Optimized production of bacteriocin ST11BR, generated by *Lactobacillus paracasei* subsp. *paracasei* ST11BR isolated from traditional South African beer

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Little is known about the production of antimicrobial peptides (bacteriocins) by lactic acid bacteria in traditional South African beer and their inhibition of food spoilage or pathogenic bacteria. In this paper, we report on bacteriocin ST11BR, produced by *Lactobacillus paracasei* subsp. *paracasei* ST11BR isolated from beer made with maize, barley, soy flour and sugar (sucrose). Bacteriocin ST11BR is a 3.2-kDa peptide with activity against *Lactobacillus casei, L. sakei, Pseudomonas aeruginosa* and *Escherichia coli.* The peptide is sensitive to proteinase K and pronase, but not to α-amylase. Glycerol in the growth medium repressed bacteriocin production. Tween 80 suppressed production by more than 50%, irrespective of the initial pH of the medium. MRS broth adjusted to pH 4.50 yielded 3200 AU/ml bacteriocin. The corresponding value at pH 5.0, 5.5, 6.0 and 6.5 was 12 800 AU/ml. The highest yield (25 600 AU/ml) was recorded in MRS broth without Tween 80, and with meat extract as the only nitrogen source, or a combination of meat extract and tryptone, or yeast extract and tryptone. Growth in the presence of tryptone as sole nitrogen source achieved only 12 800 AU/ml bacteriocin. Yeast extract, or a combination of yeast extract and meat extract, yielded 6400 AU/ml. A growth medium comprising 20.0 g/l maltose, sucrose or mannose yielded bacteriocin levels of 25 600 AU/ml, whereas the corresponding values for the same concentration of glucose or fructose were 12 800 AU/ml and 1 600 AU/ml, respectively. Lactose did not stimulate bacteriocin production — the highest yield (6 400 AU/ml) was generated in the presence of 10.0 g/l. No difference in bacteriocin activity was recorded when strain ST11BR was grown in the presence of 2.0 g/l KH_2PO_4 and 2.0–10.0 g/l K_2HPO_4. However, cyanocobalamin, thiamine and DL-6,8-thioctic acid (1.0 ppm), but not L-ascorbic acid, stimulated peptide production. This study provided valuable information on the optimal production of bacteriocin by a strain of *L. paracasei* naturally present in a traditional beer.

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Introduction

Bacteriocins are defined as proteins or protein complexes antagonistic to bacteria closely related genetically to the producer organism.[1–3] Many papers have been published on the characterization, isolation and genetics of bacteriocins produced by lactic acid bacteria, including *Lactobacillus* species.[2,4] This is not surprising, since *Lactobacillus* species are found in almost all fermented foods and many different strains are used as starter cultures.[4,5]

Lactic acid bacteria are fastidious and require rich growth media with yeast extract and protein hydrolysates for optimal growth and bacteriocin production.[6–10] The effect of medium composition on bacteriocin yield has been determined for a number of cases, e.g. enterocin AS-48, produced by *Enterococcus faecalis*[11]; enterocins 1146 and P, produced by *Enterococcus faecium*[6,12,13]; unclassified bacteriocins, produced by *Lactobacillus curvatus* L442[14]; plantaricin ST31 and plantaricin 423, produced by *Lactobacillus plantarum*[10,15]; sakacin P, produced by *Lactobacillus sakei*[3]; unclassified bacteriocins, produced by *Leuconostoc mesenteroides*[14,16]; pediocin AcH and unclassified bacteriocins, produced by *Pediococcus acidilactici*[17,18]; pediocin PD-1, produced by *Pediococcus damnosus*[19]; and nisin, produced by *Lactococcus lactis* subsp. *lactis*.[8,20,21]

Bacteriocin production is strongly dependent on pH, nutrient sources and incubation temperature; but activity levels do not always correlate with cell mass or growth rate of the producer strain.[22,23] Enhanced bacteriocin levels are often obtained at temperatures and a pH, and with nutrient sources different from those required for optimal growth of the producer strain.[3,9,10,12,16,24–26]

Little information is available on the optimal fermentation of bacteriocins produced by *Lactobacillus* spp. A few papers in this regard have been published on the proteins generated by strains

of *Lactobacillus plantarum*,[10,15,27–29] *L. helveticus*,[30] *L. acidophilus*,[31] *L. sakei*[32,33] and *L. casei*.[34] Several bacteriocin-producing strains of *Lactobacillus paracasei* subsp. *paracasei* have been isolated from various sources, namely, raw milk,[35] cheese,[36–39] and healthy oral cavities.[40]

In this paper we report on bacteriocin ST11BR, produced by *L. paracasei* subsp. *paracasei* ST11BR isolated from a traditional fermented South African beer, and optimization of the growth medium to create enhanced levels of activity.

