ISSN 1330-9862
(FTB-2157)

original scientific paper

Evaluation of Potential Probiotic Properties of *Enterococcus mundtii*, Its Survival in Boza and *in situ* Bacteriocin Production

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Received: September 23, 2008
Accepted: February 4, 2009

Summary

Boza is a low-pH and low-alcohol cereal-based beverage produced in the Balkan Peninsula. Barley was cooked and prepared according to a traditional recipe and inoculated with *Enterococcus mundtii* ST4V (a potential probiotic and bacteriocin-producing strain), commercially produced boza, *Saccharomyces cerevisiae*, and a combination of strain *E. mundtii* ST4V and *Saccharomyces cerevisiae*. Fermentation was carried out at 37 °C for 3 h. The organoleptic properties of fermented products were evaluated by a qualified taste panel. No significant differences in rheological properties were observed, suggesting that *E. mundtii* ST4V had no effect on the quality of the final product. Microbial cell numbers remained relatively unchanged during one week of storage. The preservative properties of bacteriocin ST4V were evaluated by contaminating boza with *Lactobacillus sakei* DSM 20017. Changes in microbial populations were monitored by using classical microbiological methods, PCR with species-specific primers and denaturing gradient gel electrophoresis (DGGE). Adsorption of bacteriocin ST4V to target cells is pH-dependent, with the highest adsorption (88 %) recorded at pH=8.0 and pH=10.0. Maximum adsorption of bacteriocin ST4V (75 %) to *Enterococcus faecalis* and *Listeria innocua* was recorded at 25 to 37 °C. Growth of *E. mundtii* ST4V was inhibited only by a few antibiotics and anti-inflammatory medicaments, suggesting that the strain may be used as a probiotic by individuals receiving medical treatment.

Key words: Enterococcus mundtii, boza, probiotic, bacteriocin ST4V

Introduction

Boza is a low-alcohol beverage produced from the fermentation of barley, oat, millet, maize, wheat or rice. Cooked cereal is strained to remove most of the solids, sugar is added to taste and inoculated with a starter culture, either yoghurt or sourdough. The sludge is fermented at 30 °C for 24 h, cooled and kept refrigerated for 3–5 days (*1*,*2*). Although a number of *Lactobacillus*, *Lactococcus*, *Leuconostoc*, *Pediococcus*, *Oenococcus* and *Weissella* spp. have been isolated from boza (*3*–*8*), only one paper (*2*) addressed the selection of starter cultures. Lactic acid bacteria, and presumably yeast, produce a number of vitamins (*9*) and increase the nutritional value of the product.

By definition, bacteriocins produced by lactic acid bacteria are proteinaceous antibacterial compounds that exhibit bactericidal or bacteriostatic activity against genetically closely related bacteria (*10*,*11*). Ivanova *et al.* (*12*) were the first to report on bacteriocins produced by lactic acid bacteria isolated from boza and described

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bacteriocin B14, produced by *Lactococcus lactis* ssp. *lactis* isolated from Bulgarian boza. Bacteriocin B14 inhibits the growth of Gram-positive and Gram-negative bacteria (*12*). Since 2000, a number of lactic acid bacteria from boza have been isolated, and mesentericin ST99 produced by *Leuconostoc mesenteroides* ssp. *dextranicum* (*5*), pediocin ST18 from *Pediococcus pentosaceus* (*6*), bacteriocins ST194BZ, ST414BZ and ST664BZ from *Lactobacillus plantarum*, bacteriocin ST712BZ from *Lactobacillus pentosus*, bacteriocins ST461BZ and ST462BZ from *Lactobacillus rhamnosus*, bacteriocins ST242BZ and ST284BZ from *Lactobacillus paracasei* (*7*), bacteriocins JW3BZ and JW6BZ from *L. plantarum* and bacteriocins JW11BZ and JW15BZ from *Lactobacillus fermentum* (*8*) have been described. Bacteriocins ST194BZ, ST414BZ, ST664BZ, ST712BZ, ST461BZ, ST462BZ, ST242BZ and ST284BZ are active against a number of Gram-positive bacteria, *Escherichia coli* and *Pseudomonas aeruginosa* (*7*). Bacteriocin JW15BZ is active against *Klebsiella pneumoniae* (*8*).

Criteria for selection of probiotic strains have only recently been formulated by the Food and Agriculture Organization of the United Nations and the World Health Organization (*13*). Some of the most important criteria are gastric and bile acid resistance, adhesion to mucus and human epithelial cells, competition with pathogens for adhesion sites, growth inhibition of potentially pathogenic bacteria, bile salt hydrolase activity and, in the case of vaginal applications, resistance to contraceptives (*13*).

Enterococcus mundtii ST4V isolated from soybeans produces a broad-spectrum bacteriocin active against Gram-positive and Gram-negative bacteria and has antiviral activity (*14*). The aim of this study is to determine the probiotic properties of strain *E. mundtii* ST4V, evaluate its survival in boza, and study the antimicrobial activity of the strain *in situ*.

Materials and Methods

Strains and growth media

E. mundtii ST4V was isolated from soybeans (*14*). *E. mundtii* ST4V, *Lactobacillus sakei* DSM 20017, *L. fermentum* ATCC 14931, *Enterococcus faecalis* LMG 13566 and *L. plantarum* ATCC 14917^T were cultured in MRS medium (Biolab, Biolab Diagnostics, Midrand, South Africa) and served as reference strains in denaturing gradient gel electrophoresis (DGGE) and PCR analyses. All other strains were cultured as indicated in Table 1. Cultures were stored at –80 °C in respective growth media, supplemented with glycerol (15 %, final volume concentration).

Bacteriocin bioassay

E. mundtii ST4V was grown in MRS broth (Biolab, Biolab Diagnostics, Midrand, South Africa) at 37 °C. After 24 and 36 h, cells were harvested (7000×*g*, 15 min, 4 °C), the pH of the cell-free supernatant adjusted to 6.0 with sterile 1 M NaOH, heated for 10 min at 80 °C, and then filter-sterilized (0.20 μm, Minisart®, Sartorius, Goettingen, Germany). Bacteriocin activity was tested against target organisms (Table 1) by using the agar spot test (*15*). Antimicrobial activity was expressed as arbitrary units (AU) per mL (*15*).

Table 1. Inhibitory spectra of bacteriocin ST4V produced by *E. mundtii* ST4V

Test microorganism	Medium and incubation temperature/°C*	Number of tested strains	Number of sensitive strains
Acinetobacter baumanii	BHI, 37	2	0
Bacillus cereus	BHI, 37	1	1
Bacteroides fragilis	BHI, 37	1	0
Clostridium sporogenes	RCM, 37	1	0
Clostridium tyrobutyricum	RCM, 37	1	1
Enterobacter cloacae	BHI, 37	2	0
Enterobacter spp.	BHI, 37	2	2
Enterococcus faecalis	BHI, 30	8	8
Enterococcus faecium	BHI, 30	2	2
Escherichia coli	BHI, 37	5	4
Klebsiella pneumoniae	BHI, 37	5	1
Lactobacillus acidophilus	MRS, 30	2	0
Lactobacillus bulgaricus	MRS, 30	2	0
Lactobacillus casei	MRS, 30	1	0
Lactobacillus curvatus	MRS, 30	2	0
Lactobacillus helveticus	MRS, 30	1	0
Lactobacillus fermentum	MRS, 30	2	0
Lactobacillus plantarum	MRS, 30	3	0
Lactobacillus paraplantarum	MRS, 30	1	0
Lactobacillus pentosus	MRS, 30	3	0
Lactobacillus reuteri	MRS, 30	2	0
Lactobacillus sakei	MRS, 30	2	2
Lactobacillus salivarius	MRS, 30	3	1
Leuconostoc mesenteroides ssp. *cremoris*	MRS, 30	1	0
Listeria innocua	BHI, 37	1	1
Listeria ivanovii ssp. *ivanovii*	BHI, 37	1	1
Listeria monocytogenes	BHI, 37	4	4
Pediococcus pentosaceus	MRS, 30	2	0
Propionibacterium spp.	GYE, 37	2	1
Proteus mirabilis	BHI, 37	2	1
Pseudomonas aeruginosa	BHI, 37	16	4
Pseudomonas spp.	BHI, 37	3	2
Salmonella enterocolitica	BHI, 37	2	0
Salmonella typhimurium	BHI, 37	2	0
Staphylococcus aureus (MRSA)	BHI, 37	2	0
Staphylococcus aureus	BHI, 37	19	3
Streptococcus uberis	BHI, 37	2	0
Streptococcus agalactiae	BHI, 37	1	1
Streptococcus caprinus	BHI, 30	2	2
Streptococcus pneumoniae	BHI, 37	8	2
Streptococcus thermophilus	MRS, 37	1	0

*BHI=brain heart infusion, RCM=reinforced clostridial medium, GYE=glucose yeast extract (all Biolab, Biolab Diagnostics, Midrand, South Africa)

Growth of E. mundtii ST4V at different pH and bile concentrations

E. mundtii ST4V was grown in MRS broth (Biolab, Biolab Diagnostics, Midrand, South Africa), adjusted to pH=3.0, 4.0, 5.0, 6.0, 7.0, 9.0, 11.0 and 13.0 with 1 M HCl or 1 M NaOH before autoclaving. Resistance to bile was tested by growing the cells in MRS broth (Biolab, Biolab Diagnostics, Midrand, South Africa), supplemented with 0.3, 0.6, 0.8, 1.0, 2.0 and 5.0 % (by mass per volume) of ox bile (Oxoid, Basingstoke, UK). All tests were conducted in sterile Sterelin™ microtiter plates. Each well was filled with 180 μL of medium containing ox bile and inoculated with 20-μL culture ($A_{600\ nm}$=0.3). Absorbance readings were recorded every hour for 10 h. The strain ST4V grown in MRS broth (Biolab, Biolab Diagnostics, Midrand, South Africa) without ox bile served as control. Experiments were done in triplicate.

