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Safety Assessment of *Lactobacillus plantarum* 423 and *Enterococcus mundtii* ST4SA Determined in Trials with Wistar Rats

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Abstract Colonization of *Lactobacillus plantarum* 423 and *Enterococcus mundtii* ST4SA in the gastro-intestinal tract was determined by using Wistar rats as model. The strains were administered through intragastric gavage over 14 days. FISH with strain-specific oligonucleotide probes indicated that *Lact. plantarum* 423 adhered to the surfaces of the ileum and the cecum. *Enterococcus mundtii* ST4SA, on the other hand, adhered to the surfaces of the cecum and colon. Results obtained by DGGE have shown that strains 423 and ST4SA excluded *Enterobacteriaceae*, but not lactic acid bacteria, from the cecum and colon. No signs of perforation of epithelial cells by strains 423 and ST4SA were detected. The spleen and liver appeared healthy and blood counts were normal, suggesting that the strains are not pathogenic. Both strains produce antimicrobial peptides active against a number of pathogens and may be considered as probiotics.

Keywords Lactic acid bacteria · Safety assessment · In vivo

Introduction

Lactic acid bacteria are generally regarded as safe (GRAS) and have been used as starter cultures in the fermentation of many food products [5]. Most probiotic bacteria are from the genera *Lactobacillus* and *Bifidobacterium* [5]. Various claims have been attributed to probiotics, e.g., reduction or prevention of gastrointestinal disorders, including inflammatory bowel disease, alleviation of lactose intolerance, lowering of serum cholesterol levels, stimulation of the immune system, and anti-tumor properties [7, 29]. A probiotic strain is often evaluated by its ability to colonize the intestinal tract or its ability to compete against pathogens for adhesion sites [13]. Type 1 fimbriated *Escherichia coli* adhere to mannose residues on the epithelial cells of humans and rats [2, 11]. Damage of the intestinal barrier increases the permeability which may lead to intestinal inflammation [20] and bacteremia [15, 31]. Mannose-specific adherence has also been demonstrated in many strains of *Lactobacillus plantarum* [26]. Adhesion of probiotic strains to host intestinal tissue may thus prevent colonization of pathogens [1].

Survival of probiotic lactic acid bacteria in the intestinal tract is normally studied by analyzing the fecal samples [17]. Information on the adherence of probiotics to intestinal tissue or mucus is usually obtained by in vitro studies on intestinal cell lines or mucus [9, 18, 24]. This is, however, not a true indication of the ability of the cells to adhere to intestinal tissue or mucus. In vivo studies on the adhesion of probiotics are difficult to perform and thus seldom reported.

Rat and mouse models are prevalent choices for in vivo evaluation of probiotic properties and have been incorporated in numerous trials [19, 25, 37]. These models are often used for evaluation of the safety and efficacy of a potential probiotic in the gut. Prior to the incorporation of a probiotic in food and pharmaceutical applications, every concern surrounding its safety for human consumption needs to be tested and resolved.

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We studied the colonization of two strains, *Lact. plantarum* 423 and *Ent. mundtii* ST4SA, in different sections of the gastro-intestinal tract of rats. Previous studies have shown that both strains survived conditions simulating the gastro-intestinal tract [4, 10, 27, 28], inhibited the growth of a number of potential pathogens, e.g., *Bacillus cereus*, *Clostridium sporogenes*, *Enterococcus faecalis*, and *Listeria monocytogenes* [14, 32], and harbor genes encoding adhesion to mucus [27, 28]. Fluorescent in situ hybridization (FISH) with strain-specific probes was used. The number of strains present in different sections of the intestinal tract was determined by using real-time PCR with strain-specific primers. Changes in the population of enteric bacteria were studied by 16S rDNA PCR and denaturing gradient gel electrophoresis (DGGE).

Materials and Methods

Approval to work with Wistar rats was obtained from the Ethics Committee of the University of Stellenbosch (ethics reference number: 2005B02003). Four-week-old male Wistar rats were divided into four groups (A, B, C, and D), having six rats per group. The rats were contained in a controlled environment (23 ± 2°C) and had free access to feed and drinking water.

Bacterial Strains and Preparation of Inoculum (Dosage)

Lactobacillus plantarum 423 and *Enterococcus mundtii* ST4SA were cultured in De Man Rogosa Sharpe (MRS) broth (Biolab, Biolab Diagnostics, Midrand, South Africa) at 37°C to $OD_{600nm} = 4.0$. Ten milliter of cultures were harvested (10000*g*, 10 min at 4°C), washed twice in sterile saline (0.85%, w/v, NaCl), resuspended in 10% (w/v) sterile skim milk, and lyophilized in separate vials. One vial lyophilized cells of each strain was resuspended in 1 ml sterile saline, serially diluted, and plated onto MRS (Biolab) agar to determine the number of viable cells. Lyophilized cells in the other vials were resuspended in a predetermined volume of sterile saline to yield 1×10^8 CFU per 200 µl.

Administration of Strains 423 and ST4SA

Viable bacterial cells were administered via intragastric gavage. Rats in group A received 200 µl *Lact. plantarum* 423, group B 200 µl *Ent. mundtii* ST4SA, and group C a combination of the two strains (100 µl each). Rats in group D served as control and received 200 µl sterile saline. Rats were dosed once a day for 14 days and were monitored for physical abnormalities such as ruffled coat, hunched posture, imbalance, tremors, coughing, irregular breathing, and discolouration of extremities. The rats were weighed every third day. Each rat weighed approximately 250 g.

