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EVALUATION OF BACTERIOCIN-PRODUCING *LACTOBACILLUS SAKEI* 1 AGAINST *LISTERIA MONOCYTOGENES* 1/2A GROWTH AND HAEMOLYTIC ACTIVITY

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ABSTRACT

Bacteriocin-producing *Lactobacillus sakei* 1 was cultivated in Brain-Heart Infusion broth (24 h at 25ºC). The culture supernatant was neutralized, filter sterilized and used to test the activity of bacteriocin against *Listeria monocytogenes* 1/2a, at 8ºC and 15ºC. Non-bacteriocinogenic *Lactobacillus sakei* ATCC 15521 was used as a negative control. *L. monocytogenes* 1/2a was inoculated in culture supernatant medium from *L. sakei* 1 and *L. sakei* ATCC 15521 and the listerial populations were determined after 0, 5 and 10 days. The bacteriocin production was quantified as arbitrary units per mL (AU/mL) using agar antagonism test. Additionally, to investigate if *L. monocytogenes* virulence pattern could be changed after bactericion exposure, the ability of *L. monocytogenes* to cause haemolysis in sheep red blood cells was determined, before and after exposure to bacteriocin at 8ºC. In the presence of the antimicrobial peptide, at 8ºC, *L. monocytogenes* population decreased, but growth of resistant cells was observed. At 15ºC, there was no difference between test and control. Furthermore, the haemolytic activity of *L. monocytogenes* 1/2a was not altered by exposure to *L. sakei* 1 bacteriocin, which suggests no change in its virulence pattern.

Key words: *Listeria monocytogenes*, *Lactobacillus sakei*, bacteriocins, virulence, resistance

INTRODUCTION

Nowadays, consumers demand more "natural" and minimally processed food. As a result, there has been great interest and research on naturally produced antimicrobials, such as the ribosomal peptides, synthesized by lactic acid bacteria (LAB), named bacteriocins (4). These natural antimicrobials present high potential to be applied in hurdle technology, which utilizes synergy of combined treatments to more effectively preserve foods (21).

Among pathogens that can be inhibited by LAB bacteriocins, *Listeria monocytogenes* is of particular concern to the food industry because of its ability to grow at refrigeration temperatures and its tolerance to preservative agents (19). This bacterium occurs widely in the environment and it has been isolated from a range of sources including vegetables, processed foods, silage and soils (2). *L. monocytogenes* can cause serious infection in pregnant women, newborn infants, children and adults whose immune systems are weakened (20).

L. monocytogenes produces several virulence factors such as phospholipases, a water soluble hemolysin called listeriolisin O (LLO), a protein (ActA) and several internalins (13,22). LLO is the primary determinant of *L. monocytogenes* pathogenesis. It aids the bacterial cells to be released from host macrophages, acting as a vacuole-specific lysin and also allows bacterial entrance into the host cytosol (11,23).

Although twelve serotypes of *L. monocytogenes* can cause disease, at least 95% of the strains isolated from human listeriosis cases belong to three serotypes: 1/2a, 1/2b and 4b (13). *L. monocytogenes* strains of serogroup 1 have been mainly isolated from foods and serogroup 4 has major association with outbreaks. Nonetheless, Lukinmaa *et al*. (15) showed that in Finland, cases of listeriosis related to *L. monocytogenes* serotype 1/2a increased from 22%, in 1990, to 67% in 2001 and

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those caused by *L. monocytogenes* serotype 4b decreased from 61% to 27%, in the same period.

Interestingly, *L. monocyto*genes appears to be able to detect specific signals from distinct habitats (warm-blooded animals *versus* soil and vegetation) and to express or repress specific virulence markers (13). The reason why *L. monocytogenes* serotype 1/2a is mainly found in food in contrast with *L. monocytogenes* serotype 4b being more common in human cases is not completely understood.

Epidemiological data and *L. monocytogenes* subtyping studies have demonstrated that the pathogen virulence markers are attenuated in all, or at least in the majority of contaminating food strains (13).

In this work, we evaluated the effect of bacteriocin produced by *L. sakei* 1 on growth and virulence marker (haemolytic activity) of *L. monocytogenes*.

MATERIALS AND METHODS

Bacterial strains

Bacteriocin-producing, *Lactobacillus sakei* 1, belonged to our culture collection (Laboratório de Microbiologia de Alimentos, Faculdade de Ciências Farmacêuticas de Ribeirão Preto – Universidade de São Paulo). The source of this strain was vacuum packed pork sausage (6,8). *Lactobacillus sakei* ATCC 15521, non-bacteriocin producing, was used as a negative control. Both LAB strains were kept at -70ºC in Man Rogosa Sharpe broth (MRS – Oxoid) containing 20% glycerol.