Autoaggregation and coaggregation

E. mundtii ST4V was grown in MRS broth (Biolab, Biolab Diagnostics, Midrand, South Africa) for 24 h at 37 °C. The cells were harvested (7000×*g*, 10 min, 20 °C), washed, resuspended in sterile saline (0.85 % NaCl) and diluted to $A_{660\ nm}$=0.3. After 60 min at 37 °C, the cell suspension was centrifuged (300×*g*, 2 min, 20 °C) and the A_{60} of the supernatant determined. Autoaggregation was determined using the following equation (*16*):

$$\text{Autoaggregation}=\frac{A_0 - A_{60}}{A_0}\cdot 100 \qquad /1/$$

where A_0 is absorbance at the beginning of incubation, A_{60} is absorbance recorded after 60 min of incubation at 37 °C. The experiment was performed in triplicate.

Coaggregation of *E. mundtii* ST4V with *Enterococcus faecium* T8, *Listeria innocua* LMG 13568, *Listeria ivanovii* ssp. *ivanovii* ATCC 19119, *Pediococcus pentosaceus* ST3HA, *E. faecium* ST5HA, *L. plantarum* AMA-K and ST8KF, *Lactobacillus salivarius* 241 and *L. sakei* DSM 20017 was determined in a similar manner. Lactic acid bacteria were grown in MRS broth and BHI (Biolab, Biolab Diagnostics, Midrand, South Africa) at 37 °C for 24 h. Cells were harvested (7000×*g*, 10 min, 20 °C), washed, suspended in sterile saline and then diluted to $A_{660\ nm}$=0.3. Cell suspensions (500 μL of each) were combined, incubated at 37 °C for 60 min, then harvested (300×*g*, 2 min, 20 °C) and the absorbance (at 600 nm) of the supernatant was determined. Coaggregation was calculated using the following equation (*16*):

$$\text{Coaggregation}=\frac{A_{\text{Tot}} - A_{\text{S}}}{A_{\text{Tot}}}\cdot 100 \qquad /2/$$

where A_{Tot} refers to absorbance immediately after the strains were paired and A_{S} refers to the absorbance of the supernatant after 60 min of incubation and centrifugation at 300×*g* for 2 min at 20 °C. Experiments were conducted in triplicate on two separate occasions.

Hydrophobicity

The ability of *E. mundtii* ST4V to adhere to hydrocarbons was determined according to the method of Pérez *et al.* (*17*), with a few modifications. Cells of *E. mundtii* ST4V were harvested (7000×*g*, 5 min, 4 °C) at the stationary phase, washed twice with 10 mL of sterile 50 mM phosphate buffer (pH=6.5) and resuspended in 10 mL of the same buffer. A volume of 3 mL of each cell suspension, adjusted to $A_{560\ nm}$=1.0 with sterile 50 mM phosphate buffer, was added to 0.6 mL of *n*-hexadecane and vortexed for 2 min. After 1 h of incubation at 37 °C, the aqueous phase was carefully removed and the absorbance (at 560 nm) was determined. The percentage of cell surface hydrophobicity was calculated as:

$$\text{Hydrophobicity}=\frac{A_0 - A}{A_0}\cdot 100 \qquad /3/$$

where A_0 and A refer to the absorbance readings before and after the extraction with *n*-hexadecane, respectively.

Adsorption of bacteriocin ST4V to target cells

Bacteriocin ST4V was prepared according to Todorov *et al.* (*14*). Adsorption of bacteriocin ST4V to target cells was performed according to the method described by Yıldırım *et al.* (*18*). The target strains (Table 2) were grown overnight in 10 mL of the respective growth media at 30 or 37 °C and then harvested (8000×*g*, 15 min, 4 °C). Cells were washed twice with sterile 5 mM phosphate buffer (pH=6.5) and resuspended to the original volume in the same buffer. The pH was adjusted to 6.5 with sterile 0.1 M NaOH. Each cell suspension was mixed with an equal volume of bacteriocin ST4V (12 800 AU/mL, pH=6.5) and incubated at 37 °C for 1 h. After the removal of cells (8000×*g*, 15 min, 25 °C), the activity of unbound bacteriocin ST4V in the supernatant was determined as described before. All experiments were done in duplicate.

The percentage adsorption of bacteriocin ST4V to target cells was calculated according to the following equation:

$$\text{Adsorption}=$$

$$=100-\left(\frac{\text{Bacteriocin activity after contact with cell}}{\text{Original bacteriocin activity}}\cdot 100\right) \qquad /4/$$

Table 2. Comparison between sensitivity to bacteriocin ST4V and adsorption of the bacteriocin to target strains

Target strain	Sensitive to bacteriocin ST4V	Adsorption of bacteriocin ST4V/%
Enterococcus faecalis 1071	+	17
E. faecalis FAIR E-92	+	33
Enterococcus faecium HKLHS	+	17
Lactobacillus curvatus DF38	–	33
Lactobacillus paraplantarum ATCC 700211^T	–	17
Lactobacillus pentosus NCFB 363^T	–	17
Lactobacillus plantarum 423	–	17
L. plantarum LMG 13556	–	0
Lactobacillus sakei DSM 20017	+	33
Lactobacillus salivarius 241	+	17
Listeria innocua LMG 13568	+	50
Streptococcus caprinus ATCC 700066	+	33

+ = inhibition zone of at least 5 mm in diameter, – = no growth inhibition

Effect of pH and temperature on the adsorption of bacteriocin ST4V to target cells

Bacteriocin ST4V was added to *L. innocua* LMG 13568 and *E. faecalis* FAIR E-92, as described before, and incubated for 1 h at 4, 10, 25, 30, 37, 45 and 60 °C (pH=6.0), and at 37 °C at pH=2.0, 4.0, 6.0, 8.0 and 10.0. Cells were harvested (8000×*g*, 15 min, 25 °C) and the pH of the cell-free supernatant adjusted to 6.0 with sterile 1 M NaOH. Bacteriocin activity in the supernatant was determined as described before. The experiments were done in duplicate.

Effect of inorganic salts and organic compounds on adsorption of bacteriocin ST4V to target cells

Cells of *L. innocua* LMG 13568 and *E. faecalis* FAIR E-92 were treated with 1 % (by mass per volume) NaCl, K_2HPO_4, KH_2PO_4, $MgCl_2$, KCl, KI, Tris-HCl, $(NH_4)_3C_6H_5O_7$ (ammonium citrate), CH_3COONa, Na_2CO_3 and EDTA ($C_{10}H_{16}O_8N_2$), 1 % (by volume) Triton X-100, Triton X-114 and β-mercaptoethanol, and 80 % ethanol, methanol and chloroform. The pH of all samples was adjusted to 6.5 with 1 M NaOH or 1 M HCl. Bacteriocin ST4V was added to the treated cells as described before, and incubated for 1 h at 37 °C. The cells were harvested (8000×*g*, 15 min, 25 °C) and the activity of bacteriocin ST4V in the cell-free supernatant was determined as described before. The experiments were done in duplicate.

Effect of bacteriocin ST4V on cell membrane permeability and cell lysis

Cells of *L. innocua* LMG 13568 and *E. faecalis* FAIR E-92 were harvested by centrifugation (8000×*g*, 15 min, 4 °C), washed twice with sterile 5 mM phosphate buffer (pH=6.5) and bacteriocin ST4V (12 800 AU/mL, prepared from lyophilized cell-free supernatant) was added at a ratio of 0.1:1.0. After incubation at 37 °C for 1 h, the cells were harvested (8000×*g*, 15 min, 4 °C) and the supernatant filtered through a 0.20-μm membrane (Minisart®, Sartorius, Goettingen, Germany). The presence of nucleic acids was determined by recording the absorbance of the cell-free supernatant at 260 nm. Controls were *L. innocua* LMG 13568 and *E. faecalis* FAIR E-92 suspended in 5 mM phosphate buffer without bacteriocins and in the same buffer containing only bacteriocin ST4V (without bacterial cells), respectively. The experiment was conducted in duplicate.