Screening for Strains 423 and ST4SA in Feces and their Presence in Different Sections of the Intestinal Tract

At the end of the 14-days trial, the rats were anaesthetized with an overdose (1 ml) of sodium pentobarbitone (200 mg ml^{-1}). Fecal samples (1 g), collected from the colon of each rat, were suspended into 10 ml MRS Broth (Biolab) and BHI (Biolab), respectively, and incubated at 37°C to favor bacterial growth. After 18 h, cells were harvested (8000*g*, 15 min, 4°C), washed in sterile STE buffer, suspended in 10 ml of the same buffer containing 50 µl lysozyme (100 mg ml^{-1}), and incubated for 2 h at 37°C.

The duodenum, jejunum, ileum, cecum, and colon of rats from each group were aseptically removed and dissected into sections of 2 cm. One section from each part of the intestine was inoculated into 10 ml MRS Broth (Biolab) and BHI (Biolab), respectively, and incubated at 37°C for 18 h to favor bacterial growth. The sections were aseptically removed from the test tubes, the cells harvested, washed, and treated with lysozyme as described before.

Plasmid DNA was isolated from cells using the Qiagen QIAprep Spin Miniprep Kit (Qiagen GmbH, Hilden, Germany), according to the manufacturer's instructions, and amplified with primers designed from the genes encoding bacteriocin 423 and antimicrobial peptide ST4SA (Table 1). Each reaction mixture contained 300 ng DNA, 200 µM dNTPs, 0.5 µM of each primer, and 2.5 U TaKaRa Ex Taq™ (Takara Shoza Co. Ltd, Biomedical Group, Shiga, Japan). The following cycles were used: 30 s at 94°C, 30 s at 54°C, 45 s at 72°C, repeated for 30 times, and once for 7 min at 72°C. Amplicons were separated by agarose gel electrophoresis, according to the method described by Sambrook et al. [30]. Lambda DNA digested with *Eco*RI and *Hin*dIII (Promega, Madison, USA) was used as molecular weight marker. DNA amplified from pure cultures of *Lact. plantarum* 423 and *Ent. mundtii* ST4SA served as controls.

In another experiment, 2 cm sections of the ileum, cecum, and colon from rats in each group were placed in cassettes and immersed in 4% paraformaldehyde in 0.2 M PBS (0.2 mol l^{-1} NaH_2PO_4, 0.2 mol l^{-1} Na_2HPO_4, pH 7.2) for 24 h. The tissue samples were then dehydrated in a series of 70%, 90%, 95%, and 98% (v/v) ethanol for 12.5 h, followed by 3.5 h of clearing in xylol and 4 h of impregnation in paraffin wax (Histosec®, Merck, Darmstadt, Germany). The tissues were sectioned with a R Jung microtome (Heidelberg, Germany) in 5 µm increments and placed on poly-L-lysine coated microscope slides (Lasec, Johannesburg, South Africa). The sections were deparaffinized and rehydrated by

Table 1 Strain-specific primers used in the design of probes to detect *Lact. plantarum* 423 and *Ent. mundtii* ST4SA

Primer set	Sequence 5′–3′	Size of amplicon (bp)
Bac 423F RT	GACATTTACATTGAGTAGGAACTAG	147
Bac 423R RT	GTAACCCCATTACCATAGTATTTAC	
Mund ST4SA F	ATGTCACAAGTAGTAGGTGG	123
Mund ST4SA R	AGCTAAATTCGCAGCAGA	

Primers were also used for RT-PCR

submersion in xylene for 5 min, followed by submersion in 98% (v/v) ethanol for 3 min and then in 95% (v/v) ethanol for 3 min. This was followed by a 3 min submersion in 80% (v/v) and 50% (v/v) ethanol, respectively. The slides were rinsed in sterile distilled water and submerged for 20 min in 0.1% (w/v) trypsin in PBS. Incubation was at 37°C. Slides were then rinsed with sterile distilled water, dried for 20 min at 46°C and treated with 20 μl lysozyme (20 mg ml^{-1}). After 20 min incubation at room temperature, the slides were rinsed with sterile distilled water and air dried. Each slide was covered with 50 μl hybridization buffer (5 mol l^{-1} NaCl; 1 mol l^{-1} Tris–HCl pH 8.0; 0.01%, w/v, SDS; and 25%, v/v, formamide) containing 5 μl strain-specific probe (50 ng $μl^{-1}$).

Probes were prepared by amplifying plasmid DNA with primers listed in Table 1, as described before. PCR amplicons were labeled using the universal labeling system of the ULYSIS nucleic acid labeling kit (Molecular Probes™, Invitrogen Corporation, Carlsbad, California, USA). Alexa Fluor 568 dye (Invitrogen) with a maximum absorbance at 576 nm and emission at 600 nm was used. Hybridisation was for 2.5 h at 46°C in a moist chamber. Slides were submerged in 50 ml wash buffer (5 mol l^{-1} NaCl; 1 mol l^{-1} Tris–HCl pH 8.0; 0.01%, w/v, SDS; and 0.5 mol l^{-1} EDTA, pH 8.0) for 10 min at 48°C, followed by a final rinse with sterile distilled water and air dried. Preparations were covered with fluorescent mounting medium (Dako Cytomation) to prevent photobleaching and were stored in the dark at −20°C. The presence of *Lact. plantarum* 423 and *Ent. mundtii* ST4SA on the intestinal tissue samples was observed by using an Olympus cell^R system attached to an IX-81 inverted fluorescence microscope, equipped with a 40× lens (LucPlan FLN, Olympus) and a 60× lens (PlanApoN 1.42 NA oil immersion) (Olympus Biosystems GMBH, Germany). Samples were excited by using a 572 nm filter and visualized with 400× and 600× magnification. Cell^R imaging software was used for image acquisition and analysis.

