soy Broth supplemented with 0.6% (w/v) Yeast Extract (TSA-YE, Oxoid). Both media were supplemented with 25% (v/v) glycerol at -80°C. Prior to use, the strains were recovered in their specific culture media, incubated at 30°C for 24h, and diluted to a turbidity similar to MacFarland 1, which corresponds to approximately $3x10^8$ colony forming units per mL (CFU/mL).

To determine the effect of enzyme solutions, pH, and heat treatments on bacteriocin activity, Lc08 culture was prepared and diluted ten-fold with 0.85% (w/v) NaCl to obtain an inoculum with an approximate concentration of 10^2 CFU/mL. One millilitre aliquots of the obtained culture were added to 20mL of modified MRS (mMRS, which has the same composition as MRS but contains 0.5g dextrose per 100mL). After incubation at 25°C for 48h (optimised condition for bacteriocin production, as described by Perin *et al*. 2012), the culture was centrifuged at 6,800*g* for 20min at 4°C. Aliquots of the cell-free supernatants were then submitted to the following treatments: enzyme solutions, pH and heat (considering the observations of Gálvez *et al.*, 2010).

The enzyme solutions used were α-chymotrypsin, proteinase K, trypsin TPCK, and papain (all from Sigma-Aldrich, St. Louis, MO, USA, at a final concentration of 1mg mL/L) and were added to the aliquots of the cell-free supernatants. After homogenisation, the treated aliquots were stored at 30°C for 2h.

The pH of the cell-free supernatant aliquots was adjusted with NaOH or HCl (both at 1mol/L) to 2.0, 4.0, 6.0, 8.0, 10.0, and 12.0, as measured with an electronic pH meter (Hanna Instruments, Smithfield, RI, USA).

Finally, the cell-free supernatant aliquots were heated at 65°C for 30min, 75°C for 15s, and 121°C for 15min. Then, all treated cell-free supernatant aliquots were cooled at 4°C.

Untreated cell-free supernatants were used as controls for all tests. All extracts (except those used for the pH test) were previously neutralised with NaOH (at 1 mol/L) to pH 6.0 to eliminate antimicrobial activity due to acid production. Next, all of the cell-free supernatant aliquots were filter-sterilised (Millex SLGP033RS, 0.22µm, Millipore, Bedford, MA, USA), and antimicrobial activity was evaluated with by the well-diffusion assay (Tagg and McGiven, 1971). Briefly, 10µL aliquots of the treated and filtered cell-free supernatants were transferred to wells cut in mMRS agar (Oxoid) previously inoculated with *L. monocytogenes* ATCC 7644 (at 10^5 CFU mL/L). After incubation at 35°C for 24h, the presence of inhibition halos around the wells indicated resistance due to the production of bacteriocins after the tested conditions. The tests were conducted in triplicate.

The antimicrobial activity of Lc08 and Lc25 against *L. monocytogenes* in reconstituted skim milk was also tested. Lc08, Lc25, and *L. monocytogenes* ATCC 7644 cultures were inoculated in different combinations (individual or co-cultures) in twelve flasks containing reconstituted skim milk (10%, w/v, 100 mL per flask; Molico, Nestlé Brasil Ltda., São Paulo, SP, Brazil). Each treatment was prepared for two flasks as follows: Treatment 1, only Lc08 at 10^6 CFU/mL; Treatment 2, only Lc25 at 10^6 CFU/mL; Treatment 3, only *L. monocytogenes* at 10^3 CFU/mL; Treatment 4: Lc08 (at 10^6 CFU/mL) and *L. monocytogenes* (at 10^3 CFU/mL); Treatment 5, Lc25 (at 10^6 CFU/mL) and *L. monocytogenes* (at 10^3 CFU/mL); and Treatment 6, no strains inoculated, used as an autochthonous microbiota control. One set was incubated at 25°C for 48h (during which the inoculated strains were enumerated every 12h), and one set was incubated at 7 °C for 96 h (during which the inoculated strains were enumerated every 24h).