Table 1. Spectrum of antimicrobial activity recorded for bacteriocin ST11BR.

Indicator strain	Source	Culture medium and incubation temperature	Bacteriocin activity[a]
Enterobacter cloacae 24	Human middle ear	BHI, 37°C	–
Enterococcus faecalis E77, E80, E90, E92, FA2	Pig faeces	MRS, 30°C	–
E. faecalis 20	Human middle ear	BHI, 37°C	–
Escherichia coli 8	Mastitic milk	BHI, 37°C	+
Lactobacillus casei LHS	Wine	BHI, 30°C	++
Lactobacillus curvatus DF38	Salami	MRS, 37°C	–
Lactobacillus delbrueckii subsp. *bulgaricus*	Yoghurt	MRS, 30°C	–
Lactobacillus plantarum 423	Sorghum beer	MRS, 30°C	–
Lactobacillus sakei DSM 20017	Sake	MRS, 30°C	++
Lactobacillus salivarius 241	Pig intestine	MRS, 30°C	–
Listeria innocua LMG 13568	Meat	BHI, 30°C	–
Pseudomonas aeruginosa 7	Human middle ear	BHI, 37°C	+
Staphylococcus aureus 2	Human middle ear	BHI, 37°C	–
Streptococcus agalactiae 9	Mastitic milk	BHI, 37°C	–
Streptococcus caprinus ATCC 700065, ATCC 700066	Goat	BHI, 30°C	–
Streptococcus pneumoniae 4	Human middle ear	BHI, 37°C	–
Streptococcus sp. TL1, TL2R, TL2W	Goat intestine	BHI, 37°C	–
Streptococcus uberus 12	Mastitic milk	BHI, 37°C	–

[a]–, no inhibition zone; + and ++ refer to the level of antimicrobial activity, as recorded in zone size: +, less than 10 mm in diameter; ++, 11–20 mm in diameter.

Materials and methods

Bacterial strains and growth media. Strain ST11BR was isolated from traditional South African beer produced from the fermentation of maize, barley, soy flour and sugar (sucrose). The strain was identified as *Lactobacillus paracasei* subsp. *paracasei* based on physiological and biochemical characteristics as described by Schillinger and Lücke,[41] Cogan *et al.*,[42] Stiles and Holzapfel[43] and Collins *et al.*[44] Sugar fermentation reactions were confirmed by using the API 50 CHL system (Biomérieux, Marcy-l'Etoile, France).

Strain ST11BR was grown in MRS medium (Merck, Darmstadt) and incubated at 30°C. The growth media, growth temperature and origin of the other strains included in this study are listed in Table 1. Strains were stored at –80°C in MRS broth containing 15% (v/v) glycerol.

Bacteriocin bioassay. Bacteriocin was screened using the agar spot test, as described by van Reenen *et al.*[45] Adjusting the cell-free supernatant to pH 6.0 with 1 M NaOH prevented the inhibitory effect of lactic acid. Antimicrobial activity was expressed as arbitrary units (AU) per ml. One AU was defined as the reciprocal of the highest dilution showing a clear zone of growth inhibition.[45] The indicator strains are listed in Table 1.

Molecular size of bacteriocin ST11BR. Strain ST11BR was grown in MRS broth for 20 h at 30°C. The cells were harvested by centrifugation (8000 × *g*, 10 min, 4°C) and the bacteriocin precipitated from the cell-free supernatant with 80% saturated ammonium sulphate. The precipitate was resuspended in one-tenth volume 25 mM ammonium acetate buffer (pH 6.5), desalted using a 1000-Da cut-off dialysis membrane (Spectrum Inc., California) and subjected to tricine-SDS-PAGE, as described by Schägger and Von Jagow.[46] Low molecular weight markers with sizes ranging from 2.35 to 46.0 kDa (Amersham International, U.K.) were used. The gels were fixed and one half stained with Coomassie Blue R250 (Saarchem, Krugersdorp, South Africa), as described by Van Reenen *et al.*[45] The position of the active bacteriocin was determined by overlaying the other half of the gel (not stained and extensively pre-washed with sterile distilled water) with viable cells of *Lactobacillus casei* LHS (approximately 10^6 cfu/ml), embedded in Brain Heart Infusion (BHI) agar (1.0% agar, w/v). The overlaid gel was incubated for 24 h at 30°C.