L. monocytogenes IAL 633, serotype 1/2a was maintained at -70ºC, in Brain Heart Infusion broth (BHI - Oxoid) or Trypticase Soy Broth (Oxoid) plus 20% (v/v) of glycerol (Merck).

Determination of the antilisterial activity of *Lactobacillus sakei* 1 bacteriocin

50 mL of BHI broth containing ca. 10^5 *L. sakei* 1 CFU/mL were incubated for 24 h at 25ºC and then centrifuged at 4ºC at 6,720 g for 25 minutes (Sorvall RC Plus – Du Pont, USA). The supernatants were neutralized (pH 7.0) and then, filter-sterilized through a 0.22 mm membrane with low capacity of binding proteins (GVWP, Millipore). The filtrates obtained were denominated "*L. sakei* 1 culture supernatant medium". For non-bacteriocin producing, *L. sakei* ATCC 15521, we followed similar procedure.

An overnight culture of *L. monocytogenes* 1/2a prepared in BHI broth was added (0.01% v/v) to cultures supernatants media of *L. sakei* 1 and *L. sakei* ATCC, to yield ca. 10^5 cells of *L. monocytogenes* /mL. These preparations were incubated at 8ºC (refrigeration temperature) and at 15ºC (to simulate temperature abuse). *L. monocytogenes* populations were enumerated at 0, 5 and 10 days of incubation, by spiral-plating serial decimal dilutions ("Spiral Plater", model D, K & R Technology, USA) on Tryptone Soy Agar (Oxoid) plus 0.6% yeast extract (Oxoid) – (TSAYE).

L. sakei 1 bacteriocin was quantified against *L. monocytogenes* 1/2a in the culture supernatant medium after 0 and 10 days, according to the method of critical dilution (17). Bacteriocin title was defined as the reciprocal of the highest dilution causing a clear zone of inhibition in the indicator listerial lawn and expressed in arbitrary units per ml (AU/mL).

To determine if resistant cells to *L. sakei* 1 bacteriocin had been selected after ten days of bacteriocin exposure, *L. monocytogenes* cells were harvested by centrifugation and washed twice with sodium chloride 0.85% (w/v) to remove traces of the inhibitory peptide. Those *L. monocytogenes* cells were used as indicator microorganisms in an additional critical dilution assay performed with a fresh culture supernatant medium.

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

Evaluation of *L. monocytogenes haemolytic* activity

We determined the haemolytic activity of *L. monocytogenes* 1/2a cells obtained after ten days of exposure to *L. sakei* 1and *L. sakei* ATCC culture supernatants media at 8ºC. As a control, we measured the haemolytic activity of *L. monocytogenes* 1/2a from stock. According to Kim *et al.* (14), optical density studies were performed at 650 nm, comparing differences among hemolyzed and non-hemolyzed samples tested. Three independent replicates were done for each tested condition.

Statistical analysis

Comparison among test and control groups were performed using three-way analysis of variance (ANOVA). Post-Hoc Tuckey-HSD test for comparing group means (9) was also used. The level of significance was set at $p<0.05$ for all tests.

Results of haemolytic activity tests were evaluated using T Student test at the significance level of 5% (5).

RESULTS

Assays performed at 8ºC

Initial counts of *L. monocytogenes* 1/2a in culture supernatant media were of 5.1 (±0.1) log CFU/mL for both strains (*L. sakei* 1 and *L. sakei* ATCC). Bacteriocin activity was quantified as 1,067±377 AU/mL in the broth prepared with *L. sakei* 1. However, spots of growth of *L. monocytogenes* were noted inside the halos of inhibition of critical dilution assay plates, suggesting the occurrence of survival and/or resistant cells of the pathogen.

After five days of incubation at 8ºC, *L. monocytogenes* population decreased to 4.0 (± 0.3) log CFU/mL in culture supernatant medium containing the *L. sakei* 1 bacteriocin. In the negative control, *L. monocytogenes* 1/2a reached 6.5 (±0.1) log CFU/mL.

On the tenth day of assay at 8ºC, *L. monocytogenes* population was 7.2 (±0.3) in *L. sakei* 1 culture supernatant medium

and 8.7(±0) CFU/mL in the control prepared with *L. sakei* ATCC, respectively. Bacteriocin title remained stable (1,067±377 AU/mL) in *L. sakei* 1 culture supernatant medium for up to ten days.