The number of *Lact. plantarum* 423 and *Ent. mundtii* ST4SA cells that adhered to tissue samples was determined by using real-time PCR (RT-PCR). Plasmid DNA and RT-PCR was performed in duplicates for each strain on each of the tissue samples. PCR conditions were as follows: initial denaturation at 95°C for 15 min, followed by 40 cycles of amplification at 50°C for 10 s and 72°C for 15 s. Reactions were performed in a LightCycler instrument (Roche, Mannheim, Germany), with Relative Quantification Software (version 5.32; Roche) and SYBR Green technology (Sigma, Missouri, USA). Each 20 μl reaction mixture consisted of 10 μl SYBR Green Supermix (Sigma), 1 μl of each primer (Table 1) at 5 μM, 7 μl nuclease-free water, and 1 μl diluted plasmid DNA. Five dilutions of plasmid DNA were prepared. A negative control with sterile Milli Q water was included in each run. At the end of each run a melting curve was performed from 95°C to 45°C (0.2°C s^{-1}). Standard curves were used for quantification and consisted of Log template concentration of DNA plotted against the C_t-value (crossing points of different standard dilutions). Efficiencies were calculated by $E = 10^{(-1/S)} - 1$, where $S =$ slope of the standard curve.

Changes in the Population of Intestinal Microbiota Recorded with Denaturing Gradient Gel Electrophoresis (DGGE)

Homogenized tissue samples from different sections of the intestinal tract (300 μl) were inoculated into 10 ml MRS broth (Biolab) and 10 ml MacConkey broth (Biolab) for isolation of lactic acid bacteria and *Enterobacteriaceae*, respectively. Incubation was at 37°C to $OD_{600nm} = 1.8$ to favor bacterial growth and thus enhance the sensitivity of DGGE. Amplification of 16S rDNA was with primers 341FGC (5′-CGCCCGCCGCGCGCGGCGGGCGGGGCGGGGGCACGGGGGGCCTACGGGAGGCAGCAG-3′) and 534R (5′-ATTACCGCGGCTGCTGG-3′), designed to target conserved 16S rDNA sequences [23]. Primers were selected to produce 193 bp PCR amplicons encompassing the V3 region of the 16SrDNA gene. The forward primer was modified with a GC-clamp to impart melting stability to the DNA fragments for efficient DGGE analysis.

The PCR reaction, performed in a Gene Amp® PCR 9700 system (Applied Biosystems, California, USA) contained the following: 5 μl of 10× buffer, 3 μl of 25 mM $MgCl_2$, 5 μl of 5 μM 341FGC, 5 μl of 5 μM 534R, 4 μl of 2.5 mM

DNTP's, 0.5 µl of *Taq* polymerase enzyme (Takara, Shiga, Japan), and 1 µl of genomic DNA (100 ng μl^{-1}). Each reaction was adjusted to a final volume of 50 µl with sterile milli Q water.

DGGE was performed in a 10% (w/v) polyacrylamide gel (37.5:1, acrylamide:bisacrylamide), with a gradient ranging from 30% to 60%. The 30% gradient contained 5 ml 40% (w/v) polyacrylamide, 1 ml 10× TAE buffer (20 mM Tris–acetate pH 7.4, 10 mM sodium acetate, and 0.5 mM EDTA, pH 7.4), 2.4 ml formamide, and 2.5 g urea, adjusted to 20 ml with sterile distilled water. The 60% gradient contained 5 ml 40% (w/v) polyacrylamide, 1 ml 10× TAE buffer, 4.8 ml formamide, and 5.0 g urea, adjusted to 20 ml with sterile distilled water. Polymerization was catalyzed by 140 µl 10% ammonium persulfate and 14 µl TEMED. Electrophoresis was at a constant 100 V for 16 h at 60°C using the DCode™ Universal Mutation Detection System (Bio-Rad, Laboratories, USA). Electrophoresis buffer consisted of 0.5× TAE.

Blood Analyses and Histology

Blood was drawn via cardiac puncture of the right ventricle and 100 µl spread-plated onto MRS agar and Brain Heart Infusion (BHI) Agar (Biolab). The plates were incubated at 37°C for 24 h and examined for microbial growth. White and red blood cells counts were determined with an automated hematology counter (Beckman Coulter Hematology Analyser LH750).

Samples collected from the liver, spleen, ileum, and colon were fixed in 4% formaldehyde for 20–24 h, embedded in paraffin, sectioned, and stained with hematoxylin and eosin. The method used to prepare the slides was similar to the method used for FISH. All samples were analyzed by PathCare veterinary pathologists (Cape Town, South Africa).

Results

No physical abnormalities, abnormal behavior, abnormal movements, or changes in bodyweight were recorded for any of the rats over the 14-days trial.

Screening for Strains 423 and ST4SA

Amplification of DNA isolated from fecal microorganisms yielded fragments of 147 bp and 123 bp in size (Table 1), corresponding to the structural genes encoding bacteriocin 423 and antimicrobial peptide ST4SA, respectively. *Lact. plantarum* 423 was detected in the ileum, cecum, and colon. *Ent. mundtii* ST4SA, on the other hand, was only detected in the cecum and colon.

