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BACTERIOCINOGENIC POTENTIAL OF LACTIC ACID BACTERIA ISOLATED FROM ARTISANAL COLONIAL TYPE-CHEESE

(*Potencial bacteriocinogênico de bactérias ácido-láticas isoladas de queijo artesanal tipo colonial*)

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ABSTRACT: Autochthonous microbiota from artisanal cheeses is predominantly composed of lactic acid bacteria (LAB), which are able to produce antimicrobial compounds, such as bacteriocins, suggesting their application in food biopreservation. Knowledge about LAB growth and bacteriocin production during food production and conservation is essential to determine their use. In this way, the study aimed at isolating bacteriocinogenic LAB from twenty-one artisanal Colonial-type cheeses obtained from the western region of Parana state, Brazil, determining the best conditions for growth and bacteriocin production (25°C, 30°C, and 37°C/24h); bacteriocin stability under different ranges of pH (2, 4, 6, 8, and 10 for 2h) and temperature (60°C/2h; 80°C/2h; 121°C/15min). Their activity against different target microorganisms was also evaluated. A total of 34 LAB strains presented characteristics compatible with bacteriocin production. Most of them presented better results for bacteriocin production when cultured at 25ºC and 30ºC. Bacteriocins remained active against *L. monocytogenes* when exposed from pH 4 to 8 and a wide temperature range; some bacteriocins were even resistant to sterilization temperatures. Bacteriocins produced were able to inhibit spoilage and pathogenic microorganisms, such as *L. monocytogenes, B. cereus*, and *P. fluorescens*. These results indicated that isolated bacteriocinogenic LAB present potential to be used as food biopreservatives.
Keywords: Antimicrobial compounds; autochthonous microbiota; bacteriocins; food safety.

RESUMO: A microbiota autóctone de queijos artesanais é predominantemente composta de bactérias ácido lácticas (BAL), capazes de produzir compostos antimicrobianos, como as bacteriocinas, sugerindo sua aplicação na biopreservação de alimentos. O conhecimento sobre a multiplicação de BAL e produção de bacteriocinas durante a produção e conservação de alimentos é essencial para determinar seu uso. Nesse sentido, o trabalho objetivou isolar BAL bacteriocinogênicas de vinte e um queijos artesanais do tipo Colonial, obtidos na região oeste do Paraná, Brasil, determinando as melhores condições de multiplicação e produção de bacteriocinas (25°C, 30°C e 37°C/24h); estabilidade das bacteriocinas em diferentes faixas de pH (2, 4, 6, 8 e 10 por 2h) e temperatura (60°C/2h; 80°C/2h; 121°C/15min). Sua atividade contra diferentes micro-organismos também foi avaliada. Um total de 34 isolados de BAL apresentou características compatíveis com a produção de bacteriocinas. A maioria desses isolados apresentou melhores resultados para a produção de bacteriocinas quando cultivadas a 25ºC e 30ºC. As bacteriocinas permaneceram ativas contra *L.*

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monocytogenes quando expostas aos pH de 4 a 8 e à ampla faixa de temperatura; algumas bacteriocinas foram resistentes a temperaturas de esterilização. As bacteriocinas produzidas foram capazes de inibir micro-organismos deterioradores e patogênicos, como *L. monocytogenes, B. cereus* e *P. fluorescens.* Esses resultados indicaram que BAL bacteriocinogênicas isoladas apresentam potencial para serem utilizados como biopreservadores alimentares.
Palavras-chave: Bacteriocinas; compostos antimicrobianos; microbiota autóctone; segurança dos alimentos.

INTRODUCTION

Artisanal cheeses are produced from raw milk and, as a result, most of the autochthonous microbiota is composed of lactic acid bacteria (LAB). Most LAB are safe for human consumption and can be used in food industry as starter cultures, probiotics, and biopreservatives. LAB have been recognized as valuable food biopreservatives due to their ability to synthetize antimicrobial substances, such as organic acids, hydrogen peroxide, and bacteriocins, which are able to inhibit spoilage microorganisms and foodborne pathogens (Favaro et al., 2015; Reis et al., 2012; Zacharof and Lovitt, 2012; Castellano et al., 2008; Nero et al., 2008; Deegan et al., 2006).