Effect of enzymes on bacteriocin ST11BR. Strain ST11BR was grown in MRS broth at 30°C for 24 h, the cells harvested by centrifugation (8000 × *g*, 10 min, 4°C), and the cell-free supernatant adjusted to pH 6.0 with 6 M NaOH. One ml cell-free supernatant was incubated for 2 h in the presence of 1 mg/ml Proteinase K (Roche, Indianapolis), pronase (Boehringer Mannheim), α-amylase (Sigma Diagnostics, St Louis, MO) and catalase (Boehringer Mannheim). Antimicrobial activity was monitored using the agar spot test method as described before.

Production of bacteriocin ST11BR in different growth media and at different initial pH values. An 18-h-old culture of strain ST11BR was inoculated (2%, v/v) in duplicate into MRS broth, BHI broth (Biolab Diagnostics, Midrand, South Africa), M17 broth (Merck), soy milk (10%, w/v, soy meal) and molasses (2 to 10%, w/v, at 2% intervals). Incubation was at 30°C and 37°C, respectively, without agitation, for 28 h. Samples were taken every hour and examined for bacterial growth [assessed by optical density (OD) at 600 nm], changes in culture pH, and antimicrobial activity (AU/ml) against *L. casei* LHS. The agar spot test method was used, as described before.

The effect of initial medium pH on bacteriocin ST11BR production was tested in a separate experiment. MRS broth, without Tween 80 (MRS basal broth, MRSbb), was divided into 300-ml volumes in 500-ml Erlenmeyer flasks, adjusted to pH 4.5, 5.0, 5.5, 6.0 and 6.5, respectively, with 6 M HCl or 6 M NaOH, and then autoclaved. Each flask was inoculated with 2% (v/v) of an 18-h-old culture of strain ST11BR and incubated at 30°C for 20 h, without agitation. Changes in culture pH and production of the bacteriocin were determined every hour, as described before.

Effect of medium composition on bacteriocin production. Strain ST11BR was grown in 10 ml MRS broth for 18 h at 30°C, the cells harvested by centrifugation (8000 × *g*, 10 min, 4°C), and the pellet resuspended in 10 ml sterile peptone water. This suspension was used to inoculate 200 ml MRSbb, supplemented with nutrients and vitamins, as listed in Table 3. An inoculum size of 2% (v/v) was used. Incubation was at 30°C for 20 h. Activity levels of bacteriocin ST11BR were determined as described above.

In a separate experiment, the vitamins cyanocobalamin (Sigma, St Louis), L-ascorbic acid (BDH Chemicals, Poole, UK), thiamine (Sigma) and DL-6,8-thioctic acid (Sigma) were filter-sterilized and added to MRSbb at 1 mg/ml (final concentration).

Results

Bacteriocin ST11BR inhibited the growth of *L. casei*, *L. sakei*, *Pseudomonas aeruginosa* and *E. coli* (Fig. 1), but none of the other species included in the study (Table 1).

According to tricin-SDS-PAGE, bacteriocin ST11BR is a small polypeptide with a molecular mass of approximately 3.2 kDa (Fig. 2). Complete inactivation or significant reduction in antimicrobial activity was observed after treatment of the cell-free supernatant with Proteinase K and pronase (results not shown). Treatment with catalase and α-amylase did not alter the antimicrobial activity of the bacteriocin (results not shown).

