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BACTERIOCIN PRODUCTION BY *LACTOBACILLUS PENTOSUS* ST712BZ ISOLATED FROM BOZA

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ABSTRACT

Bacteriocin ST712BZ (14.0kDa in size) inhibits the growth of *Lactobacillus casei*, *Escherichia coli*, *Pseudomonas aeruginosa*, *Enterococcus faecalis*, *Klebsiella pneumoniae* and *Lactobacillus curvatus*. Growth of strain ST712BZ in BHI, M17, soy milk and molasses was similar to growth in MRS, with optimal bacteriocin production (12800AU/mL) recorded in MRS after 24h. The same level of bacteriocin production (12800AU/mL) was recorded in MRS broth with an initial pH of 6.5, 6.0 and 5.5. However, MRS broth (pH 6.5) supplemented with 1mM EDTA, yielded only 6400AU/mL. Low levels of bacteriocin activity were recorded in MRS broth with an initial pH of 5.0 and 4.5. Of all media compositions tested, MRS supplemented with tryptone (20.0g/L), glucose (20.0 to 40.0g/L), mannose (20.0g/L), vitamin B_{12}, or vitamin C yielded 12800AU/mL. Glycerol concentrations of 1.0g/L and higher repressed bacteriocin production. Maximal bacteriocin activity (25600AU/mL) was recorded in MRS supplemented with Vit. B_1 or DL-6,8-thioctic acid.

Key words: Bacteriocin ST712BZ; *Lactobacillus pentosus*; boza

INTRODUCTION

Cereals are fermented in most regions of the world and a variety of raw materials and fermentation conditions are used, mostly with lactic acid bacteria and yeast as starter cultures. Lactic acid bacteria play an important role in the preservation, microbiological stability and production of aroma compounds in these products (27).

A few papers have been published on the microbial composition of boza, a beverage traditionally produced in Bulgaria and prepared from a combination of different cereals (14,12,3,35). Most of the lactic acid bacteria that have been isolated from boza belong to the genera *Lactobacillus* spp., *Lactococcus* spp. and *Leuconostoc* spp. and as many as 33 strains revealed antibacterial activity against a number of Gram-positive bacteria, including *Listeria innocua*, and a few Gram-negative bacteria, including *Escherichia coli* (14). In most of these studies, the antimicrobial properties of the strains have been ascribed to the production of bacteriocins (27,33), defined as small proteins or peptides with bactericidal or bacteriostatic activity against genetically closely related species (17).

Few bacteriocin-producing strains of *Lactobacillus pentosus* have been reported, namely bacteriocins produced by strains 191 and 204, isolated from sucuk (8); bacteriocins ST151BR and ST112BR produced by strains ST151BR and ST112BR, respectively (29,30); a bacteriocin produced by a commercial starter culture (25); and pentocin TV35b, produced by strain TV35b isolated from vaginal secretions (26).

In recent papers (20,23), specific environmental conditions, including those found in food, have been studied to determine their effect on the production of bacteriocins. Bacteriocin production changes dramatically upon altering of environmental conditions and optimum production may require a specific combination of environmental parameters (19). Little is known about the interactions these factors have on the production of a bacteriocin, especially in a complex food environment.

Two papers have reported on the effect of nitrogen and carbon in the medium on the production of bacteriocins ST151BR and ST112BR produced by *L. pentosus* (29,30).

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Studies on bacteriocins from other lactic acid bacteria, e.g. pediocin PD-1 (24), enterocin AS-48 (2), enterocin P (13), sakacinP (1), bacteriocins produced by *L. mesenteroides* L124 (21), plantaricin ST31 (31) and plantaricin UG1 (11) have suggested that production is often regulated by growth pH and temperature. In some cases, higher bacteriocin activity has been recorded at sub-optimal growth conditions (22,5,18,1,31).

The aim of this study was to determine the conditions needed for optimal production of bacteriocin ST712BZ produced by *L. pentosus* ST712BZ isolated from boza.

MATERIALS AND METHODS

Bacterial strains and growth conditions

Strain ST712BZ, isolated from boza, was classified as *L. pentosus* based on phenotypic and genotypic characteristics (33). The strain was cultured in MRS medium (Biolab, Biolab Diagnostics, Midrand, SA) at 30ºC and stored at -80ºC in spent MRS broth, supplemented with 15% (v/v) glycerol. MRS broth (Biolab) was used in all experiments, except growth optimization, in which case MRS broth (10) was modified as indicated.