In a separate experiment, extracellular levels of β-galactosidase activity were monitored. Eleven-hour-old cultures of *L. innocua* LMG 13568 and *E. faecalis* FAIR E-92 (10 mL each) were harvested, than the cells were washed twice with 0.03 M sodium phosphate buffer (pH=6.5) and resuspended in 2 mL of the same buffer. The cell suspensions were treated with 2 mL of bacteriocin ST4V (12 800 AU/mL, pH=6.0, prepared from lyophilized cell-free supernatant) for 5 min at 25 °C, followed by the addition of 0.2 mL of 0.1 M ONPG (*o*-nitrophenyl-β-D-galactopyranoside) in 0.03 M sodium phosphate buffer (pH=6.8). After 10 min at 37 °C, the reaction of β-galactosidase was stopped by the addition of 2.0 mL of 0.1 M Na_2CO_3. The cells were harvested (8000×*g*, 15 min, 25 °C) and absorbance readings of the cell-free supernatant were recorded at 420 nm. Controls were the cells prepared in the same way, but not treated with bacteriocin ST4V (*19,20*). All experiments were performed in duplicate.

In a separate experiment, 20 mL of cell-free culture supernatant containing bacteriocin ST4V (12 800 AU/mL, pH=6.0, prepared from lyophilized cell-free supernatant) were filter-sterilized (0.20 μm, Minisart®, Sartorius, Goettingen, Germany) and added to 100 mL of 3-hour-old cultures ($A_{600\ nm}$=0.1–0.2) of *L. innocua* LMG 13568 and *E. faecalis* FAIR E-92. The cells were incubated at 37 °C. Cell density was determined hourly for 10 h by recording absorbance at 600 nm. The experiment was repeated with the cells in stationary phase.

In another experiment, 18-hour-old cultures of *L. sakei* DSM 20017, *E. faecalis* FAIR E-92 and *L. innocua* LMG 13568 were harvested (5000×*g*, 5 min, 4 °C), washed twice with sterile saline water and resuspended in 10 mL of saline water. Equal volumes of cell suspensions and filter-sterilized (0.20 μm pore size nitrocellulose membrane, Minisart®, Sartorius, Goettingen, Germany) bacteriocin ST4V-containing cell-free supernatant were mixed. Viable cell number was determined before and after the incubation for 1 h at 37 °C.

Effect of antibiotics and medicaments on the growth of E. mundtii ST4V

An overnight culture of the strain ST4V (10^6 CFU/mL) was embedded in MRS soft agar (1.0 %, by mass per volume) plates. Antibiotic discs (Oxoid, UK; Table 3) were placed on the agar surface and incubated at 37 °C for 24 h. Growth inhibition was recorded by measuring the diameter of the zones (5-mm diameter of the disc included). The experiment was repeated with commercially available medicaments, at concentrations shown in Table 4. The medicaments were dissolved in 1 mL of sterile distilled water and 10 μL were spotted onto the

Table 3. Sensitivity of *E. mundtii* ST4V to antibiotics

Antibiotic discs	Sensitivity of *E. mundtii* ST4V to antibiotics (inhibition zone in mm)
Nalidixic acid, sulphamethoxazole, neomycin, tobramycin, cefuroxime, clindamycin, cefotaxime, sulphonamide compound, oxacillin, cefepime, amikacin, ceftazidime, ceftriaxone, streptomycin, metronidazole, sulphafurazole	0
Cephazolin	13
Ofloxacin, trimethoprim	16
Sulphamethoxazole/trimethoprim	17
Furazolidone	21
Erythromycin	23
Ciprofloxacin, vancomycin	24
Fusidic acid	25
Nitrofurantoin, rifampicin	30
Chloramphenicol	33
Tetracycline	38

Table 4. Effect of commercially available medicaments on the growth of *E. mundtii* ST4V

Commercial name	γ/(mg/mL)	Composition (active substance)	Medicament group	Diameter of inhibition zone/mm
Dimenhydrinate	10	dimenhydrinate 50 mg	antiemetic, sedative, antihistaminic	0
Dehydratin Neo	5	hydrochlorothiazide 25 mg	diuretic	0
Famotidine	4	famotidine 20 mg	antiacid	0
Thioridazine	2	thioridazine hydrochloride 10 mg	neuroleptic, antipsychotic	5
Diclamina	14	cinarizine 20 mg, heptaminol acefylinate 50 mg	cardiovascular	0
Acetylcysteine 600 Stada® tabs	120	acetylcystein 600 mg	mucolytic and nephroprotective agent	0
Duoventrinetten N	100	antacid 500 mg	antiacid	0
Bisalax	1	bisacodyl 5 mg	treatment of constipation	0
Diuretidin	7.5	triamterene 25 mg, hydrochlorothiazide 12.5 mg	cardiovascular, diuretic	12
Oleum jecoris	7.5	retinol palmitate (Vit A) 3750 IU ergocalciferolum (Vit D2) 375 IU oleum jecoris aselli 37.5 mg	hepatic and pancreatic	0
Atarax	5	hydroxyzine dichlorohydrate 25 mg	antihistaminic	0
Ambro	20	ambroxol 100 mg	mucolytic agent	0
Voltaren	10	diclofenac sodium 50 mg	anti-inflammatory	4
Proalgin	100	metamizole sodium 500 mg	analgetic, anti-inflamatory	0
Novphyllin	10	aminophylline 100 mg	anti-asthmatic	0
Cerucal	2.5	metoclopramide hydrochloride 10.54 mg	gastrointestinal disorders	0
Espumisan (Simethicone)	8	simethicone 40 mg, methyl-4-hydroxybenzoate 0.28 mg	gastrointestinal disorders	0
Hepcarsil	14	silymarin 70 mg	hepatic and pancreatic	0
Ibuprofen	100	ibuprofen 500 mg	anti-inflammatory	12
Nordette	36	levonorgestrel 150 mg ethinylstradiol 30 mg	contraceptive, ovulation controlling agent	0
Microval	6	levonorgestrel	contraceptive, ovulation controlling agent	0
Triphasil	16	levonorgestrel 50 mg ethinylstradiol 30 mg	contraceptive, ovulation controlling agent	0
Biphasil	20	levonorgestrel 50 mg ethinylstradiol 50 mg	contraceptive, ovulation controlling agent	0

surface of the plates. The plates were incubated at 37 °C for 24 h and growth inhibition was recorded by measuring the diameter of the zones.

Production of boza with E. mundtii ST4V and other starter cultures

Boza was prepared by adding 525 g of barley to 3.5 L of water and boiled for 20 min. The mixture was stirred continuously. Cold water (500 mL) was added, the mixture was homogenized with a blender and filtered through a cheesecloth. Sugar (175 g) was added, the volume adjusted to 4.0 L with cold water, and divided into 250-mL samples. One sample was inoculated with 75 mg of commercial baker's yeast (*Saccharomyces cerevisiae*). Three samples were inoculated with 75 mg of baker's yeast and 0.1 % (by volume) *E. mundtii* ST4V (10^8 CFU/mL) and the other three with the same level of baker's yeast and 0.1 % (by volume) of commercial boza. The control received no starter culture. A duplicate set of samples inoculated as described here received 0.1 % (by volume) of *L. sakei* DSM 20017 (10^8 CFU/mL) as target strain. All samples were fermented for 3 h at 37 °C and then stored at 4 °C for 7 days.

After 3, 5 and 7 days at 4 °C, 10 mL from each sample were serially diluted in sterile saline and plated onto MRS agar (Biolab, Biolab Diagnostics, Midrand, South Africa), supplemented with 50 mg/L of natamycin (Gist Brocades, B.V., Delft, The Netherlands) to prevent yeast growth. Plates containing between 30 and 300 colonies were overlaid with BHI agar (1 %, by mass per volume), inoculated with *L. innocua* LMG 13568 at 10^6 CFU/mL and incubated for 24 h at 37 °C. Colonies with surrounding inhibition zones were counted and a few selected at random to test for bacteriocin ST4V production, as described previously.

Isolation of lactic acid bacteria from boza and DNA extraction

Lactic acid bacteria were isolated from boza on days 1, 5 and 7 after the fermentation. Serial dilutions were made in sterile physiological saline, plated onto MRS agar (Biolab, Biolab Diagnostics, Midrand, South Africa) supplemented with natamycin as described before, and incubated at 30 °C for 24–48 h. Colonies were harvested from plates representing 10^3 CFU/mL and suspended in 10 mL of sterile physiological saline. DNA was isolated from 2 mL of cell suspension by using the method of Dellaglio *et al*. (*21*).