At the described intervals, aliquots of each treatment were obtained under sterile conditions and diluted ten-fold with 0.85% (w/v) NaCl. Specific dilutions were selected according the probable concentration of each strain or microbial group and subjected to microbiological analyses. LAB strains were enumerated on MRS agar (Oxoid), by pour plate and duplicates, incubated at 35°C for 48h, at anaerobic conditions (GasPak EZTM Gas Generating Container Systems, Becton, Dickinson and Co., Franklin Lakes, NJ, USA) (Frank and Yousef, 2004). *L. monocytogenes* was enumerated according to ISO 11290-2 (ISO, 1996, 2004) on Chromogenic *Listeria* agar (Oxoid), by surface plating and duplicates, incubated at 35°C for 48h. The autochthonous microbiota was enumerated on Petrifilm™ Aerobic Count plates (3M Microbiology, St. Paul, MN, USA) and incubated at 35°C for 24 h. All results obtained were expressed in CFU/mL. The experiment was conducted in three repetitions.

RESULTS

Bacteriocins cell-free supernatants of Lc08 without treatments with enzyme solution, pH and heat were capable to inhibit *L. monocytogenes* growth in all tests, confirming their antimicrobial activity against this foodborne pathogen. The treatment of cell-free supernatants with α-quymotrypsin, tripsin, proteinase k and pepsin completely inactivated their anitimicrobial activity. Treatment with papain did not interfere in the antimicrobial activity. After adjusting the pH to 2.0, 4.0, 6.0, 8.0, 10.0, and 12.0, the cell-free supernatants of Lc08 were capable to inhibit *L. monocytogenes* growth, indicating that variations in pH values did not affect their antimicrobial activity. Similar results were observed after heat at 65°C for 30 min and 75°C for 15s, the antimicrobial activity of the Lc08 cell-free supernatant against *L. monocytogenes* was lost only after autoclave treatment, at 121°C for 15min.

In relation to the antimicrobial activity of Lc08 and Lc25 against *L. monocytogenes* in reconstituted skim milk, the *L. monocytogenes* populations after storage at 7 and 25°C in reconstituted skim milk systems alone or co-cultured with Lc08 or Lc25 are presented in Figure 1. The Lc08 and Lc25 populations under the same conditions (both inoculated alone or in co-cultured with *L. monocytogenes*) are presented in Figure 2.

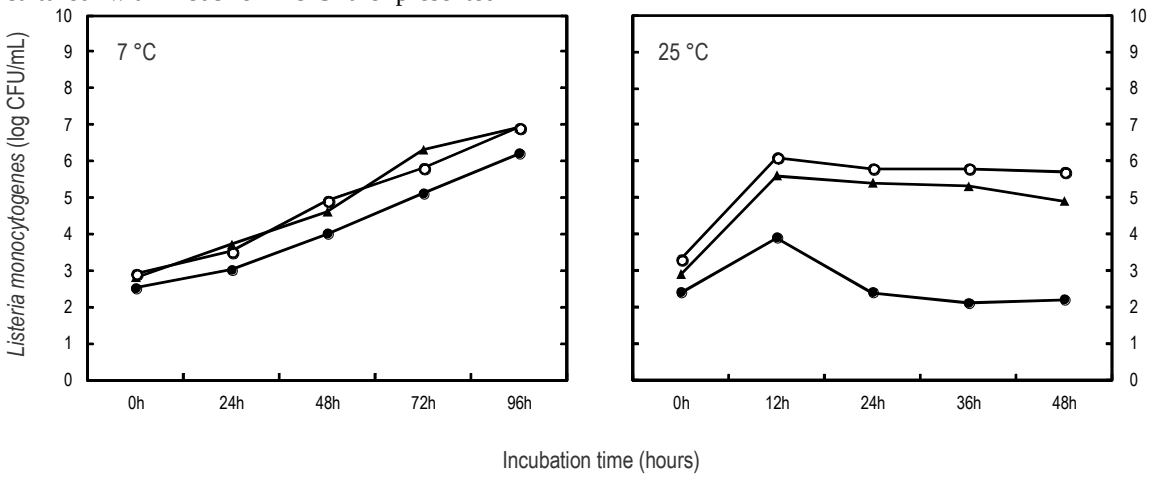

Figure 1. Mean counts of *Listeria monocytogenes* ATCC 7644 artificially inoculated in skim milk systems alone (triangle), in co-culture with Lc08 (bacteriocinogenic strain, black circles), and in co-culture with Lc25 (non-bacteriocinogenic strain, white circles), after incubation at 7 and 25°C in distinct periods.