FISH conducted on homogenized tissue samples indicated that *Lact. plantarum* 423 adhered to the epithelial lining of the cecum (Fig. 1a). Similar results were recorded for the ileum (not shown). Strain 423 did not bind to the epithelium of the colon and remained in the lumen (Fig. 1b). Higher cell numbers of *Lact. plantarum* 423 were recorded in the ileum and cecum, compared to the duodenum and colon of rats in group A (Table 2). Identical results were obtained for all six rats in group A. Low fluorescent signals were recorded in the cecum of rats from group D, the control group (Fig. 1c). Similar results were recorded in the ileum and colon (not shown). Identical results were obtained for all six rats in group D.

Enterococcus mundtii ST4SA colonized the cecum and, to a lesser intensity, the colon (Table 2). In comparison to *Lact. plantarum* 423, ST4SA cells did not adhere to the epithelial lining of the cecum, but were translocated across the membrane and concentrated in the cytoplasm of the cells (Fig 1d). Identical results were recorded for colon cells (not shown). The same results were recorded for all six rats in group B.

Denaturing Gradient Gel Electrophoresis (DGGE)

Colonization of *Lact. plantarum* 423 and *Ent. mundtii* ST4SA in the intestinal tract significantly altered the population of enteric bacteria. Both strains lowered the numbers of *Enterobacteriaceae* in the cecum and colon, as indicated by changes in DNA profiles recorded with DGGE (Fig. 2a, b). The population of lactic acid bacteria remained unchanged in the presence of strains 423 and ST4SA (Fig. 2c, d).

Blood Analyses and Histology

No viable cells of *Ent. mundtii* ST4SA and *Lact. plantarum* 423 were detected in any of the blood samples. Macroscopic examination of the liver, spleen, colon, and ileum (Fig. 3) did not show any morphological abnormalities. The number of white and red blood cells in rats that were administered with *Lact. plantarum* 423, *Ent. mundtii* ST4SA, or a combination of the two strains, were identical to that recorded for rats in the control group.

Discussion

Screening for Strains 423 and ST4SA

The fact that no physical abnormalities, abnormal behavior, abnormal movements, or changes in bodyweight were recorded suggests that *Lact. plantarum* 423 and *Ent. mundtii* ST4SA are not pathogenic. *Lactobacillus plantarum* 423