DNA amplification

The V2–V3 variable region (approx. 200 base pairs) of the 16S rRNA gene in lactic acid bacteria was amplified by using primers 534 (5′ ATTACCGCGGCTGCTGG 3′) and 341FGC (5′ CGCCCGCCGCGCGCGGCGGGCGGGGCGGGGGCACGGGGGGCCTACGGGAGGCAGCAG 3′) (*22*). The PCR reaction was performed in 50 μL of solution containing 0.5 μM of each primer, 200 μM dNTP (Takara Bio Inc., Shiga, Japan), 0.5 U Taq DNA polymerase (Takara Bio Inc., Shiga, Japan), 1×PCR buffer (Takara Bio Inc., Shiga, Japan) and 10 μL of DNA. The following conditions were used: initial denaturation at 94 °C for 4 min, followed by 35 cycles of denaturation at 94 °C for 30 s, annealing at 56 °C for 30 s and elongation at 72 °C for 8 min. PCR reactions were performed in a MyCycler™ Thermal Cycler Firmware (Bio-Rad Laboratories, USA). Amplicons were analysed on 2 % (by mass per volume) agarose gels with ethidium bromide and 0.5×TBE electrophoresis buffer. DNA fragments were visualised under UV light (Vilber Lourmat, Torcy, France).

Amplicons were separated by DGGE using the Bio-Rad DCode™ Universal Mutation Detection System (Bio-Rad Laboratories, USA). A volume of 35 μL of DNA was loaded onto an 8 % (by mass per volume) polyacrylamide gel. Separation was in a linear denaturing gradient gel between 40 and 60 % in 0.5×TAE buffer. The 100 % denaturing solution contained 40 % (by volume) formamide (Saarchem, Krugersdorp, South Africa) and 7.0 M urea (Merck, Germany). Electrophoresis was performed with a constant voltage of 130 V at 60 °C for 5 h. The gel was stained with ethidium bromide for 30 min and the fragments were visualised under UV light (Vilber Lourmat, France).

Species- and genus-specific PCR

Species-specific PCR was performed to validate the results obtained by DGGE. The following species-specific primers were used: planF (5′ CCGTTTATGCGGAACACCTA 3′) and pREV (5′ TCGGGATTACCAAACATCAC 3′) for *L. plantarum* (*23*), Ls (5′ ATGAAACTATTAAATTGGTAC 3′) and 16 (5′ GCTGGATCACCTCCTTTC 3′) for *L. sakei* (*24*), FERM1 (5′ GTTGTTCGCATGAACAACGCTTAA 3′) and LOWLAC (5′ CGACGACCATGAACCACCTGT 3′) for *L. fermentum* (*25*), and Ent1 (5′ TACTGACAAACCATTCATGATG 3′) and Ent2 (5′ AACTTCGTCACCAACGCGAAC 3′) for *Enterococcus* sp. (*26*). The presence of *E. mundtii* ST4V was determined by using primers ST4Forward: TGAGAGAAGGTTTAAGTTTTGAAGAA and ST4Reverse: TCCACTGAAATCCATGAATGA, designed from the sequence of antimicrobial peptide ST4SA (*27*). *L. plantarum* ATCC 14917^T, *L. fermentum* ATCC 14931, *L. sakei* DSM 20017^T and *E. mundtii* ST4V served as controls.

Sensory analysis

Descriptive sensory analysis was performed on boza produced with different combinations of lactic acid bacteria as starter cultures. The sensory panel consisted of nine panelists, trained according to the consensus method of Lawless and Heymann (*28*). A 100-mm unstructured line scale was used for attribute intensity evaluation, with the left side of the scale corresponding to the lowest intensity (zero) and the right side corresponding to the highest intensity (100).

A consensus list of attributes that describe boza includes yeasty aroma, yeasty flavour (taste), bitterness, sweetness and acidity. The panelists were seated in individual booths in a temperature- (21 °C) and light-controlled (artificial daylight) room. Samples were presented in a complete randomized order in four sessions. Purified water and unsalted fat-free crackers were given to the panelists between samples. Data were subjected to the appropriate analyses of variance (ANOVA) using SAS version 8.2 statistical software (*29*). Shapiro-Wilk tests were performed to test non-normality (*30*).

Results and Discussion

Spectrum of activity

Cell–free supernatants from 24- and 36-hour-old cultures of *E. mundtii* ST4V (pH neutralized) inhibited the growth of *Enterobacter* spp., *E. faecalis*, *E. faecium*, *E. coli*, *K. pneumoniae*, *L. sakei*, *L. salivarius*, *L. innocua*, *L. ivanovii* ssp. *ivanovii*, *L. monocytogenes*, *P. aeruginosa*, *Pseudomonas* spp., *Proteus mirabilis*, *Propionibacterium* spp., *Staphylococcus aureus*, *Streptococcus uberis*, *Streptococcus agalactiae*, *Streptococcus caprinus*, *Streptococcus pneumoniae*, *Bacillus cereus* and *Clostridium tyrobutyricum* (Table 1).

Only a few bacteriocins of lactic acid bacteria with activity against Gram-negative bacteria have been reported, *viz.* thermophilin 81 (4.5 kDa) produced by *Streptococcus thermophilus*, a bacteriocin produced by *L. lactis* KCA2386 (8.1 kDa), plantaricin 35d (4.5 kDa) produced by *L. plantarum*, lacticin NK24 (between 3.0 and 3.5 kDa) produced by *L. lactis* NK24, and bacteriocins ST28MS (5.5 kDa) and ST26MS (2.8 kDa) produced by *L. plantarum* ST28MS and ST26MS, respectively (*15,31–34*).

Growth of E. mundtii ST4V at different pH and bile concentrations

E. mundtii ST4V grew well in the absence of ox bile (Fig. 1). Ox bile at 0.8 % (by mass per volume) and higher repressed the growth of the strain ST4V. The growth was less affected by 0.3 and 0.6 % (by mass per volume) ox bile. Good growth was recorded in MRS broth with initial pH value of 6.0, but it was repressed at pH=3.0, 4.0 and 5.0 (Fig. 1). Similar results were recorded for *L. plantarum* 423, *L. salivarius* 241, *L. curvatus* DF38 and *L. lactis* ssp. *lactis* HV219 when strains were

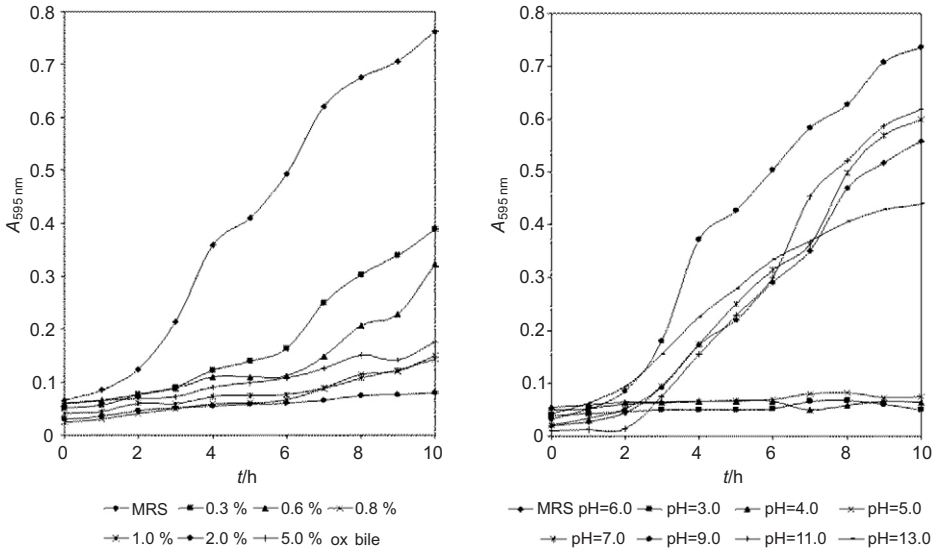

Fig. 1. Growth of *E. mundtii* ST4V in MRS broth supplemented with different concentrations of ox bile at different pH. Each data point represents an average of three readings

grown in the presence of 0.3–5.0 % (by mass per volume) ox bile and at pH=3.0–13.0 (*35*,*36*). Variable results were recorded for strains of *L. plantarum* exposed to HCl (pH=2.0) and bile salts (*37*). As many as 10 % of *L. plantarum* cells, but less than 0.001 % of *L. sakei* and *L. paracasei* cells survived these conditions (*37*). Although these assays cannot predict patterns of behaviour in the human body, the results are valuable in selecting *Lactobacillus* spp. for probiotic applications (*38*).