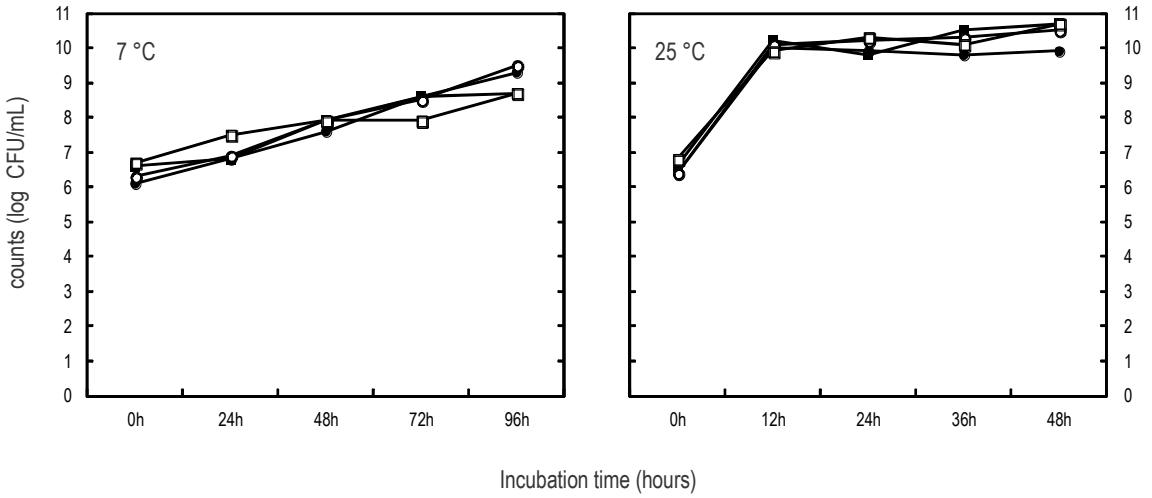

Figure 2. Mean counts of *Lactococcus lactis* isolates (Lc08, bacteriocinogenic strains, circles, and Lc25, non-bacteriocinogenic strain, squares) artificially inoculated in skim milk systems alone (black symbols), and in co-culture with *Listeria monocytogenes* ATCC 7644 (white symbols), after incubation at 7 and 25°C in distinct periods.

DISCUSSION

The enzymatic sensitivity of the bacteriocins produced by Lc08 indicate that they could be used as biopreservatives in foods because they would be easily degraded in the human digestive system without interfering with natural microbiota (Sharma *et al.*, 2006). The Lc08 was previously described as nisin Z producer and capable to inhibit some foodborne pathogens present in dairy products, including *L. monocytogenes* (Perin *et al*., 2012). The bacteriocin cell-free supernatant of Lc08 was

sensitive to all of the tested proteolytic enzymes, except papain. Other studies have demonstrated a similar pattern of nisin sensitivity to these tested enzymes (Moreno *et al.*, 2000; Tuncer, 2009), and nisin Z was previously reported to be resistant to papain (Olasupo *et al.*, 1999). It is important to test the interference of enzymes in bacteriocins because proteolytic enzymes naturally present in foods could limit the use of specific LAB or their bacteriocins as biopreservatives (de Arauz *et al.*, 2009).

The bacteriocins produced by Lc08 did not lose their antimicrobial activity after the pH was adjusted at distinct values. Nisin usually maintains its antimicrobial activity at a variety of pH values and exhibits higher inhibitory activity at low pH (Olasupo *et al.*, 1999; Campos *et al.*, 2006; Deegan *et al.*, 2006; de Arauz *et al.*, 2009; Castro *et al.*, 2011; Dal Bello *et al.*, 2012). When the pH is lowered, the solubility and stability of nisin tend to increase drastically; conversely, under neutral and alkaline conditions, nisin is nearly insoluble (de Arauz *et al.*, 2009). Some studies have demonstrated reduced inhibitory activity of nisin at high pH (Olasupo *et al.*, 1999; Deegan *et al.*, 2006; de Arauz *et al.*, 2009)