Bacteriocin bioassay

Bacteriocin screening was performed by using the agar-spot-test method (32). Correction of the cell-free supernatant to pH 6.0 with 1M NaOH prevented the inhibitory effect of lactic acid. Antimicrobial activity was expressed as arbitrary units (AU/mL), calculated as a^bx100, where "a" represents the dilution factor and "b" the last dilution that produces an inhibition zone of at least 2mm in diameter. Activity is expressed per mL by multiplication with 100. One AU is defined as the reciprocal of the highest dilution showing a clear zone of growth inhibition (32). *Lactobacillus casei* LHS was used as indicator strain.

Molecular size of the bacteriocins

L. pentosus ST712BZ was cultured in MRS broth (Biolab) for 20h at 30ºC. The cells were harvested (8000x*g*, 10 min, 4ºC) and the bacteriocins precipitated from the cell-free supernatants with 60% saturated ammonium sulphate. The precipitate was re-suspended in one tenth volume 25mM ammonium acetate (pH 6.5), desalted against distilled water by using a 1000Da cut-off dialysis membrane (Spectrum Inc., CA, USA) and then separated by tricine-SDS-PAGE, as described by Schägger and Von Jagow (28). A low molecular-weight marker with sizes ranging from 2.5 to 45.0kDa (Amersham International) was used. The gels were fixed and one half overlaid with *L. casei* LHS (10^6CFU/mL), embedded in Brain Heart Infusion (BHI) agar (Biolab) to determine the position of the active bacteriocin, as described by Todorov and Dicks (29).

Bacteriocin production in different growth media and at different initial growth pH

An 18h-old culture of strain ST712BZ was inoculated (2%, v/v) into MRS broth (Biolab), BHI broth, M17 broth (Merck), soy milk (10%, w/v, soy flour) and molasses (10%, w/v), respectively. Incubation was at 30ºC and 37ºC, respectively, without agitation, for 25h. Samples were taken every hour and examined for bacterial growth (OD at 600 nm), changes in culture pH, and production of bacteriocins (AU/mL). The agar-spot-test method was used, with *L. casei* LHS as target organism.

In a separate experiment, the effect of initial medium pH on the production of bacteriocin ST712BZ was determined. Volumes of 300 mL MRS broth were adjusted to pH 4.5, 5.0, 5.5, 6.0 and 6.5, respectively, with 6M HCl or 6M NaOH and then autoclaved. Each flask was inoculated with 2% (v/v) of an 18h-old culture of *L. pentosus* ST712BZ and incubated at 30ºC for 24h, without agitation. Changes in culture pH and production of bacteriocin ST712BZ, expressed as AU/mL, were determined every hour as described elsewhere. All experiments were done in triplicate.

Effect of medium composition on bacteriocin production

L. pentosus ST712BZ was grown in 10 mL MRS broth (Biolab) for 18h at 30ºC, the cells harvested by centrifugation (8000x*g*, 10 min, 4ºC), and the pellet re-suspended in 10 mL sterile peptone water. Four ml of the cell suspension was used to inoculate 200 mL of the following media: (a) MRS broth (10), without organic nutrients, supplemented with tryptone (20.0 g/L), meat extract (20.0 g/L), yeast extract (20.0 g/L), tryptone (12.5 g/L) plus meat extract (7.5 g/L), tryptone (12.5 g/L) plus yeast extract (7.5 g/L), meat extract (10.0 g/L) plus yeast extract (10.0 g/L), or a combination of tryptone (10.0 g/L), meat extract (5.0 g/L) and yeast extract (5.0 g/L), respectively; (b) MRS broth, i.e. with 20.0 g/L D-glucose; (c) MRS broth without D-glucose, supplemented with 20.0 g/L fructose, sucrose, lactose, mannose, and maltose, respectively; (d) MRS broth with 1.0 to 40.0 g/L glucose as sole carbon source; (e) MRS broth with 2.0 to 100.0 g/L K_2HPO_4; and (f) MRS broth supplemented with 1.0 to 50.0 g/L glycerol.