Bacteriocins are protein substances with inhibitory activity against closely related microorganisms (Reis et al., 2012). Due to their antimicrobial potential, bacteriocins are valuable tools for food safety, especially when it comes to cheeses produced with raw milk. Besides that, their use as biopreservatives can help to reduce the need for chemical additives and rigorous thermal treatments used in food preservation (Favaro et al., 2015).

Several intrinsic and extrinsic factors of food, such as pH, available nutrients, water activity, microbial diversity, and storage temperature can positively or negatively influence bacteriocin production and activity (Galvez et al., 2007; Batdorj et al., 2006). Artisanal Colonial-type cheese is made from raw milk and is the main cheese produced by rural families of the southern region of Brazil. In this study, we aimed to isolate potential bacteriocinogenic LAB from artisanal Colonial-type cheeses, determining the influence of temperature in bacteriocin production. Additionally, the spectrum of activity of these compounds and their stability under different values of pH and temperature were also evaluated in the present study.

MATERIALS AND METHODS

Sampling and isolation of LAB with antimicrobial potential

Twenty-one artisanal Colonial-type cheeses were obtained from informal farmer's markets of three cities located in the western region of the state of Parana, Brazil. Samples were transported in isothermal containers and kept under refrigeration (3 to 8°C) for no more than 24 h, until the moment of analysis.

Cheese samples were homogenized at a 1:10 ratio with saline solution (0.85% NaCl, w/v); suspensions were diluted ten-fold in saline solution, plated on the surface of multiple plates containing 10 mL of MRS (de Man, Rogosa and Sharpe) agar, and incubated at 37°C for 24 h. All analyses were performed in duplicate.

The triple-layer method was performed in order to pre-select LAB with potential to produce antimicrobial compounds, as described by Todorov and Dicks (2004). For this purpose, *Listeria monocytogenes* ATCC 7644 and *L. monocytogenes* 422 were used as target microorganisms. Briefly, colonies on MRS agar plates were overlaid with bacteriological agar. After that, plates with less than 50 colonies were overlaid with 10 mL of semi-solid BHI agar containing active growing cells from target microorganisms (approximately 10^6 CFU/mL), and then incubated at 37^oC for 24 h. Colonies with inhibition zones larger than 2 mm were re-streaked in MRS agar, and single colonies were submitted to Gram staining and catalase test. Gram-positive and catalase negative strains were selected as LAB with antimicrobial potential.

Evaluation of bacteriocinogenic activity

Selected isolates were grown on MRS at 30°C for 24h, and the cell-free supernatant was obtained by centrifugation at 14,000 × *g*, for 15 min. The pH of the supernatant was adjusted to about 6.5 with 1M NaOH in order prevent the inhibitory effect caused by lactic acid production. After that, supernatants were treated for 10 min at 80°C to inactivate hydrogen peroxide (H_2O_2). Then, the agar spot method was used. In this method, 10 µl of each supernatant were spotted on plates containing agar BHI supplemented with the target microorganisms cited above (about 10^6 CFU/mL). Plates were incubated at 35ºC for 24 h, and inhibition zones around the colonies were recorded as positive results (Todorov and Dicks, 2004).

In order to confirm the proteinaceous nature of the antimicrobial compounds, cell-free supernatants from the selected isolates, obtained as described above, were treated with 0.1 mg/mL (final concentration) proteinase K 1 mg/mL (Sigma-Aldrich, Darmstadt, Germany) at 30°C for 2 h, followed by deactivation of the enzyme by thermal treatment at 98°C for 2 min. Antimicrobial activity was determined by the agar spot test against *L. monocytogenes* ATCC 7644 and *L. monocytogenes* 422, as described above. Loss of antimicrobial activity was recorded as the positive result for bacteriocin production.