L. monocytogenes cells harvest by centrifugation from culture supernatant media after bacteriocin exposure, were used as indicators in an additional test of critical dilution. Bacteriocin produced by *L. sakei* 1 was ineffective against those cells of *L. monocytogenes*.

The *L. monocytogenes* cells that had been recovered after exposure to bacteriocin of *L. sakei* 1 at 8ºC were additionally assayed for their haemolytic activity. The results showed there were no statistical differences between test and control (Fig. 1).

Assays performed at 15ºC

At 15ºC, initial *L. monocytogenes* 1/2a population was 5.0 (±0) log CFU/mL in *L. sakei* 1 culture supernatant medium and 5.1 (±0.1) log CFU/mL for negative control. Title of 1,067±377 AU/mL was measured for *L. sakei* 1 bacteriocin. However, spots of growth of *L. monocytogenes* inside inhibition halos of critical dilution assay were observed.

On the fifth day of incubation at 15ºC, *L. monocytogenes* population had sharply increased, regardless the presence of bacteriocin and reached 8.9 (±0.1) log CFU/mL in *L. sakei* 1 supernatant medium and 9.0 (±0.1) log CFU/mL in negative control.

After 10 days, the counts of *L. monocytogenes* remained steady, but the activity of the bacteriocin decreased (533±189 AU/mL).

Critical dilution assay was also performed with *L. monocytogenes* cells recovered after bacteriocin exposure as indicator. No inhibition was observed, suggesting that a bacteriocin resistant population had been selected.

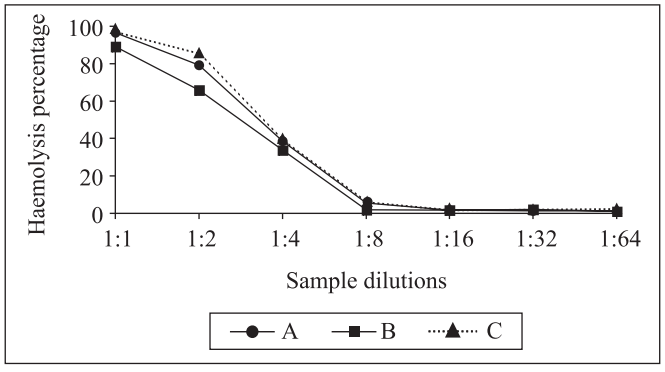

Figure 1. Haemolytic activity of *L. monocytogenes* 1/2a towards sheep red blood cells (expressed as hemolysis percentage). **A**: working-culture of *L. monocytogenes* 1/2a made from stock; **B**: *L. monocytogenes* 1/2a recovered from *L. sakei* ATCC 15521 culture supernatant medium incubated at 8ºC for 10 days and **C**: *L. monocytogenes* 1/2a recovered from *L. sakei* 1 culture supernatant medium incubated at 8ºC for 10 days.

Statistically significant lower *L. monocytogenes* populations were obtained in the presence of bacteriocin at 8ºC when compared to the absence of the peptide. However, statistical test showed that, at 15ºC, *L. monocytogenes* growth was not affected by either LAB bacteriocin after five and ten days of incubation.

DISCUSSION

Bacteriocin activity was stable in *L. sakei* 1 culture supernatant medium inoculated with *L. monocytogenes* and incubated for ten days at 8ºC, but not after incubation at 15ºC.

In a previous study (16), we tested preparations with *L. sakei* 1 against *L. monocytogenes* 4b and the bacteriocin presented higher activity (12,800 UA/mL). This indicates that *L. monocytogenes* 4b was more sensitive to *L. sakei* 1 bacteriocin than *L. monocytogenes* 1/2a. Similar results were described by Buncic *et al.* (3). Those authors evaluated the behavior of *L. monocytogenes* 4b and 1/2a strains isolated from clinical and food samples, after exposition to two LABs: *L. sakei* 265 and *L. sakei* 706. They evidenced that *L. monocytogenes* 4b strains, were more sensitive to both bacteriocins when compared to *L. monocytogenes* 1/2a. Those authors concluded that *L. monocytogenes* 1/2a could present competitive advantage over serotype 4b in food kept at refrigeration temperature containing bacteriocin-producing LAB as intrinsic microbiota. *L. monocytogenes* serotype 1/2a would probably survive and surpass *L. monocytogenes* 4b growth rates.