Low bacteriocin activity (200 AU/ml) was recorded when strain ST11BR was grown in BHI and M17 broth, despite good growth. Similar results were recorded in the presence of 10% (w/v) soy milk. Good growth was also recorded in 2% and 10% (w/v) molasses, but low levels of the peptide were produced (400 AU/ml for each). The highest yield of bacteriocin ST11BR activity (25 600 AU/ml) was recorded after 17 h in MRSbb, and only when incubated at 30°C. During 28 h of growth in MRSbb, the pH decreased from 6.50 to 3.58 (results not shown) and the

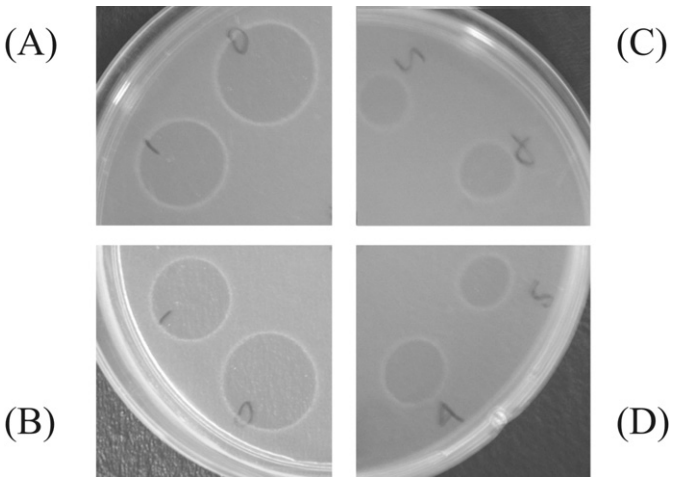

Fig. 1. Bacteriocin ST11BR activity against (**A**) *L. casei* LHS, (**B**) *L. sakei* DSM 20017, (**C**) *E. coli* 8 and (**D**) *P. aeruginosa* 7.

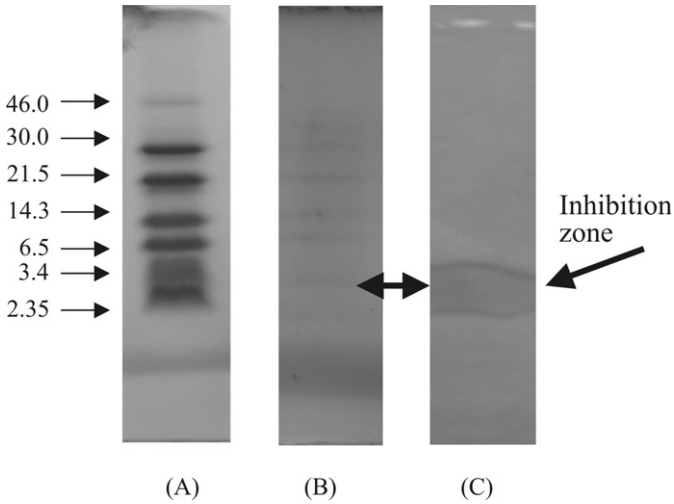

Fig. 2. Tricine-SDS-PAGE of bacteriocin ST11BR. Lane A: molecular weight markers (in kDa). Lane B: peptide band stained with Coomassie Blue. Lane C: zone of growth inhibition, corresponding to the position of the peptide band in lane B. The gel was covered with viable cells of *L. casei* LHS (approximately 10^6 cfu/ml), embedded in MRS agar. Incubation was at 30°C for 24 h.

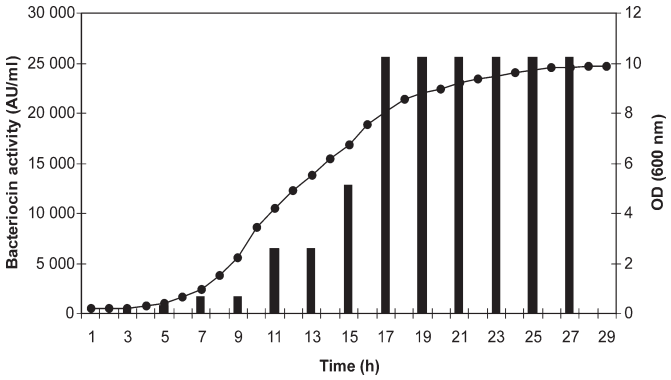

Fig. 3. Growth of strain ST11BR and corresponding bacteriocin production in MRS broth at 30°C. The pH was not regulated. Cell growth was recorded as change in optical density ($OD_{600\ nm}$; -●-) and bacteriocin production in activity units (AU/ml, bars).

cell density increased from 0.05 to 9.95 (Fig. 3).