Temperature optimization for bacteriocin production

Selected isolates were recovered in 10 mL of MRS broth and incubated at 25°C, 30°C, and 37°C for 24 h. The cell-free supernatant was obtained by centrifugation, pH adjustment and thermal treatment, as described before. Then, supernatants were submitted to the critical dilution method in 10 mM phosphate buffered saline (PBS) at pH 6.5 (Mayr-Harting et al., 1972). Bacteriocin activity against *L. monocytogenes* ATCC 7644 and *L. monocytogenes* 422 was tested using the agar spot test (Todorov and Dicks, 2004). Bacteriocinogenic activity was expressed as arbitrary units (AU/mL), calculated as a^bx100, where "a" corresponded to the dilution factor, and "b" corresponded to last dilution that produced an inhibition zone greater than 2 mm in diameter. Supernatants were obtained based on the best conditions for bacteriocin production, and used in the tests described next.

Effect of pH and temperature on bacteriocin activity

The effect of pH on bacteriocin activity was determined by adjusting the cell-free supernatants to pH 2, 4, 6, 8, and 10 with sterile HCl 1N or NaOH 1N. After 1 h incubation at 30°C, the cell-free supernatants were adjusted again to pH 6.0. The effect of temperature on the bacteriocins was tested by incubating the cell-free supernatants at 7°C, 42°C, 60°C, and 80°C for 2 h, as well as at 121°C/15 min.

In both cases, bacteriocin activity was determined by critical dilution and the agar spot test, as described above, using *L. monocytogenes* ATCC 7644 and *L. monocytogenes* 422 as the targets. The results were expressed as AU/mL. Cell-free supernatant with pH adjusted to 6.0 without any specific treatment was used as the control.

Inhibitory spectrum

To evaluate the inhibitory spectrum of bacteriocins produced by selected isolates, each supernatant obtained and treated as previously described was tested against the following targets: *Staphylococcus aureus* ATCC 25923, *S. aureus* ATCC 29213, *S. aureus* ATCC 23235, *S. aureus* ATCC 13565, *S. aureus* ATCC 27664, *S. aureus* ATCC 19095, *S. aureus* ATCC 14458, *S.*

epidermidis ATCC 12228*, L. monocytogenes* ATCC 7644, *L. monocytogenes* 422*, Pseudomonas aeuruginosa* ATCC 2785*, P. fluorescens* ATCC 13525, *Lactobacillus sakei* ATCC 15521*, Bacillus cereus* NVH 0173/05, *B. cereus* 1600075/95, *Samonella* Thyphimurium ATCC 14028; *Samonella* Enteritidis, *Yersinia enterocolítica* ATCC 9610, *Escherichia coli* ATCC 8730, and *E. coli* ATCC 25922. Plates containing about 10^6 CFU/mL of each target microorganism were prepared, and the agar spot test was used (Todorov and Dicks, 2004). Plates were incubated in the best conditions for growth of each target microorganism. Inhibition zones larger than 2 mm were recorded as positive results.

RESULTS

From the 21 samples, 264 LAB presented antimicrobial activity against *L. monocytogenes* strains used in this study. *L. monocytogenes* ATCC 7644 showed to be more sensitive than the field strain (*L. monocytogenes* 422*)*, as 161 BAL strains were able to inhibit *Listeria monocytogenes* ATCC 7644*,* but only 103 BAL strains were able to inhibit the *L. monocytogenes* field strain. From the 264 BAL strains with antagonist activity against *L. monocytogenes*, 34 LAB strains (12.9%) obtained from six cheeses were confirmed as bacteriocin producers. The other BAL strains isolated presented inhibiting potential against *Listeria monocytogenes* probably due to other antimicrobial compounds, such as lactic acid or hydrogen peroxide.