Autoaggregation and coaggregation

Values of (41.34±1.15) % were recorded for autoaggregation of *E. mundtii* ST4V (Fig. 2). Various degrees of coaggregation were observed when cells were paired with *L. ivanovii* ssp. *ivanovii* ATCC 19119, *L. innocua* LMG 13568, *P. pentosaceus* ST3HA, *E. faecium* ST5HA, *E. faecium* T8, *L. plantarum* AMA-K, *L. plantarum* ST8KF, *L. sakei* DSM 20017 and *L. salivarius* 241 (Fig. 2). Coaggregation is not indicative of biofilm formation. However, coaggregation as observed between *E. mundtii* ST4V and *L. innocua* LMG 13568 or *L. ivanovii* ssp. *ivanovii* ATCC 19119 may play an important role in eliminating pathogens from the gastrointestinal tract (GIT). Some lactic acid bacteria isolated from the urogenital tract and GIT coaggregate with *E. coli* (*39*,*40*). According to Reid *et al.* (*39*), coaggregation may be beneficial to lactic acid bacteria that produce antimicrobial compounds, as it would force the cells into closer contact.

Lactic acid bacteria have a number of genes encoding surface proteins that could function in recognition of, or binding to, compounds in the environment. Several of these genes are homologous to proteins with predicted functions such as binding to mucus, promotion of aggregation and intracellular adhesion (*41*,*42*).

Hydrophobicity

A relatively low hydrophobicity value (5.57 %) was recorded for the strain ST4V. A hydrophobicity of 55 % was reported for *L. rhamnosus* GG (*43*).

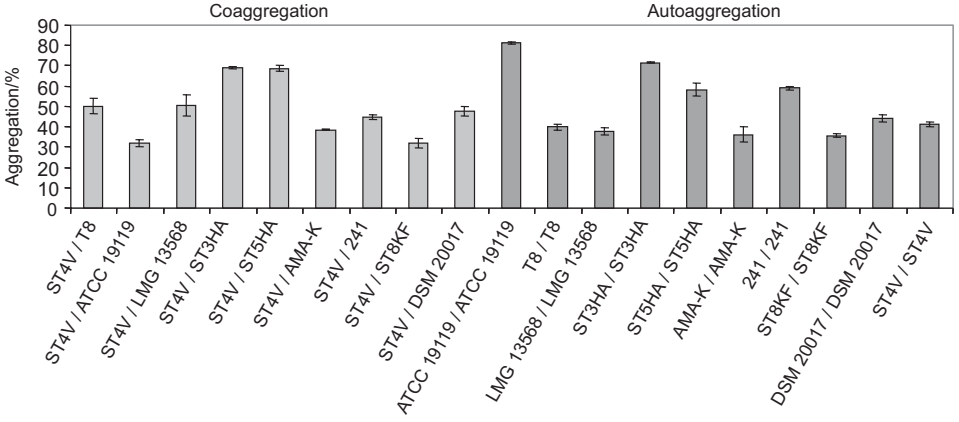

Fig. 2. Co- and autoaggregation of *E. mundtii* ST4V with different strains, expressed as percentage value. ST4V=*E. mundtii*, T8=*E. faecium*, ATCC 19119=*L. ivanovii* ssp. *ivanovii*, LMG 13568=*L. innocua*, ST3HA=*P. pentosaceus*, ST5HA=*E. faecium*, AMA-K=*L. plantarum*, 241=*L. salivarius*, ST8KF=*L. plantarum* and DSM 20017=*L. sakei*. The experiments were done in triplicate

Cell surface hydrophobicity is a nonspecific interaction between microbial cells and host cells. The initial interaction may be weak, is often reversible and precedes subsequent adhesion processes mediated by more specific mechanisms that involve cell surface proteins and lipoteichoic acid (*44–46*). Bacterial cells with high hydrophobic properties usually form strong interactions with mucosal cells.

Hydrophobicity varies among species genetically closely related and even among strains of the same species (*47*). Strains with a high cell surface hydrophobicity do not always adhere to HT-29 cells at a high level. Strain *L. pentosus* ST712BZ, characterised by a relatively low hydrophobicity (38 %), adhered to HT-29 cells at 63 %. Only 32 % adherence was recorded for *L. rhamnosus* GG, which has a hydrophobicity level of 53 % (*43*). Hydrophobicity may assist in adhesion, but is not a prerequisite for colonization (*43*).

Adsorption of bacteriocin ST4V to target cells

Bacteriocin ST4V adsorbed to sensitive and resistant cells of Gram-positive bacteria (Table 2). Adsorption to cells sensitive to bacteriocin ST4V ranged from 17 % for *L. salivarius* 241, *E. faecalis* 1071 and *E. faecium* HKLHS to 50 % for *L. innocua* LMG 13568. Bacteriocin ST4V did not adsorb to *L. plantarum* LMG 13556, which is resistant to bacteriocin ST4V. In general, bacteriocin ST4V adsorbed more strongly to sensitive cells (Table 2).

Similar results had been reported for pediocin N5p (*48*), *viz.* 100 % adsorption to sensitive cells of *Oenococcus oeni* X2L, 80 % to *Lactobacillus hilgardii* and *O. oeni* L10, and 70 % to *L. hilgardii* 6D (*48*). Adsorption of pediocin N5p to resistant bacteria was below 20 % (*48*). Buhnericin LB adsorbed 100 % to sensitive cells of *L. plantarum*, *Pediococcus dextrinicus*, *O. oeni* and *E. faecalis*, but also to an insensitive strain of *Pediococcus cerevisiae* (*18*). In the case of plantaricin 423, adsorption ranged from 17 % for *S. caprinus* ATCC 700066 to 67 % for *L. plantarum* LMG 13556, *L. curvatus* DF38, *L. innocua* LMG 13568 and *L. sakei* DSM 20017 (*49*). Strains sensitive to plantaricin 423 adsorbed the peptide more strongly (*49*).

Adsorption of bacteriocins to the target cells is the first step in the interaction. Receptors such as lipid II are involved in the interaction with nisin. It is highly possible that several other receptors are involved in the 'recognition' and adsorption of bacteriocins to cell walls of target microorganisms.

Effect of pH and temperature on the adsorption of bacteriocin ST4V

Optimal adsorption of bacteriocin ST4V to *L. innocua* LMG 13568 was recorded at pH=6.0 and pH=8.0, and to *E. faecalis* FAIR E-92 at pH=8.0 and pH=10.0 (Table 5). Temperatures above 45 °C and below 25 °C had a negative effect on the adsorption of bacteriocin ST4V to *L. innocua* LMG 13568 (Table 3). Optimal adsorption of bacteriocin ST4V to *L. innocua* LMG 13568 was recorded between 25 and 45 °C. Similar results had been recorded for adsorption of the peptide to *E. faecalis* FAIR E-92, with optimum adsorption between 25 and 37 °C (Table 5). Optimal adsorption at body temperature could provide the peptide with an advantage. In the case of buchnericin LB, identical adsorption to the cells of *L. plantarum* was recorded after treatment at 0, 10, 25, 50 and 80 °C (*18*). Changes in temperature had no effect on the adsorption of plantaricin 423 to *E. faecium* HKLHS (*49*).

Table 5. Effect of temperature, pH and selected chemicals on the adsorption of bacteriocin ST4V to *L. innocua* LMG 13568 and *E. faecalis* FAIR E-92

	Adsorption to *L. innocua* LMG 13568/%	Adsorption to *E. faecalis* FAIR E-92/%
Temperature/°C		
4, 10	33	17
25, 30, 37	50	33
45	50	17
60	33	17
pH		
2.0	17	17
4.0	17	33
6.0	50	33
8.0	50	50
10.0	33	50
Chemicals (1 %)		
NaCl, CH_3COONa	67	33
K_2HPO_4, KH_2PO_4, KI, Na_2CO_3, chloroform	50	50
$MgCl_2$, KCl, ammonium citrate, methanol	33	33
Tris-HCl	67	50
EDTA (Na), 80 % ethanol	50	33
SDS	33	50
Triton X-100	83	50
Triton X-114, β-mercaptoethanol	50	67
Control (no treatment)	50	33

Different levels of adsorption of bacteriocin ST4V at specific pH values may be due to specific interaction between bacteriocin ST4V and the target strain. In the case of buchnericin LB, optimal adsorption to *L. plantarum* was recorded at pH=5.0–8.0 (*18*). Optimal adsorption of plantaricin 423 to *E. faecium* HKLHS was recorded between pH=8.0 and pH=10.0, and to *L. sakei* DSM 20017 between pH=2.0 and pH=6.0 (*49*).

Effect of inorganic salts and organic compounds on the adsorption of bacteriocin ST4V to target cells

The effect of different chemicals on the adsorption of bacteriocin ST4V to target cells is listed in Table 5. Increased adsorption of bacteriocin ST4V to *L. innocua* LMG13568 was detected in the presence of NaCl, Tris-HCl, CH_3COONa and Triton X-100. Adsorption of bacteriocin ST4V to *E. faecalis* FAIR E-92 increased in the presence of K_2HPO_4, KH_2PO_4, KI, Tris-HCl, Na_2CO_3, sodium dodecyl sulphate (SDS), Triton X-100, Triton X-114, 2-mercaptoethanol and chloroform.