Low levels of the bacteriocin were recorded in MRS broth adjusted to pH 4.50 (3 200 AU/ml; Table 2). In the same medium adjusted to pH 5.0, 5.5, 6.0 and 6.5, respectively, the corresponding yield was 12 800 AU/ml. The final pH of the cultures was essentially the same (3.40–3.73), irrespective of the initial growth pH (Table 2).

Inclusion of Tween 80 in the growth medium reduced bacteriocin ST11BR production by more than 50% (results not shown). When meat extract was added to MRSbb as the only nitrogen source, the yield was 25 600 AU/ml (Table 3). In the presence of tryptone as the only nitrogen source, much lower levels (12 800 AU/ml) were produced. However, when meat extract and tryptone, or yeast extract and tryptone, were added, yield increased to 25 600 AU/ml. Yeast extract, or a combination of yeast extract and meat extract, produced only 6400 AU/ml. The corresponding value for a combination of all three nitrogen sources (tryptone, meat extract and yeast extract) was 12 800 AU/ml (at 20 h).

Growth in the presence of 20.0 g/l maltose, sucrose or mannose yielded bacteriocin levels of 25 600 AU/ml (Table 3). The same concentration of glucose or fructose generated only 12 800 AU/ml and 1600 AU/ml, respectively. Concentrations of 25 600 AU/ml were recorded in the presence of 15.0, 20.0, 30.0 and 40.0 g/l lactose, respectively. Lactose at 10.0 g/l yielded 6 400 AU/ml, whereas concentrations below 10.0 g/l gave only 800 AU/ml.

No difference in bacteriocin activity was recorded when strain ST11BR was grown in the presence of 2.0 g/l KH_2PO_4 and 2.0–10 g/l K_2HPO_4 (Table 3). Concentrations of 20 g/l and higher of K_2HPO_4 repressed bacteriocin output.

Peptide production was the highest (25 600 AU/ml) in the absence of glycerol (Table 3). Moreover, glycerol concentrations of 1.0–50 g/l repressed bacteriocin production (Table 3).

Cyanocobalamin, thiamine and DL-6,8-thioctic acid in MRS broth (1.0 ppm) yielded 25 600 AU/ml bacteriocin (Table 3). However, L-ascorbic acid reduced yields to 12 800 AU/ml.

Discussion

Bacteriocin ST11BR inhibited the growth of the genetically closely related species *L. casei* and *L. sakei,* and in this regard conforms to the description of a bacteriocin as defined by Klaenhammer.[1] The activity of bacteriocin ST11BR as recorded against Gram-negative bacteria (*E. coli* and *P. aeruginosa*) is an unusual phenomenon. Only a few bacteriocins of lactic acid bacteria with activity against Gram-negative bacteria have been reported, namely, termophilin 81, produced by *Streptococcus thermophilus,* a protein produced by *L. paracasei* subsp. *paracasei* L126 and L134, and one produced by *L. lactis* KCA2386.[39,47,48]

The molecular size of bacteriocin ST11BR (3.2 kDa) is within the range of most of these proteins reported for the genus *Lactobacillus*.[4] Resistance to treatment with α-amylase suggests that bacteriocin ST11BR is not glycosylated and is in accordance with most bacteriocins thus far described. Any sensitivity to α-amylase would have implied the presence of an essential

Table 2. Effect of initial medium pH on the production of bacteriocin ST11BR.[a]

Initial pH	4.50	5.00	5.50	6.00	6.50
Final pH after 20 h	3.40	3.53	3.59	3.64	3.73
Bacteriocin activity (AU/ml) after 20 h	3 200	12 800	12 800	12 800	12 800

[a]Medium: MRS broth, without Tween 80.

Table 3. Influence of nitrogen, carbohydrates, potassium and vitamins on bacteriocin ST11BR production.

Component	Concentration (g/l)	Activity (AU/ml)
Tryptone	20.0	12 800
Meat extract	20.0	25 600
Yeast extract	20.0	6 400
Tryptone + meat extract	12.5 + 7.5	25 600
Tryptone + yeast extract	12.5 + 7.5	25 600
Meat extract + yeast extract	10.0 + 10.0	6 400
Tryptone + meat extract + yeast extract	10.0 + 5.0 + 5.0	12 800
Maltose, or saccharose, or mannose	20.0	25 600
Glucose	20.0	12 800
Fructose	20.0	1 600
Lactose	0.5, 1.0, 2.0, 3.0, 4.0, 5.0	800
"	10.0	6 400
"	15.0, 20.0, 30.0, 40.0	25 600
KH_2PO_4	2.0	25 600
K_2HPO_4	2.0, 5.0, 10.0	25 600
"	20.0	12 800
"	50.0	800
Glycerol	0	25 600
"	1.0	12 800
"	2.0, 5.0	6 400
"	10.0, 20.0	1 600
"	50	400
	Concentration (ppm)	
Cyanocobalamin (vit. B_{12})	1.0	25 600
Thiamine (vit. B_1)	1.0	25 600
DL-6,8-thioctic acid	1.0	25 600
L-ascorbic acid (vit. C)	1.0	12 800
Control	0	12 800