Eighteen LAB strains showed greater bacteriocin production when incubated at 25°C, while at 30°C greater production was demonstrated by 20 strains, and at 37°C, only four strains showed improved bacteriocin activity (Table 1). It was noticed that some LAB strains were able to produce the same levels of bacteriocins, when incubated at different temperatures (Table 1).

Regarding the exposure of bacteriocins to different conditions, it was noticed that pH changes were more influential than temperature in bacteriocin activity. In different pH conditions, bacteriocins produced by five (14.7%) LAB strains (named 1, 2, 20, 21, and 23) lost their activity against the target microorganisms. Differently, bacteriocins produced by five other strains (10, 11, 16, 27, and 32) were able to inhibit the target microorganisms in all pH values analyzed, as shown in Table 2. Considering temperature, bacteriocins produced by 30 LAB strains (88.2%) were able to inhibit *L. monocytogenes* after exposure to 7°C, 42°C, 60°C, and 80°C. Additionally, bacteriocins produced by four strains (11.8%) kept their activity even after exposure to sterilization temperatures (Table 2).

Bacteriocins produced by isolated LAB strains showed a diverse spectrum of inhibition. From 34 cell-free supernatants evaluated, 13 were active against *L. monocytogenes* ATCC 7644, and 22 were active against *L. monocytogenes* 422, while only one strain (24) produced bacteriocins able to inhibit both *Listeria* strains tested. Eight strains were able to produce bacteriocins active against more than one target strain tested (Table 3). From these, bacteriocins produced by strains 09, 22, 24, 25, and 34 were active against Gram-positive and Gram-negative targets, such as *L. monocytogenes*, *B. cereus, L. sakei,* and *P. fluorescens*. None of the bacteriocins produced by LAB showed activity against *S. aureus*, *S. epidermidis, P. aeuruginosa, S.* Thyphimurium, *S.* Enteritidis, *Y. enterocolítica,* and *E. coli*.

Table 1. Bacteriocinogenic activity against *L. monocytogenes* expressed by Lactic Acid Bacteria strains isolated from artisanal cheeses incubated at different temperatures (25°C, 30°C, or 37°C).

Bacteriocin identification	Incubation temperature[1]		
	25°C	30°C	37°C
1	<100	<100	400
2	<100	100	100
3	<100	100	100
4	<100	100	100
5	<100	400	400
6	<100	100	100
7	<100	100	<100
8	<100	<100	100
9	<100	100	<100
10	100	100	<100
11	400	100	<100
12	100	<100	<100
13	100	800	<100
14	800	1600	<100
15	400	800	<100
16	400	800	<100
17	400	800	<100
18	400	100	<100
19	400	<100	<100
20	400	100	<100
21	400	100	<100
22	400	<100	<100
23	400	100	<100
24	200	<100	100
25	100	100	100
26	400	<100	<100
27	400	400	<100
28	<100	400	<100
29	100	100	<100
30	100	100	<100
31	100	<100	<100
32	400	100	<100
33	100	100	<100
34	100	100	<100

[1]Quantification of bactericinogenic activity expressed as Arbitrary Units per milliliter (AU/mL).

DISCUSSION

Several intrinsic and extrinsic factors in foods can influence LAB activity by reducing or increasing the production of antimicrobial compounds, especially bacteriocins. Selection of well-adapted strains for food production and storage conditions are essential for successful use of LAB as biopreservation tools (Galvez et al., 2007).

Among storage factors during food production, temperature is important to be considered, as it can directly influence LAB growth, metabolism, and bacteriocin production. LAB strains isolated in this study showed different behaviors when cultured at different temperatures, and it was noticed that at 25°C and 30°C, bacteriocin production was greater than at 37°C. These results corroborate findings about higher levels of bacteriocin production by *L. plantarum* cultured at 30°C than at 37°C (Todorov and Dicks, 2006). However, it was observed by other authors that 30 °C and 37 °C are better temperatures for bacteriocin production in *L. plantarum*,

Lactococcus lactis, Enterococcus durans, and *Enterococcus faecium* (Cavicchioli et al., 2015; Todorov et al., 2010; Todorov, 2008).