In a separate experiment, the vitamins cyanocobalamin (Sigma, St. Louis, Mo.), L-ascorbic acid (BDH Chemicals Ltd), thiamine (Sigma) and DL-6,8-thioctic acid (Sigma) were filter-sterilised and added to MRS broth at 1.0 mg/mL (final concentration). All cultures were incubated at 30ºC for 24h. Activity levels of bacteriocin ST712BZ were determined as described elsewhere. All experiments were done in triplicate.

Plasmid isolation

Plasmid DNA was isolated according to the method described by Burger and Dicks (6), followed by CsCl density gradient centrifugation (4). The DNA was separated on an agarose gel, according to Ausubel *et al.* (4).

RESULTS AND DISCUSSION

All data represent an average of three repeats. The values recorded in each experiment did not vary by more than 5% and single data points are presented in the figures without standard deviation bars.

The cell-free supernatant of *L. pentosus* ST712BZ inhibited the growth of *L. casei*, *E. coli*, *Pseudomonas aeruginosa*, *Enterococcus faecalis*, *Klebsiella pneumoniae* and *Lactobacillus curvatus* (33). According to tricine-SDS-PAGE, bacteriocin ST712BZ is approximately 14.0kDa (Fig. 1). No plasmid DNA was isolated.

Growth of *L. pentosus* ST712BZ in BHI, M17, soy milk and molasses was very similar to growth in MRS (Fig. 2). The cell density of both strains increased from OD_{600nm} 0.02 to approximately 9.0 (dilution factor taken into calculation) during 25h (Fig. 2). Low levels of bacteriocin ST712BZ activity (less than 200AU/mL) were detected after 6h of growth in MRS broth

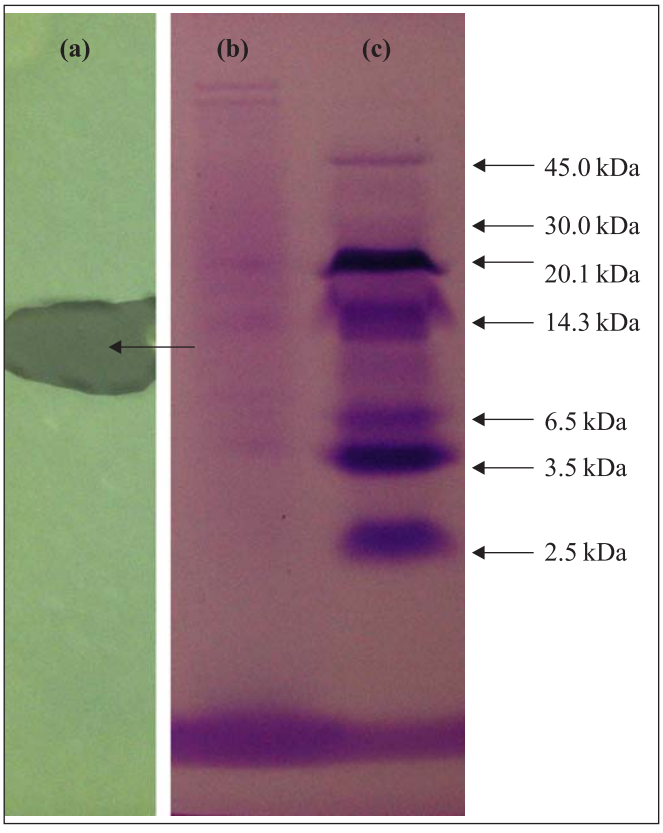

Figure 1. Tricine-SDS-PAGE of bacteriocin ST712BZ. Lane (a): Zone of growth inhibition, corresponding to the position of the bacteriocin band. Lane (b): Peptide band stained with Coomassie Blue R250. Lane (c): Molecular weight marker. The gel was covered with viable cells of *L. casei* LHS (approximately 10^6 CFU/mL), imbedded in BHI agar. Incubation was at 30ºC for 24 h.