Only autoclaving resulted in the loss of antimicrobial activity of the nisin produced by Lc08. Other studies have reported that nisin activity is only maintained after autoclave treatment when the pH is low or neutral (Olasupo *et al.*, 1999; Campos *et al.*, 2006). Heat treatments similar to pasteurisation (high temperature, short time and low temperature, long time) did not affect the antimicrobial activity of the Lc08 bacteriocin. The relevant functional properties of nisin and its variants, such as acid tolerance, thermostability at low pH and specific mode of action, may be a consequence of the lanthionine amino acids in their structures (de Vuyst and Vandamme, 1992; de Arauz *et al.*, 2009). The maintenance of antimicrobial activity at distinct pH values and heat treatments supports the application of Lc08 or their bacteriocin as biopreservatives in processed dairy products. And to verify if these results were reproduced *in situ*, Lc08 was tested against *L. monocytogenes* in reconstituted skim milk at different temperatures.

When skim milk treatments were stored at 7°C, *L. monocytogenes* exhibited similar growth in all treatments (alone or co-cultured with Lc08 and Lc25) (Figure 1). At 25°C, however, the growth of *L. monocytogenes* was similar only in treatment 3 and 5 (alone and co-cultured with Lc25, non-bacteriocinogenic strain); in treatment 4 (co-cultured with bacteriocinogenic Lc08) the counts of *L. monocytogenes* increased between 0 and 12 h (lower growth than other treatments), but after 12 h its population decreased 2 logs, being controlled as the initial level (Figure 1). Perin *et al*. (2012) observed that Lc08 exhibited maximum antimicrobial performance when incubated at 25°C after 12h, as observed in the present study in the milk system adopted to demonstrate this inhibitory activity (Figure 1). Nisin production by Lc08 under these conditions was consistent with its development pattern, in which the exponential phase occurred in the first 12h of incubation at 25°C (Figure 2).

Based on the comparison of the *L. monocytogenes* counts observed in the treatments (Figure 1), and the ability of Lc08 in producing nisin Z, the inhibitory activity can be attributed to this bacteriocin production by such strain. The distinct behaviour of *L. monocytogenes* in the treatments added to the non-bacterocinogenic strain support this inhibitory activity of Lc08. Nisin Z was previously shown to exhibit antimicrobial activity against spoilage bacteria in milk, reducing their counts to an undetectable level after a period of incubation between 8 and 20 h (Mitra *et al.*, 2011). Other studies have already demonstrated the inhibitory activity of nisin or its producer against *L. monocytogenes* in food systems (Benkerroum *et al.*, 2003; Jamuna *et al.*, 2005; Dal Bello *et al.*, 2012).

Antimicrobial activity was absent when Lc08 was incubated at 7°C (Figure 1). Only a limited number of LAB strains are known to produce bacteriocins at low temperatures (Castro *et al.*, 2011). Bacteriocin production is usually induced depending on the cell density of the bacteriocinogenic strain, achieving maximum levels at the end of the exponential phase or at the beginning of the stationary phase (Parente and Ricciardi, 1994; Fields, 1996). At 7°C, Lc08 was not able to reach these growth phases (Figure 2), preventing adequate bacteriocin production.

Although the addition of bacteriocins to milk may lack practical application, this food serves as an important system to evaluate the influence of milk components on bacteriocin activity (Bizani *et al.*, 2008). With the presented results it is possible to infer that Lc08 could be used as a biopreservative culture in dairy products stored at temperatures up to 25°C, such as ripened cheeses. The antimicrobial potential of Lc08 could be reached in dairy products stored at low temperatures by purifying the produced bacteriocins by Lc08 and applying them to foods. However, the use of purified bacteriocins is not always attractive to the food industry because their use as additives in foods requires regulatory approval. Alternatively, the bacteriocinogenic strain could be used in a fermented ingredient or starter culture that does not require regulatory approval (Deegan *et al.*, 2006). *Lactococcus* species are often used in the dairy industry as starter cultures, and their capacity to produce bacteriocins would increase interest in their use (Gálvez *et al.*, 2010).

CONCLUSION

The nisin Z produced by Lc08 was sensible to different proteases and resistant to papain and was stable against distinct values of pH and pasteurisation heat treatments. Given these results and the antimicrobial activity against *L. monocytogenes* in a milk system incubated at 25°C, Lc08 is a good candidate for use as a biopreservative in dairy products. Further studies are necessary to determine their dynamic when inoculated in cheese systems.

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