An increase in the adsorption of plantaricin 423 to *E. faecium* HKLHS was observed in the presence of Triton X-100, Triton X-114 and chloroform (*49*). Adsorption of bacHV219 to *E. faecalis* E88 increased in the presence of CH_3COONa, Na_2CO_3, Triton X-100, 80 % ethanol, methanol, K_2HPO_4, KH_2PO_4, $MgCl_2$, KCl, Tris-HCl and ammonium citrate ($(NH_4)_3C_6H_5O_7$) (*36*). *L. sakei* DSM 20017 treated with NaCl, K_2HPO_4, KH_2PO_4, $MgCl_2$, KCl, KI, Tris-HCl, $(NH_4)_3C_6H_5O_7$, Na_2CO_3, SDS, β-mercaptoethanol, 80 % ethanol and methanol led to a reduction in the adsorption of plantaricin 423 (*49*). No change in adsorption was observed in the presence of sodium acetate or EDTA, whereas an increase in adsorption was observed in the presence of Triton X-100, Triton X-114 and chloroform (*49*). Adsorption of buchnericin LB to *L. plantarum* was reduced by NaCl, NH_4Cl, $MgCl_2$, KCl, KI and Tris-HCl. Treatment of cells with $(NH_4)_3C_6H_5O_7$, CH_3COONa, $NaCO_3$, EDTA SDS, Triton X, β-mercaptoethanol, 80 % ethanol and 80 % methanol had no effect on the adsorption of buchnericin LB to *L. plantarum* (*18*). Adsorption of pediocin N5p to *P. pentosaceus* E5p increased in the presence of $MgCl_2$, $MgSO_4$, $MnCl_2$, $MnSO_4$, whereas NaCl, KCl, KI, NH_4Cl, $CaCl_2$, Na_3PO_4, Na_2SO_4, EDTA and ethanol had no affect on the adsorption (*48*). Organic salts and sodium acetate reduced pediocin N5p adsorption to target cells. Adsorption of pediocin N5p increased for 25 % in the presence of SDS (*48*).

Temperature, pH, salts, organic compounds and lipids play an important role in the adsorption of a bacteriocin to target cells. These factors may have an effect on the structure of the bacteriocin, thereby changing the active sites, or may affect the receptor sites on the surface of the target organism.

Mode of action of bacteriocin ST4V

Cells of *L. innocua* LMG 13568 and *E. faecalis* FAIR E-92 in early log phase treated with bacteriocin ST4V resulted in immediate and complete growth inhibition for at least 10 h (Fig. 3), suggesting that the mode of action is bactericidal. Similar results were recorded for bacteriocin HV219 against *E. faecium* HKLHS and *E. faecalis* E88 (*36*), plantaricin 423 against *O. oeni* 19Cl (*50*), *E. faecium* HKLHS and *L. sakei* DSM 20017 (*49*), pediocin N5p against *P. pentosaceus* E5p (*48*), and buchnericin LB against *L. monocytogenes* and *Bacillus cereus* (*18*).

Treatment of stationary phase cells of *L. innocua* LMG 1358 with bacteriocin ST4V (25 600 AU/mL) in non-growing physiological conditions led to a decrease in the cell number (from $8.9 \cdot 10^9$ to $8.5 \cdot 10^6$ CFU/mL) over 1 h. *E. faecalis* FAIR E-92 treated with the same concentrations of bacteriocin ST4V (25 600 AU/mL) led to a decrease from $5.3 \cdot 10^{10}$ to $5.1 \cdot 10^3$ CFU/mL over 1 h. These results suggest that bacteriocin ST4V activity towards stationary phase cells is bactericidal. Treatment of stationary phase cells of *E. faecium* HKLHS and *E. faecalis* E88 with bacteriocin HV219 led to a 3-log decrease in viable cell numbers within 1 h (*36*). Cell numbers of *L. monocytogenes* treated with 640, 1280 and 2560 AU/mL buhnericin LB decreased by log 0.8, 1.9 and 3.1, respectively (*18*). *L. sakei* DSM 20017 treated with plantaricin 423 decreased by two log cycles within 1 h (*49*), which is lower compared to the results obtained for *E. faecium* HKLHS and *E. faecalis* E88.

Treatment of *L. innocua* LMG 13568 with bacteriocin ST4V (25 600 AU/mL) resulted in the leakage of nucleic acids (detected at 260 nm). Similar results were recorded when *E. faecalis* FAIR E-92 was treated with bacteriocin ST4V (data not shown). Low levels of external β-galactosidase were detected when *L. innocua* LMG 13568 and *E. faecalis* FAIR E-92 were treated with bacteriocin ST4V (data not shown), proving the hypothesis that bacteriocin ST4V disorganized the cell wall of the target cells. Treatment of *L. sakei* DSM 20017 and *Enterococcus* sp. HKLHS with bacteriocins ST194BZ and ST23LD resulted in the leakage of intracellular material (*51*).

Effect of antibiotics and medicaments on the growth of E. mundtii ST4V

Growth of *E. mundtii* ST4V was repressed in the presence of nitrofurantoin, ciprofloxacin, fusidic acid, furazolidone, rifampicin, tetracycline, ofloxacin, cephazolin, erythromicin, chloramphenicol, vancomycin, sulphamethoxazole/trimethoprim and trimethoprim (Table 3) and was inhibited by anti-inflammatory medicaments containing diclofenac sodium, triamterene/hydrochlorothiazide, ibuprofen and thioridazine hydrochloride (Table 4).

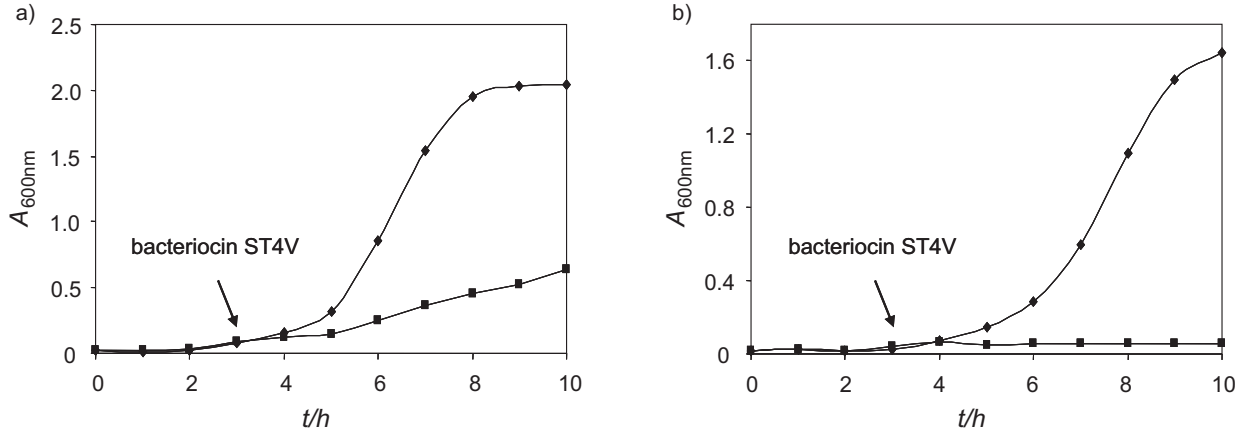

Fig. 3. The effect of bacteriocin ST4V on the growth of (a) *L. innocua* LMG 13568 and (b) *E. faecalis* FAIR E-92. (■) Growth in the presence of bacteriocin, (◆) cells not treated with bacteriocin ST4V. The arrow indicates the point at which bacteriocin ST4V was added

Patients taking probiotics are often treated for other illnesses. It is thus important to determine the effect of medicaments on the growth of probiotic strains, especially if the product (such as boza in this case) is considered as a possible probiotic product. Growth of *L. plantarum* ST194BZ, ST414BZ and ST664BZ, *L. paracasei* ST242BZ and ST284BZ, *L. rhamnosus* ST461BZ and ST462BZ, and *L. pentosus* ST712BZ was inhibited by similar medicaments containing diclofenac, triamterene/hydrochlorothiazide, ibuprofen and thioridazine hydrochloride (*43*). Dimenhydrinate inhibited the growth of *L. rhamnosus* ST462BZ and *L. plantarum* ST664BZ (*43*). Diclofenac and ibuprofen inhibited the growth of *L. lactis* ssp. *lactis* HV219 (*36*). It is, however, important to mention that the concentration of these substances is critical. The same cells treated with ibuprofen produced by a different company were not inhibited (*36*).