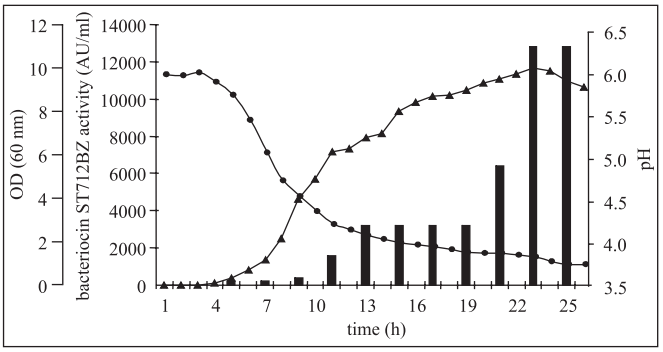

Figure 2. Growth of strain ST712BZ (- ▲ -), production of bacteriocin ST712BZ (- bars -) and changes in pH (- ● -) in MRS broth (Biolab).

(Fig. 2). Optimal production of bacteriocin ST712BZ (12800AU/mL) was recorded after 24h (Fig. 2), and only when incubated at 30ºC. Only 6400AU/mL for ST712BZ was recorded when the cells were incubated at 37°C (not shown). Bacteriocin ST712BZ is a primary metabolite. Similar results have been reported for bacteriocin ST151BR (29), plantaricin Y (7) and bacteriocins produced by *P. acidilactici* (25).

The activity of bacteriocin ST712BZ did not decrease during 72h of incubation at 25ºC, suggesting that extracellular proteases have not been produced. Optimal bacteriocin production (12800AU/mL) was recorded in MRS broth with an initial pH of 6.5, 6.0 and 5.5 (Table 1). In MRS broth (pH 6.5) supplemented with 1 mM EDTA, bacteriocin ST712BZ production was 6400AU/mL. Low levels of bacteriocin activity were recorded when the strains were cultured in MRS broth with an initial pH of 5.0 and 4.5 (Table 1). The culture pH after 24h growth was between 3.52 and 3.75. Similar results have been reported for other bacteriocins produced by *L. pentosus* (29,30) and *L. plantarum* (9,15,31).

Table 1. Influence of initial medium pH on production of bacteriocin ST712BZ in MRS broth (Biolab).

	Initial pH				
	4.5	5.0	5.5	6.0	6.5
Final pH	3.5	3.6	3.7	3.7	3.8
Δ pH	1.0	1.4	1.9	2.3	2.8
Bacteriocin activity (AU/mL)	3200	6400	12800	12800	12800
OD_{600nm}	8.3	8.8	9.2	9.1	9.4
Specific bacteriocin activity ($AU/mL/OD_{600nm}$)	386	727	1391	1407	1362
Changes in bacteriocin activity (%)*	25	50	100	100	100

*Compared to 12 800AU/mL recorded in MRS broth (Biolab).

Growth of strain ST712BZ in BHI broth or M17 broth adjusted to pH 6.5 yielded only 400AU/mL of bacteriocin ST712BZ (Table 2). No bacteriocin production was recorded in 10% (w/v) soy milk (Table 2). Low levels of bacteriocin ST712BZ (200AU/mL) were recorded when the strains were grown in 10% (w/v) molasses (Table 2). Specific nutrients are required for the production of the bacteriocin ST712BZ. This phenomenon has been observed for other bacteriocins, e.g. bacteriocins ST151BR and ST112BR (29,30).

Tryptone (20 g/L), or a combination of tryptone and yeast extract (1:0.6), added to basal MRS medium yielded a bacteriocin level of 12800AU/mL (Table 2). Growth in the presence of a combination of tryptone and meat extract (1:0.6) reduced bacteriocin production with 50%. Growth in the presence of

Table 2. Influence of organic nitrogen, carbohydrates, growth medium and potassium on the production of bacteriocin ST712BZ