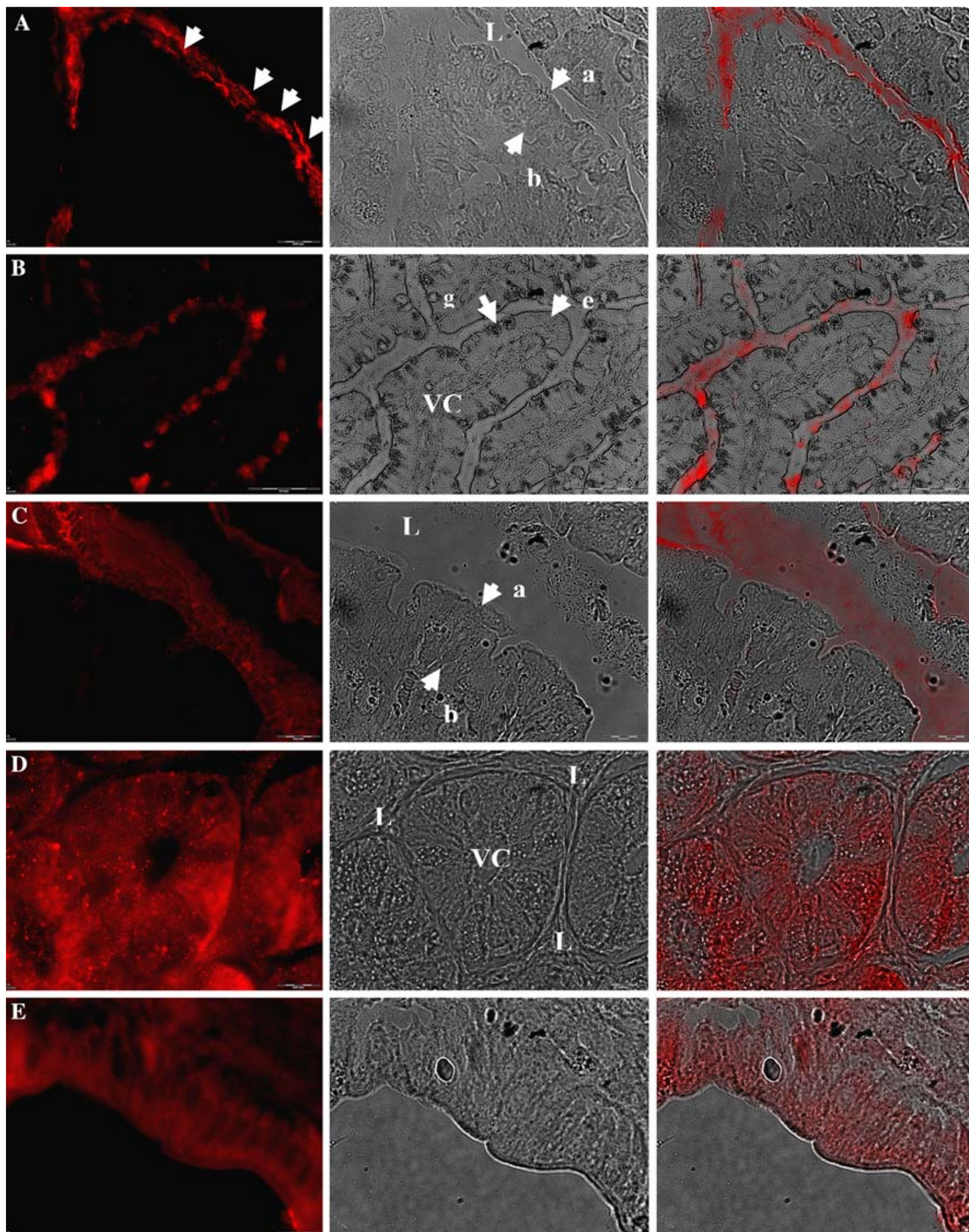

Fig. 1 FISH images of *Lact. plantarum* 423 and *Ent. mundtii* ST4SA adhered to rat intestinal tissue. Magnification: 600×. Left lane = fluorescent images, middle lane = phase images, right lane = overlayed images. **a** *Lact. plantarum* 423 adhering to cecum (*arrows* indicate strong fluorescent signals recorded along the epithelial lining); **b** Strain 423 in the lumen of the colon, with no binding to colonic tissue; **c** Strain 423 on cecum tissue of control (group D) rats; **d** *Ent. mundtii* ST4SA in the cytoplasm of cecum cells (indicated with arrows); and **e**: Strain ST4SA on cecum tissue of group D rats. *L* lumen, *a* apical side of the enterocyte, *b* basal side of enterocyte, *VC* villus core, *g* goblet cell, *e* enterocyte

colonizes the lower section of the small intestine, whereas *Ent. mundtii* ST4SA prefers more anaerobic conditions in the large intestine. Both strains were secreted in the feces. Although the results obtained cannot be used to determine the number of viable cells in the feces or in the different sections of the intestinal tract, valuable information was

Table 2 Quantification of strains by using real time PCR

Strain	Section	Av. Ct	Quant.[a]	Set to calibrator[b]
423	Duodenum	30.76	2.8	1
	Ileum	25.99	60	22
	Cecum	25.76	70	26
	Colon	30.84	2.7	1
ST4SA	Jejenum	27.01	1.3	1
	Ileum	26.29	2.1	1.6
	Cecum	19.21	251	193
	Colon	22.26	32	24

[a] Calculation of the quantity of each bacteriocin gene in specific tissue sections were determined by equations obtained from a standard curve

[b] Samples that contained the lowest quantity of the bacteriocin gene served as the calibrator sample

obtained regarding the distribution of the strains. PCR with strain specific primers proved more accurate than viable cell counts, as *Lact. plantarum* 423 and *Ent. mundtii* ST4SA could not be differentiated from each other or other lactic acid bacteria based on colony morphology.

FISH has been used to detect lactobacilli in pig mucus and epithelial tissue [21]. The technique has also been used to detect and quantify predominant groups of anaerobic bacteria in human fecal samples [6]. Results obtained by FISH confirmed that strain 423 colonized the ileum and cecum. The low cell numbers recorded in the colon suggests that the cells are not contained and that they are secreted with fecal material.

The reason for detecting *Lact. plantarum* 423 in the intestine of rats that have not been treated with *Lact. plantarum* 423 (e.g., the cecum, Fig. 1c) is not known. The probe used in this study is based on the sequence of the structural gene encoding bacteriocin 423 and is thus strain specific. Conditions used in the PCR reaction were stringent and only one fragment of 147 bp was amplified (Table 1) and used as a probe. However, the probe could have hybridized with DNA from another strain containing a gene similar in sequence to the gene encoding bacteriocin 423. The possibility of horizontal gene transfer cannot be ruled out, as the operon for bacteriocin 423 is located on a plasmid [32]. It is, however, also possible that rats in group D were contaminated with *Lact. plantarum*