Table 2. Effects of pH (2, 4, 6, 8, and 10) and temperature (7, 42, 60, 80, and 121°C) on bacteriocinogenic activity expressed by 34 LAB strains isolated from artisanal cheeses using *L. monocytogenes* as the target microorganism.

Bacteriocin identification	pH (AU/mL)[1]					Temperature (AU/mL)[1]				
	2	4	6	8	10	7°C	42°C	60°C	80°C	121°C
1	<100	<100	<100	<100	<100	1600	1600	1600	800	<100
2	<100	<100	<100	<100	<100	1600	800	1600	800	<100
3	<100	<100	100	<100	<100	800	400	1600	800	<100
4	<100	<100	100	100	100	<100	800	1600	800	<100
5	<100	100	<100	<100	<100	800	800	1600	800	<100
6	<100	<100	100	100	<100	400	400	800	1600	<100
7	<100	100	100	100	100	400	800	800	800	<100
8	<100	<100	100	<100	<100	800	100	100	100	<100
9	<100	100	100	100	100	100	800	400	800	200
10	100	100	100	100	100	800	800	400	400	400
11	100	100	100	400	100	200	200	200	100	<100
12	100	<100	100	100	100	100	100	100	<100	<100
13	<100	<100	100	100	100	200	100	200	100	<100
15	<100	100	<100	100	<100	200	200	200	100	<100
16	100	100	100	100	100	200	100	100	100	<100
17	<100	100	100	100	100	200	100	100	100	<100
18	<100	100	100	100	100	100	100	100	100	<100
19	<100	100	100	100	100	100	100	100	100	<100
20	<100	<100	<100	<100	<100	200	400	200	200	<100
21	<100	<100	<100	<100	<100	200	100	200	100	<100
22	<100	100	400	100	100	100	100	100	100	<100
23	<100	<100	<100	<100	<100	200	200	200	200	<100
24	<100	<100	<100	100	100	400	800	800	200	200
25	<100	<100	100	<100	<100	400	800	400	400	400
26	<100	<100	100	<100	<100	200	100	200	200	<100
27	100	100	100	100	100	200	200	200	200	<100
28	<100	100	100	100	<100	200	100	100	200	<100
29	100	100	100	100	<100	200	200	200	200	<100
30	100	100	100	100	<100	200	200	200	200	<100
31	100	100	100	100	<100	200	100	200	200	<100
32	100	100	100	100	100	200	200	200	100	<100
33	<100	100	100	100	<100	200	200	400	100	<100
34	<100	100	100	<100	100	100	100	100	<100	<100

[1]Results expressed as Arbitrary Units per milliliter (AU/mL)

The stability of bacteriocins produced by LAB can also be influenced by intrinsic and extrinsic factors of different types of food, such as pH, temperature, composition, and resident microbiota (Galvez et al., 2007). The pH can exert an important influence in bacteriocin activity, which may be variable according to the producing species and the target microorganism, as observed by different authors. In a study performed to evaluate the influence of pH on bacteriocinogenic activity against *L. monocytogenes,* authors observed greater inhibitory effect at pH between 4 and 6 (Furtado et al., 2014). Similar results were observed for bacteriocin HV219, produced by *Lactococcus lactis* ssp. *lactis,* which presented greater activity against *E. faecium* HKLHS and *E. faecalis* E88 in pH between 2 to 6 (Todorov and Dicks, 2006). The bacteriocin AMA-K, produced by *Lactobacillus plantarum,*

was more effective in inhibiting *L. innocua*, *L. ivanovii* subsp. Ivanovii, and *L. monocytogenes* Scott A when pH was adjusted to 7, while bacteriocins produced by *Lactococcus* sp. and *Enterococcus* sp. isolated from goat milk were able to keep their activity in pH varying from 2 to 10 (Cavicchioli et al., 2016; Todorov, 2008).