Component	Concentration (g/L)	pH	Bacteriocin ST712BZ	
			Activity (AU/mL)	Changes in bacteriocin activity (%)*
Tryptone	20.0	3.8	1280	100
Meat extract	20.0	3.7	1600	13
Yeast extract	20.0	3.7	3200	25
Tryptone + meat extract	12.5 + 7.5	3.7	6400	50
Tryptone + yeast extract	12.5 + 7.5	3.7	12800	100
Meat extract + yeast extract	10.0 + 10.0	3.8	3200	25
Tryptone + meat extract + yeast extract	10.0 + 5.0 + 5.0	3.8	12800	100
Glucose	20.0	3.7	12800	100
Fructose	20.0	4.0	1600	13
Sucrose	20.0	3.8	6400	50
Maltose	20.0	3.8	3200	25
Mannose	20.0	3.7	12800	100
Lactose	20.0	4.0	3200	25
Gluconate	20.0	5.2	3200	25
Glycerol	0	3.8	12800	100
	1.0	3.8	6400	50
	5.0	3.8	6400	50
	10.0	3.8	6400	50
	20.0	3.8	3200	25
	50.0	3.8	3200	25
MRS	50.0	3.8	12800	100
BHI	37.0	6.1	400	3
M17	42.5	6.0	400	3
Soy flour	100.0	4.3	0	0
Molasses	100.0	4.1	200	2
K_2HPO_4	2.0	3.8	12800	100
KH_2PO_4	2.0	3.8	12800	100
	(mg/mL)			
Cyanocobalamin (Vit. B_{12})	1.0	4.0	12800	100
Thiamine (Vit. B_1)	1.0	4.0	25600	200
DL-6,8-thioctic acid	1.0	4.0	25600	200
L-ascorbic acid (Vit. C)	1.0	4.0	12800	100
Control	0	3.9	12800	100

*Compared to 12 800 AU/mL recorded in MRS broth (Biolab).

either yeast extract, or a combination of meat extract and yeast extract (1:1), resulted in bacteriocin production of 3200AU/mL (Table 2). Growth in the presence of only meat extract yielded only 1600AU/mL.

Tryptone is the key nitrogen source needed for optimal production of bacteriocin ST712BZ. Similar results have been reported for the production of plantaricin 423 (34) and for bacteriocins ST151BR and ST112BR (29,30). In the case of plantaricin 423, optimal bacteriocin production was recorded in MRS broth supplemented with bacteriological peptone, followed by casamino acids, tryptone and meat extract. Stimulation of bacteriocin production by yeast extract and meat extract has been reported for helveticin J (16). As far as we could determine, this is the first indication that tryptone is the key nitrogen source needed in the production of *L. pentosus* bacteriocins.

Growth of strain ST712BZ in the presence of glucose (20.0, 30.0 and 40.0 g/L) yielded 12800AU/mL of bacteriocin ST712BZ (Fig. 3A). Lower concentrations of glucose (1.0 g/L, 5.0 g/L and 10.0 g/L) yielded 800AU/mL, 800AU/mL and 6400AU/mL, respectively (Fig. 3A). Growth in presence of mannose (20 g/L) yielded the same activity as 20 g/L glucose (Table 2). Growth in the presence of sucrose (20.0 g/L) reduced bacteriocin production by 50%, i.e to 6400AU/mL. Gluconate, maltose and lactose (20.0 g/L), on the other hand, yielded 3200AU/mL (Table 2). Low activity (1600AU/mL) was recorded in the presence of 20.0 g/L fructose. Based on these results, the production of bacteriocin ST712BZ is stimulated when cells are grown in medium supplemented with 20.0 to 40.0 g/L glucose.

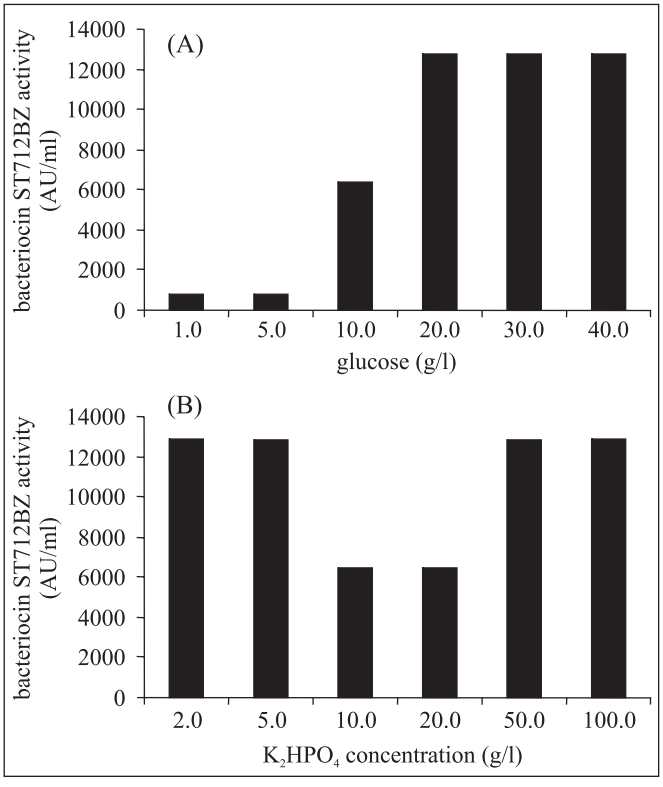

Figure 3. Effect of glucose **(A)** and K_2HPO_4 **(B)** on the production of bacteriocin ST712BZ.