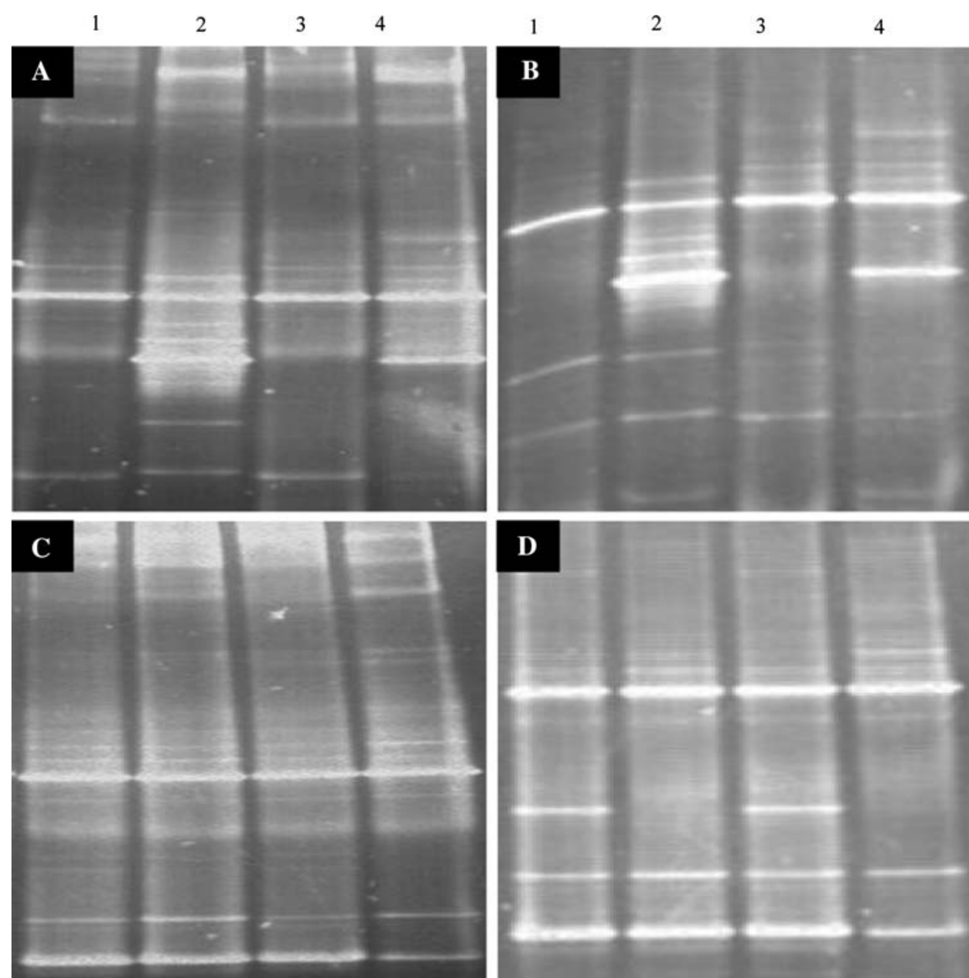

Fig. 2 Effect of *Lact. plantarum* 423 and *Ent. mundtii* ST4SA treatment on the lactic acid bacteria and *Enterobacteriaceae* population in the cecum and colon of rats. **a** *Lact. plantarum* 423 on *Enterobacteriaceae*, **b** *Ent. mundtii* ST4SA on *Enterobacteriaceae*, **c** *Lact. plantarum* 423 on lactic acid bacteria, and **d** *Ent. mundtii* ST4SA on lactic acid bacteria. Lane 1 = cecum, lane 2 = cecum control, lane 3 = colon, lane 4 = colon control

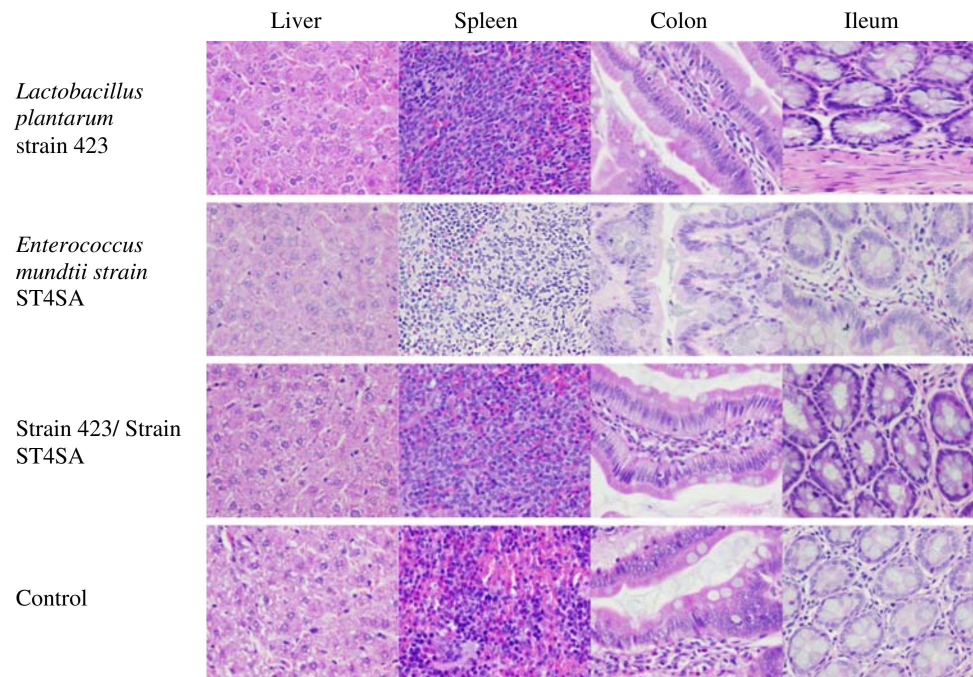

Fig. 3 Hematoxylin and Eosin stains of liver, spleen, colon, and ileum tissue from rats administered with *Lact. plantarum* 423, *Ent. mundtii* ST4SA, and a combination of the two strains. The control (placebo) group received sterile saline

423 from rats in group A, despite stringent control measurements.

It is not surprising to detect high cell numbers of *Lact. plantarum* 423 in the ileum and cecum. In a previous study [21], optimal adhesion of strain 423 was recorded to mucus isolated from the ileum and cecum of piglets. The pH in the ileum and cecum of rats is approximately 6.0 [22] and within the optimal growth range for most lactobacilli [12]. Furthermore, the *MapA* gene of *Lact. plantarum* 423, encoding the mucus adhesion-promoting protein, was up-regulated at pH 6.5 and in the presence of mucus, proportional to increasing concentrations [27]. Strain 423 also contains genes encoding the mucus-binding protein (*Mub*) and the adhesion-like factor (*EF-Tu*) [27]. The duodenum and colon are slightly more acidic, pH 5.0–5.5 [22], and the cells would be metabolically less active. Adhesion to mucosa is important for proliferation of probiotic cells, especially in the small intestine with high flow rates [33]. The expression of bacteriocin 423 is not affected by physiological stress conditions in the intestinal tract [27]. These characteristics, and the ability to survive pH conditions as low as 2.0 [27] renders strain 423 an ideal probiotic candidate.