DGGE and PCR with genus-specific and species-specific primers

Amplification of the V2-V3 variable region of the 16S rDNA gene produced fragments of approx. 200 bp in size. Separation by DGGE produced a single DNA band for *L. plantarum* and a single band for *L. fermentum*, both located at the same position (Fig. 4). A smaller DNA fragment was amplified and corresponded to that expected for *L. sakei* (Fig. 4). However, a DNA fragment of the same size was amplified from lactic acid bacteria isolated from all boza samples, irrespective of whether the samples had been inoculated with *L. sakei* or not (Fig. 4). Furthermore, *E. mundtii* ST4V could not be distinguished from *E. faecalis* (Fig. 4). DGGE could thus not be used to differentiate between *L. plantarum* and *L. fermentum* and did not allow reliable detection of *L. sakei* DSM 20017^T and *E. mundtii* ST4V.

DNA amplification with species- and genus-specific primers, and primers designed from the DNA sequence of the mature bacteriocin ST4V yielded fragments of specific sizes, characteristic for each species (Figs. 5–9). *E. mundtii* ST4V was detected in boza after 7 days of storage at 4 °C, but only in the samples that were inoculated with this strain (Fig. 5). Other *Enterococcus* spp. were present in all samples, as revealed by DNA amplification with genus-specific primers (Fig. 6). This confirmed previous findings (*43*) that enterococci are naturally present in boza. DNA amplicons characteristic for *L. fermentum* (Fig. 7) and *L. plantarum* (Fig. 8) were also recorded. This is not surprising, as strains of *L. fermentum* and *L. plantarum* have been isolated from boza (*8*). The target strain, *L. sakei* DSM 20017^T or another strain of *L. sakei*, was present in all boza samples, except in those inoculated with *E. mundtii* ST4V (Fig. 9). This suggested that adequate levels of bacteriocin ST4V were produced during fermentation and storage at 4 °C to inhibit the growth of *L. sakei* DSM 20017^T. Previous studies had shown that bacteriocin ST4V had bactericidal effect against *L. sakei* (*14*).

Sensory analysis

Sensory analysis of the six boza preparations is shown in Table 6. No significant difference ($p \leq 0.05$) was observed for yeasty aroma, yeasty flavour (taste), sweetness and bitterness among samples fermented with starter cultures *E. mundtii* ST4V, commercial boza or the naturally fermented one. However, slightly higher bitterness was recorded when boza was produced by the starter culture of commercial boza (Table 6). Similar results were obtained for this sample regarding stronger yeasty flavour of the product when commercial boza was used as starter culture (Table 6). These differences, although statistically significant, are so small they would probably not be detected by the consumers. The acid taste of all samples was relatively low; with the lowest recorded for boza prepared with *E. mundtii* ST4V. No correlation was found between the different sensory attributes tested. Fermentation of the product contributes to the acidity of the product (*2*).

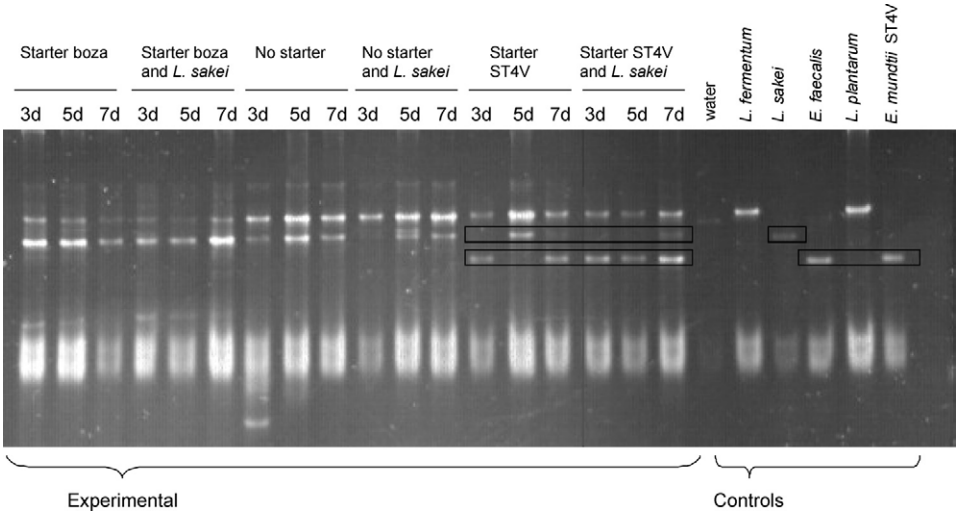

Fig. 4. DGGE analysis of boza inoculated with commercial boza (starter boza), commercial boza and *L. sakei* DSM 20017^T (starter boza and *L. sakei*), *L. sakei* DSM 20017^T only (no starter and *L. sakei*), *E. mundtii* ST4V (starter ST4V), *E. mundtii* ST4V and *L. sakei* DSM 20017^T (starter ST4V and *L. sakei*), and one sample not inoculated (no starter). All samples were fermented for 3 h at 37 °C and then stored at 4 °C. 3d, 5d and 7d refer to the number of days boza was stored at 4 °C. *L. fermentum* ATCC 14931, *L. sakei* DSM 20017^T, *E. faecalis* LMG 13566, *L. plantarum* ATCC 14917^T and *E. mundtii* ST4V served as reference strains

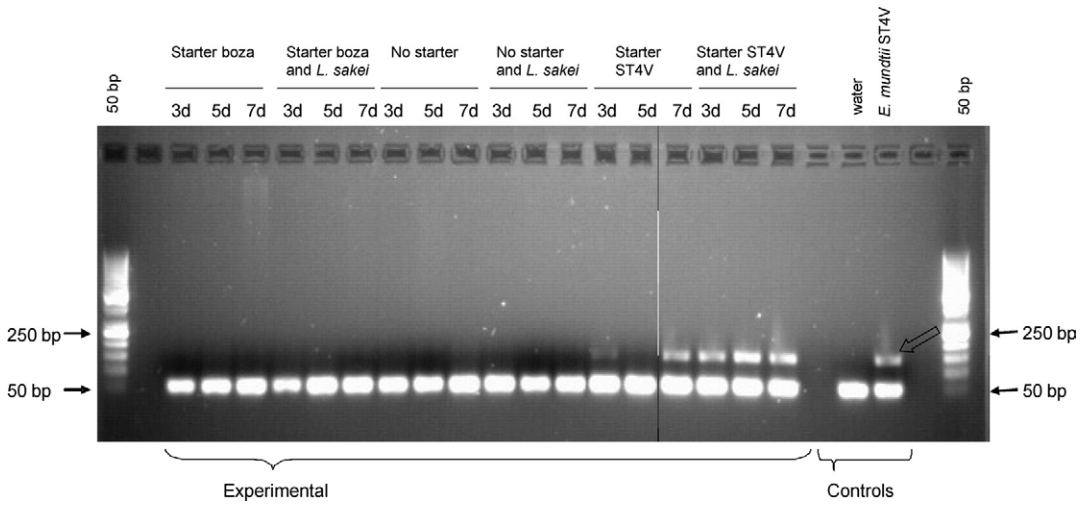

Fig. 5. DNA fragments amplified with primers designed from the structural gene encoding bacteriocin ST4V. 3d, 5d and 7d refer to the number of days boza was stored at 4 °C. The species-specific fragment is indicated by an arrow. The bands at the bottom of the gel are primer dimers

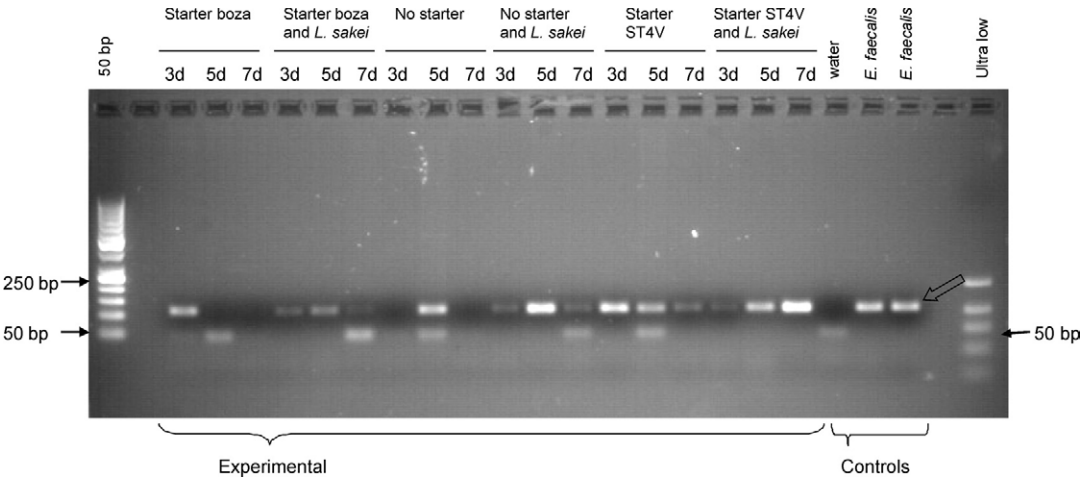

Fig. 6. DNA fragments amplified with genus-specific primers designed from the 16S rRNA sequence of *Enterococcus*. 3d, 5d and 7d refer to the number of days boza was stored at 4 °C. The bands at the bottom of the gel are primer dimers