In our work, *L. monocytogenes* 1/2a was recovered after bacteriocin exposure at 8ºC and 15ºC, and used for critical dilution assay. Under those conditions, it was observed a complete absence of the halos of inhibition. Thus, those bacterial cells were not only survivors but resistant to *L. sakei* 1 bacteriocin. Ennahar *et al.* (10) tested thirty-one *Listeria* strains for sensitivity to four class IIa bacteriocins (enterocin A, mesenterocin Y105, divercin V41, pediocin AcH and nisin A) and verified similar antimicrobial patterns ranging from highly susceptible to fully resistant strains, whereas nisin A showed a different pattern in which all *Listeria* strains were inhibited. Gravesen *et al.* (12) also verified that *L. monocytogenes* susceptibility to bacteriocins, specially for those belonging to IIa subclass, varied from high susceptibility patterns up to answers of complete resistance of the pathogenic microorganism. These evidences suggest that within a given species and without prior exposure to bacteriocin, resistant *L. monocytogenes* strains to all subclass IIa bacteriocins could naturally occur in the environment (10). Gravesen *et al.* (12) suggested that the high *L. monocytogenes* resistance level against IIa bacteriocins class and some Gram-positive bacteria may occur by a common mechanism, which would not be dependent on the bacterial strain, bacteriocin class or environmental tested conditions.

In the present study, there was no statistical significant change of the haemolytic activity of *L. monocytogenes* recovered under any condition, after ten days of exposure to 800-1,600 AU/mL *L. sakei* 1 bacteriocin.

There are few studies available in literature concerning to the possible modification of *L. monocytogenes* haemolytic activity when submitted to adverse conditions, such as antimicrobial substances or co-inoculation with microorganisms presenting inhibitory activity.

McKellar (18) studied the influence of chemical preservatives of food on *L. monocytogenes* LLO secretion and observed an inhibition of it in the presence of nitrates, sorbates and chloride. It was postulated that the action of these substances in the cytosol of the pathogen altered the production of LLO.

Alves *et al.* (1) verified a significant reduction of *L. monocytogenes* 4b e 1/2a haemolytic activity after exposure to some ingredients present in model food gravies added of the bacteriocinogenic culture *L. sakei* 1.

Alves *et al.* (1) studied the effect of *L. sakei* 1 bacteriocin produced "in situ" and not the influence of the semi-purified antimicrobial peptide as performed in our assays. The differences observed could be attributed to acid production and to bacterial competition for room, nutrients and oxygen, which could have altered *L. monocytogenes* strains haemolytic activity. Furthermore, bacteriocins are known to act on the membrane surface of target microorganisms, differently from acid antimicrobials which acts in the cytosol (7).

In conclusion, our results show that *L. monocytogenes* 1/2a was inhibited in the presence of *L. sakei* 1 bacteriocin at 8ºC, but not at 15ºC. In this work, we demonstrated that the use of LAB bacteriocin should be combined with temperature to effectively control *L. monocytogenes* in food systems. Furthermore, LLO expression in *L. monocytogenes* 1/2a was not altered by exposure to *L. sakei* 1, suggesting no modification in the pathogen virulence had ocurred.

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RESUMO

Avaliação de *Lactobacillus sakei* 1 produtor de bacteriocina frente a *Listeria monocytogenes* 1/2a e sua atividade hemolítica

Lactobacillus sakei 1 produtor de bacteriocina foi cultivado em caldo Infusão Cérebro-Coração por 24h a 25ºC. O sobrenadante da cultura foi neutralizado, esterilizado por filtração e usado para testar a atividade da bacteriocina frente a *Listeria monocytogenes* 1/2a, a 8ºC e 15ºC. *Lactobacillus sakei* ATCC 15521 não bacteriocinogênico, foi utilizado como controle negativo. *L. monocytogenes* 1/2a foi inoculada no sobrenadante da cultura de *L.sakei* 1 e *L. sakei* ATCC 15521 e as populações listeriais foram determinadas após 0, 5 e 10 dias. A produção de bacteriocina foi quantificada como unidades arbitrárias por mL (UA/mL), utilizando-se o teste de antagonismo em ágar. Adicionalmente, para investigar se o padrão de virulência de *L. monocytogenes* poderia ter mudado após exposição à bacteriocina, foi avaliada a habilidade de *L. monocytogenes* em causar hemólise em hemáceas de carneiro, antes e após exposição à bacteriocina a 8ºC. Na presença do peptídeo antimicrobiano, a 8ºC, a população de *L. monocytogenes* foi reduzida, mas o desenvolvimento de células resistentes foi observado. A 15ºC, não houve diferença entre os grupos controle e teste. Além disso, a atividade hemolítica de *L. monocytogenes* 1/2a não foi alterada pela exposição à bacteriocina de *L. sakei*, o que sugere que não houve mudança em seu padrão de virulência.

Palavras-chave: *Listeria monocytogenes*, *Lactobacillus sakei*, bacteriocinas, virulência, resistência

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