Translocation of cells across the epithelium, as observed for *Ent. mundtii* ST4SA (Fig. 1d), has been recorded for *Ent. faecalis* in mice [36]. Migration of enterococci across the intestinal barrier and vaginal epithelium has also been reported [35]. The bacteria exit through the apical side of the epithelial cells or migrate in phagocytes to mesenteric lymph nodes where they proliferate, spread to distant sites, and cause infection [34]. *Enterococcus faecium* and *Ent. faecalis* have been associated with cases of bacteremia and endocarditis [3, 8, 16]. However, it is rarely reported that species other than *Ent. faecium* and *Ent. faecalis* are involved in human pathogenesis [7]. In all of these studies patients were diagnosed with other diseases or underwent surgery, which makes it extremely difficult to prove that lactic acid bacteria were the primary source of infection [8]. Although *Ent. mundtii* ST4SA migrated across the epithelium of the cecum, no viable cells were detected in any of the blood samples, suggesting that strain ST4SA is not likely to cause bacteremia and endocarditis.

Results presented in Fig. 1 are those that have been recorded when single-strain cultures (423 or ST4SA) were administered. The same observations were made when the cells were administered in combination (423 and ST4SA in the same dosage). It was, however, difficult to differentiate between the two strains with the probes we have used. For this reason, only results recorded with pure cultures are presented.

Denaturing Gradient Gel Electrophoresis (DGGE)

Colonization of *Lact. plantarum* 423 and *Ent. mundtii* ST4SA in the intestinal tract significantly altered the population of enteric bacteria. Both strains lowered the numbers of *Enterobacteriaceae* in the cecum and colon, as indicated by changes in DNA profiles recorded with DGGE (Fig. 2a, b). The population of lactic acid bacteria remained unchanged in the presence of strains 423 and ST4SA (Fig. 2c, d). The same results were obtained when strains 423 and ST4SA were administered together in the

same dosage (not shown). Strains 423 and ST4SA have excellent probiotic properties and should be explored for human applications.

Blood Analyses and Histology

The fact that no viable cells of *Ent. mundtii* ST4SA and *Lact. plantarum* 423 were detected in any of the blood samples suggests that the cells did not migrate through the epithelium and that the strains are not likely to cause bacteremia. More importantly, macroscopic examination of the liver, spleen, colon, and ileum did not show morphological abnormalities. Furthermore, the number of white and red blood cells in rats that were administered strains 423 and ST4SA were identical to that recorded for rats in the control group, suggesting that the strains did not elicit allergic reactions.

The aim of this study was not to compare *Lact. plantarum* 423 and *Ent. mundtii* ST4SA with commercially available probiotic strains, but to study their ability to colonize the intestinal tract.

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References

1. Adawi D, Molin G, Ahrne S, Jeppsson B (1999) Modulation of the colonic bacterial flora affects differently bacterial translocation and liver injury in an acute liver injury model. Microbial Ecol Health Dis 11:47–54
2. Adlerberth I, Hanson LA, Svanborg C (1995) Adhesins of *Escherichia coli* associated with extra-intestinal pathogenicity confer binding to colonic epithelial cells. Microb Pathog 18:373–385
3. Alcid DV, Troke M, Andszewski S, John JF (1994) Probiotics as a source of *Enterococcus feacium.* Abstracts of the Infectious Diseases Society of America, Abstract no. 123
4. Botes M (2008) Survival of probiotic lactic acid bacteria in the intestinal tract, their adhesion to epithelial cells and their ability to compete with pathogenic microorganisms. PhD dissertation, University of Stellenbosch, Stellenbosch, South Africa
5. De Vuyst L, Vandamme EJ (1994) Nisin, a lantibiotic produced by *Lactococcus lactis* subsp. *lactis*: properties, biosynthesis, fermentation and application. In: de Vuyst L, Vandamme EJ (eds) Bacteriocins of lactic acid bacteria. Chapman and Hall, London, pp 151–221
6. Franks AH, Harmsen HJM, Raangs GC, Jansen GJ, Schut F, Welling GW (1998) Variations of bacterial populations in human feces measured by fluorescent in situ hybridisation with group-specific 16S rRNA-targeted oilgonucleotide probes. Appl Environ Microbiol 64:3336–3345
7. Franz CMAP, Holzapfel WH, Stiles ME (1999) Enterococci at the crossroads of food safety? Int J Food Microbiol 47:1–24
8. Franz CMAP, Stiles ME, Schleifer KH, Holzapfel WH (2003) Enterococci in foods—a conundrum for food safety. Int J Food Microbiol 88:105–122
9. Granato D, Bergonzelli GE, Pridmore RD, Marvin L, Rouvet M, Corthesy-Theulaz IE (2004) Cell surface-associated elongation factor Tu mediates the attachment of *Lactobacillus johnsonii* NCC533(La1) to human intestinal cells and mucins. Infect Immun 72:2160–2169
10. Granger M, Van Reenen CA, Dicks LMT (2008) Effect of gastro-intestinal conditions on *Enterococcus mundtii* ST4SA and production of bacteriocin ST4SA, as recorded by real-time PCR. Int J Food Microbiol 123:277–280
11. Herias MV, Midtvedt T, Hanson LA (1995) Role of *Escherichia coli* P fimbriae in intestinal colonisation in gnotobiotic rats. Infect Immun 63:4781–4789
12. Kandler O, Weiss N (1986) Genus Lactobacillus Beijerinck 1901, 212 AL. In: Sneath PHA, Mair NS, Sharpe ME, Holt JG (eds) Bergeys manual of systematic bacteriology, vol 2. The Williams & Wilkens Co., Baltimore, pp 1209–1234
13. Kirjavainen PV, Ouwehand AC, Isolauri E, Salminen SJ (1998) The ability of probiotic bacteria to bind to human intestinal mucus. FEMS Microbiol Lett 167:185–189
14. Knoetze H (2007) Characterization of a broad-spectrum antimicrobial peptide from *Enterococcus mundtii* active against bacteria associated with middle-ear infections. MSc thesis, University of Stellenbosch, Stellenbosch, South Africa
15. Kops SK, Lowe DK, Bement WM, West AB (1996) Migration of *Salmonella typhi* through intestinal epithelial monolayers: an in vitro study. Microbiol Immunol 40:799–811
16. Lara-Villoslada F, Sierra S, Martin R, Delgad S, Rodríguez JM, Olivares M, Xaus J (2007) Safety assessment of two probiotic strains, *Lactobacillus coryniformis* CECT5711 and *Lactobacillus gasseri* CECT5714. J Appl Microbiol 103:175–184
17. Lick S, Drescher K, Heller KJ (2001) Survival of *Lactobacillus delbrueckii* subsp. bulgaricus and *Streptococcus thermophilus* in the terminal ileum of fistulated Gottingen minipigs. Appl Environ Microbiol 9:4137–4143
18. Mack DR, Ahrne S, Hyde L, Wei S, Hollingsworth MA (2003) Extracellular MUC3 mucin secretion follows adherence of *Lactobacillus* strains to intestinal epithelial cells in vitro. Gut 52:827–833
19. Mangell P, Nejdfors P, Wang M, Ahrne S, Westrom B, Thorlacius H, Jeppsson B (2002) *Lactobacillus plantarum* 299V inhibits *Escherichia coli*-induced intestinal permeability. Dig Dis Sci 47:511–516
20. Mao Y, Nobaek S, Kasravi B, Adawi D, Stenram U, Molin G, Jeppsson B (1996) The effects of *Lactobacillus* strains and oat fiber on methotrexate-induced enterocolitis in rats. Gastroenterology 111:334–344
21. Mare L, Wolfaardt GM, Dicks LMT (2006) Adhesion of *Lactobacillus plantarum* 423 and *Lactobacillus salivarius* 241 to the intestinal tract of piglets, as recorded with fluorescent in situ hybridisation (FISH) and production of plantaricin 423 by cells colonised to the ileum. J Appl Microbiol 100:838–845
22. McConnell EL, Basit AW, Murdan S (2008) Measurements of rat and mouse gastrointestinal pH, fluid and lymphoid tissue, and implications for in vivo experiments. J Pharm Pharmacol 60: 63–70
23. Muyzer G, De Waal EC, Uitterlinden AG (1993) Profiling of complex microbial populations by denaturing gradient gel electrophoresis analysis of polymerase chain reaction-amplified genes coding for 16S rRNA. Appl Environ Microbiol 59:695–700
24. Ouwehand AC, Kirjavainen PV, Gronlund MM, Isolauri E, Salminen SJ (1999) Adhesion of probiotic microorganisms to intestinal mucus. Int Dairy J 9:623–630
25. Pavan S, Desreumaux P, Mercenier A (2003) Use of mouse models to evaluate the persistence, safety and immune modulation capacities of lactic acid bacteria. Clin Diagn Lab Immunol 10:696–701
26. Pretzer G, Snel J, Molenaar D, Wiersma A, Bron PA, Lambert WM, De Vos W, Van der Meer R, Smits MA, Kleerebezem M (2005) Biodiversity-based identification and functional characterisation of