carbohydrate moiety as shown for leuconocin S[49] and carnocin 54.[50]

The low activity levels of bacteriocin ST11BR recorded in M17 broth, BHI broth, soymilk and molasses, despite relatively good growth, suggests that specific nutrients are required for its production. Furthermore, with bacteriocin production recorded at 30°C and not at 37°C, temperature seems to play an important role. Indeed, growth temperature and bacteriocin production are often correlated, as observed for lactocin A,[24] enterocin 1146,[12] lactocin S,[26] amylovorin 147,[19] nisin Z,[25] and mesenterocin.[22]

Detectable levels of bacteriocin ST11BR were recorded after 3 h of growth in MRS broth, indicating that the peptide is a primary metabolite. Similar results were reported for plantaricin Y[51] and bacteriocins generated by *P. acidilactici*,[52] and *L. paracasei* subsp. *paracasei* M3.[38] Bacteriocin yield grew logarithmically and stabilized at 25 600 AU/ml during the next 10 h of slow growth at 30°C (Fig. 3). As observed for other bacteriocins (sakacin K and one produced by *E. faecium* RZS C5), extended growth does not necessarily lead to higher production or activity levels.[53] We conclude from our results that maximum levels of bacteriocin ST11BR were achieved towards the end of logarithmic growth ($OD_{600\ nm}$ = 8.50), remaining at 25 600 AU/ml for the duration of incubation (Fig. 3), suggesting that the peptide was not affected by changes in culture conditions (e.g. lowering of pH from 3.9 to 3.5). Reduced bacteriocin activity after logarithmic growth has been observed for lactacin B, mesenterocin 5, helveticin J, and enterocin 1146.[7,54–56] In many of these cases, loss of activity has been ascribed to proteolytic degradation, protein aggregation, adsorption on cell surfaces and feedback regulation.[3,9,12,24,57]

An initial growth pH of between 5.0 and 6.5 did not affect bacteriocin ST11BR production. Krier *et al.*[16] showed that the influences of pH and temperature were different for two bacteriocins produced by the same microorganism. This demonstrated that more than one growth mechanism may be involved.

The activity levels of bacteriocin ST11BR were strongly influenced by the nitrogen source added to MRS medium. Similar results were observed for the production of plantaricin ST31 by *L. plantarum* ST31.[10] In the case of the latter, highest production was obtained in MRS broth supplemented with bacteriological peptone, followed by casamino acids, tryptone and meat extract.[15]

Tween 80 reduced the activity of bacteriocin ST11BR by approximately 50%, whereas it had the opposite effect on the production of plantaricin 423,[15] pediocin AcH[17] and lactocine 705.[58]

Lactose concentrations in the range of 15–40 g/l stimulated bacteriocin ST11BR production. Different results were observed for sakacin P[3], where a glucose concentration above 40 g/l reduced its production. Similar results were observed for nisin and enterocin 1146, exposed to more than 40 g/l sucrose and 20 g/l glucose, respectively.[20,59]

Little is known about the influence of potassium ions on the production of these peptides. Whereas low concentrations of KH_2PO_4 (2 g/l) and K_2HPO_4 (2–10 g/l) made no difference to bacteriocin ST11BR activity and K_2HPO_4 concentrations of 20 g/l and higher repressed activity, 7.0 g/l K_2HPO_4 increased bacteriocin production by *L. plantarum* UG1.[60]

This study provided information to optimize bacteriocin production by *L. paracasei* subsp. *paracasei* ST11BR, a strain naturally present in traditional South African beer made from maize, barley, soy flour and sugar. Further research is needed to show if changes in the ingredients of the beer would lead to enhanced bacteriocin production *in situ* and an extended shelf life.

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