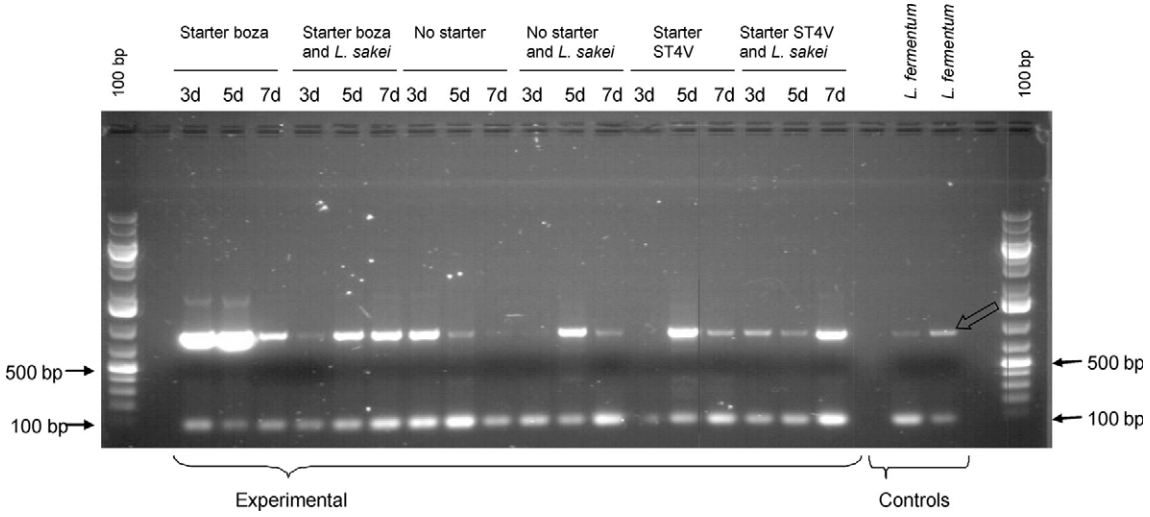

Fig. 7. DNA fragments amplified with species-specific primers designed from the 16S rRNA sequence of *L. fermentum*. 3d, 5d and 7d refer to the number of days boza was stored at 4 °C. The bands at the bottom of the gel are primer dimers

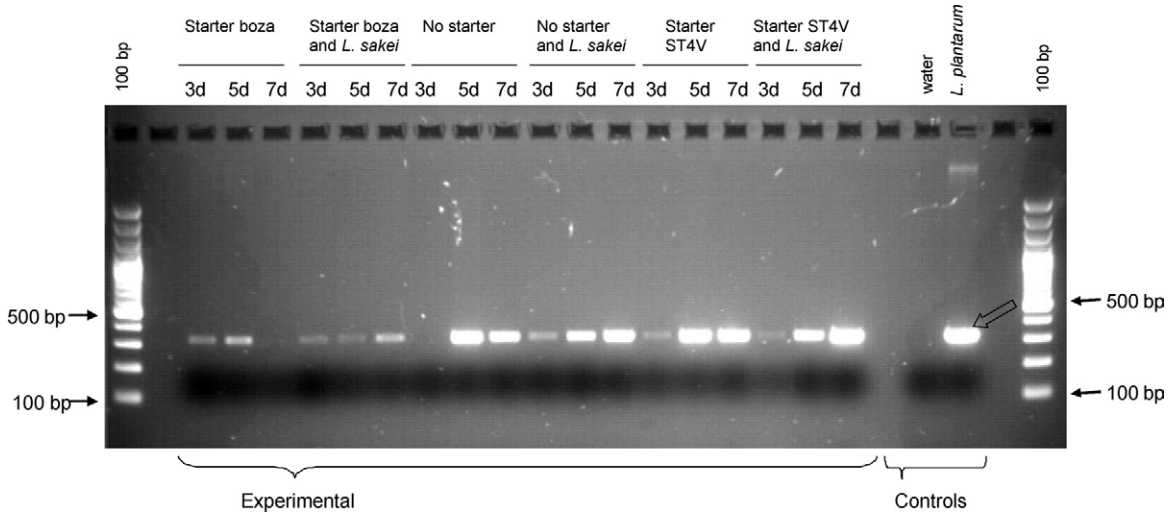

Fig. 8. DNA fragments amplified with species-specific primers designed from the 16S rRNA sequence of *L. plantarum.* 3d, 5d and 7d refer to the number of days boza was stored at 4 °C

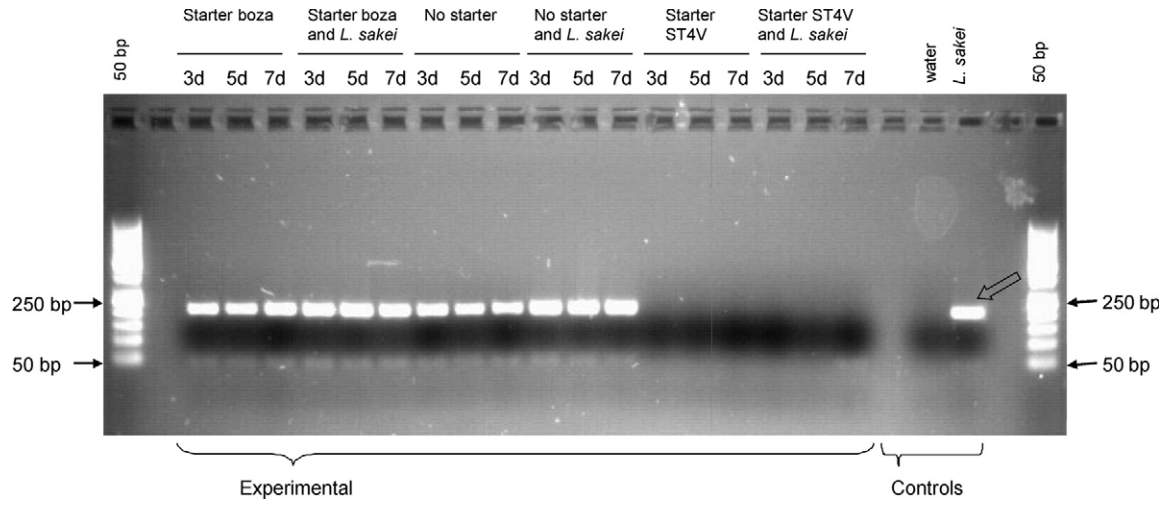

Fig. 9. DNA fragments amplified with species-specific primers designed from the 16S rRNA sequence of *L. sakei.* 3d, 5d and 7d refer to the number of days boza was stored at 4 °C. The bands at the bottom of the gel are primer dimers

Table 6. Sensorial evaluation of boza produced by natural fermentation, with commercial boza starter or *E. mundtii* ST4V

Attributes	Scale anchor	Treatment (starter culture)			LSD (p=0.005)
		Natural fermentation	With starter boza from previous batch	*E. mundtii* ST4V	
Yeasty aroma	no yeasty aroma/strong yeasty aroma	49.0280^{a,b}	48.6940^{a,b}	48.7220^{a,b}	2.2359
Bitterness	no bitter taste/prominent bitterness	2.4444^{a}	3.0278^{a}	2.6111^{a}	1.0246
Sweetness	no sweet taste/prominent sweetness	17.2430^{a}	18.3890^{a}	17.6940^{a}	1.9993
Yeasty flavour	low yeasty flavour/high yeasty flavour	40.9310^{b}	43.2860^{a}	42.9170^{a,b}	2.3015
Acidity	low acidity/high acidity	10.0580^{b,c}	11.1944^{a,b}	9.5833^{b,c}	1.8863

$^{a–c}$mean values in the same row with the same superscript are not significantly different

Conclusions

Bacteriocin ST4V has antibacterial and antiviral activity (*14*). *E. mundtii* ST4V survived 7 days in boza that was stored at 4 °C and produced bacteriocin ST4V at levels high enough to prevent the growth of the target strain *L. sakei* DSM 20017^{T}. No off-flavours or abnormal textural changes were recorded in boza during storage. Boza could thus be developed as a vector to deliver strain ST4V. The probiotic properties of strain ST4V need to be determined with *in vivo* studies.

Acknowledgements

This research was supported by the National Research Foundation (NRF), South Africa and a post-doctoral fellowship to Dr S. Todorov by Claude Leon Foundation, Cape Town, South Africa.

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