the mannose specific adhesin of *L.* plantarum. J Bacteriol 187: 6128–6136

27. Ramiah K, Van Reenen CA, Dicks LMT (2007) Expression of the mucus adhesion genes *mub* and *mapA*, adhesion-like factor *EF-Tu* and bacteriocin gene *plaA* of *Lactobacillus plantarum* 423 monitored with real-time PCR. Int J Food Microbiol 116:405–409
28. Ramiah K (2008) Characterization of the adhesion genes of probiotic lactic acid bacteria. PhD dissertation, University of Stellenbosch, Stellenbosch 7600, South Africa
29. Saarela M, Mogensen G, Fondén R, Mättö J, Mattila-Sandholm T (2000) Probiotic bacteria: safety, functional and technological properties. J Biotechnol 84:197–215
30. Sambrook JE, Fritsch F, Maniatis J (1989) Molecular cloning: a laboratory manual, 2nd edn. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY
31. Spitz J, Yuhan R, Koutsouris A, Blatt C, Alverdy J, Hecht G (1995) Enteropathogenic *Escherichia coli* adherence to intestinal epithelial monolayers diminishes barrier function. Am J Physiol 268:G374–G379
32. Van Reenen CA, Dicks LMT, Chikindas ML (1998) Isolation, purification and partial characterization of plantaricin 423, a bacteriocin produced by *Lactobacillus plantarum*. J Appl Microbiol 84:1131–1137
33. Wadstrom T (1998) Adherence traits and mechanisms of microbial adhesion in the gut. Baillier's Clin Trop Med Commun Dis 3:417–433
34. Wells CL, Maddaus MA, Simmons RL (1988) Proposed mechanisms for the translocation of intestinal bacteria. Rev Infect Dis 10:958–978
35. Wells CL, Jechorek RP, Twiggs LB, Brooker DC (1990) Recovery of viable bacteria from pelvic lymph nodes of patients with gynecologic tumors. J Infect Dis 162:1216–1218
36. Wells CL, Erlandsen SL (1991) Localisation of translocating *Escherichia coli*, *Proteus mirabilis* and *Enterococcus faecalis* within cecal and colonic tissues of monoassociated mice. Infect Immun 59:4693–4697
37. Zhou JS, Shu Q, Rutherfurd KJ, Prasad J, Gopal PK, Gill HS (2000) Acute oral toxicity and bacterial translocation studies on potentially probiotic strains of lactic acid bacteria. Food Chem Toxicol 38:153–161