Table 3. Inhibitory spectrum of bacteriocins produced by lactic acid bacteria (LAB) strains isolated from artisanal cheeses with simultaneous activity against more than one target microorganism tested.

Bacteriocin	Bacteriocinogenic activity against the target microorganisms tested[1]					
	LM	LM-UFV	BCNVH	BC	LS	PF
7	+	-	+	+	-	-
8	+	-	+	+	-	-
9	+	-	+	+	+	+
10	+	-	+	+	+	-
22	-	+	-	-	-	+
24	+	+	+	+	-	+
25	+	-	+	+	-	+
34	+	-	+	+	-	+

[1]LM = *Listeria monocytogenes* ATCC 7644; LM-UFV = *Listeria monoytogenes* 422; BCNVH = *Bacillus cereus* NVH 0173/05; BC = *Bacillus cereus* 160 0075/95; LS = *Lactobacillus sakei* ATCC 15.521; PF = *Pseudomonas fluorescens* ATCC 27.843

In our study, bacteriocin activity against *L. monocytogenes* remained stable in from pH 4 to 8, indicating that despite loss of activity in extreme conditions, bacteriocins produced by LAB strains were active in a wide range of pH. This result indicates that the acid conditions found in fermented foods may not affect bacteriocin activity and, in some cases, could even enhance its effect, highlighting an important finding for food technology, especially dairy products. Temperature may also act as a determinant factor in bacteriocin activity. Bacteriocins produced by LAB in this study remained active after exposure to temperatures from 7°C up to 80°C, and similar to findings were reported by Cavicchioli et al. (2015). In addition, bacteriocins produced by four strains remained active even after being exposed to 121°C, showing the high stability of these compounds. Most bacteriocins are considered thermostable, resisting thermal treatment at high temperatures, such as bacteriocin DF04Mi, and nisin, produced by *Lactococcus lactis* (Furtado et al., 2014; Noonpakdee et al., 2003). The stability of bacteriocins in different conditions may be a good standard to determine their use as food biopreservatives under several processing conditions, including pasteurization and other thermal treatments; addition of acids; freezing; or prolonged storage periods (Batdorj et al., 2006).

Bacteriocins produced by LAB are usually effective against closely related microorganisms, and the activity against *L. monocytogenes, Staphylococcus aureus, Lactobacillus* sp., and other Gram-positive microorganisms has been constantly reported. Although not usual, some recent studies also demonstrated the inhibitory potential of bacteriocins produced by LAB against Gram-negative species, such as *Escherichia coli*, *Salmonella* Typhimurium, *Shigella dysenteriae, Pseudomonas aeruginosa,* and *Klebsiella pneumoniae* (Zouhir et al., 2011; Cheikhyoussef et al., 2010; Gong et al., 2010). Besides antibacterial activity, some bacteriocins were also reported as inhibitory compounds against some viruses and some yeast genera, such as *Candida* and *Saccharomyces* (Todorov et al., 2005;

Atanassova et al., 2003). In this context, is important to highlight that bacteriocins produced by five LAB strains (9, 22, 24, 25, and 34) in this study presented a broad spectrum of activity, and were able to inhibit Gram-positive microorganisms, such as *L. monocytogenes*, *B. cereus,* and *L. sakei*, as well as Gram-negative microorganisms, such as *P. aeruginosa*.

CONCLUSIONS

Artisanal Colonial-type cheeses produced in the western region of the state of Parana can be considered important sources of bacteriocinogenic LAB. The antimicrobial substances produced by these strains were active in different processing conditions and against spoilage and pathogenic microorganisms, such as *L. monocytogenes* and *Pseudomonas fluorescens*. Additional studies are necessary to identify and characterize the strains and the antimicrobial substances they produce in order to evaluate their potential as alternative tools for food biopreservation.

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