Bacteriocin ST712BZ production remained at 12800AU/mL when strain ST712BZ was grown in the presence of K_2HPO_4 or KH_2PO_4 (Fig. 3B). Growth in the presence of 10.0 g/L or 20.0 g/L yielded 6400AU/mL bacteriocin ST712BZ (Fig. 3B). In the case of plantaricin UG1, 7.0g/L K_2HPO_4 resulted in increased activity (11). Concentrations of 10.0 and 20.0g/L K_2HPO_4 lowered bacteriocin ST151BR activity by 50% (29). In the case of bacteriocin ST112BR, higher levels of activity were recorded when the medium contained 5.0 g/L, 10.0 g/L and 20.0 g/L KH_2PO_4 (30). The optimal concentration of K_2HPO_4 required for plantaricin ST31 production was between 2.0 g/L and 5.0 g/L (31).

Production of bacteriocin ST712BZ was the highest (12800AU/mL) in the absence of glycerol (Table 2). Glycerol concentrations of 1.0 g/L and higher (up to 50.0 g/L) led to decreased levels of bacteriocin ST712BZ production (Table 2). Similar results were reported for the production of bacteriocins ST151BR, ST112BR and plantaricin ST31 (29,30,31). An increase in glycerol leads to a lowering in water activity. The production of bacteriocin ST712BZ may be influenced by osmotic stress.

Bacteriocin ST712BZ production of 12800AU/mL was recorded in the presence or absence of Vit. B_{12} or Vit. C (Table 2). A increase in production levels was recorded in MRS supplemented with Vit. B_1 or DL-6,8-thioctic acid up to 25600AU/mL (Table 2). In the case of bateriocin ST151BR, produced by *L. pentosus* ST151BR, the latter vitamins had no effect on production levels (29). However, these vitamins led to a 50% decrease in the production of bacteriocin ST112BR, produced by *L. pentosus* ST112BR (30).

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RESUMO

Bacteriocina produzida por *Lactobacillus pentosus* ST712BZ isolad de boza

A bacteriocina ST712BZ produzida par *Lactobacillus pentosus* (peso molecular de 14,0kDa) inibe o crescimento de *Lactobacillus casei*, *Escherichia coli*, *Pseudomonas aeruginosa*, *Enterococcus faecalis*, *Klebsiella pneumoniae* e *Lactobacillus curvatus*. O crescimento de *L. pentosus* ST712BZ

em BHI, M17, leite de soja e melaços foi semelhante ao observado em MRS, registando-se a produção máxima de bacteriocina (12800UA/mL) em MRS após 24 h. Observou-se o mesmo nível de produção de bacteriocina (12800UA/mL) em caldo MRS com pH inicial de 6,5, 6,0 e 5,5. No entanto, em caldo MRS (pH 6,5) suplementado com 1 mM de EDTA a produção apenas atingiu 6400UA/mL. Os níveis de atividade bacteriocinogênica detectados em caldo MRS com um pH inicial de 5,0 e 4,5 foram baixos. De todas as fórmulas de meios de cultura testadas a que apresentou a atividade máxima 12800UA/mL foi MRS suplemento de triptona (20,0g/L), glicose (20,0 e 40,0 g/L), manose (20.0 g/L), vitamina B_{12} e vitamina C. A produção de bacteriocinas foi inibida por concentrações de glicerol superiores ou iguais a 1.0 g/L. Verificou-se a actividade bacteriocinogênica máxima (25600UA/mL) em caldo MRS suplementado com vitamina B_1 ou ácido DL-6,8-thioctic.

Palavras-chave: Bacteriocina ST712BZ, *Lactobacillus pentosus*, boza

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