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Evaluation of lactic acid bacteria from kefir, molasses and olive brine as possible probiotics based on physiological properties

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Abstract - A combination of eight strains comprising of *Lactobacillus plantarum*, *Enterococcus faecium* and *Leuconostoc mesenteroides* subsp. *mesenteroides* isolated from molasses, olives, beer and kefir were studied for growth at low pH and ox-bile resistance. pH neutralised cell-free supernatants from 24-h-old cultures inhibited the growth of *Enterococcus faecium, Lactobacillus sakei, Lactococcus lactis* subsp. *lactis, Listeria innocua* and *Listeria ivanovii* subsp. *ivanovii*. Good growth was recorded in MRS broth supplemented with 0.3% (w/v) ox-bile. *Lactobacillus plantarum* ST28MS and ST26MS, *Enterococcus faecium* ST311LD and *Leuconostoc mesenteroides* subsp. *mesenteroides* ST33LD grew well in the presence of 0.6% (w/v) ox-bile. All eight strains grew well in MRS broth adjusted to pH 7.0. Good growth of *Enterococcus faecium* ST311LD, *Leuconostoc mesenteroides* subsp. *mesenteroides* ST33LD and *Lactobacillus plantarum* 423 was recorded in MRS broth with an initial pH of 4.0. Auto cell-aggregation ranged from 74.3% for *Lactobacillus plantarum* ST23LD to 95.4% for *Lactobacillus plantarum* ST28MS. Different levels of co-aggregation were recorded between the eight strains and *Enterococcus faecium* HKLHS, *Lactobacillus sakei* DSM 20017, *Lactococcus lactis* subsp. *lactis* HV219, *Listeria innocua* LMG 13568 and UWC N27, and *Listeria ivanovii* subsp. *ivanovii* ATCC 19119. Growth of the eight strains was inhibited by several antibiotics and anti-inflammatory medicaments containing ibuprofen, hydrochlorothiaziden and thioridazine hydrochlorid. Sodium diclofenac inhibited the growth of *Lactobacillus plantarum* ST8KF and ST341LD, *Enterococcus faecium* ST311LD and *Leuconostoc mesenteroides* subsp. *mesenteroides* ST33LD. Dimenhydrinate inhibited the growth of only *Lactobacillus plantarum* ST8KF. Adherence to Caco-2 cells ranged from 8.0 to 1.3%. All eight strains contain the *Mub, MapA* and *EF-Tu* genes, as determined by amplification with gene-specific primers.

Key words: probiotics, bacteriocins*, Lactobacillus plantarum, Enterococcus faecium, Leuconostoc mesenteroides* subsp. *mesenteroides.*

INTRODUCTION

Probiotics are defined as viable microorganisms with a beneficial effect on the host by improving the balance of the intestinal microflora (Guipta *et al*., 1996; Saarela *et al.*, 2002). Probiotic lactic acid bacteria may prevent the use of certain antibiotics in animal feeds (Park *et al.*, 2002) and if carefully selected, control the proliferation of pathogenic bacteria that may lead to diarrhoea and other clinical disorders such as cancer and inflammatory bowel disease (Fooks *et al.*, 1999).

The human intestinal tract harbours a number of microorganisms which forms a uniquely balanced ecosystem. Lactic acid bacteria colonise the intestinal mucus and epithelial cells and prevent the adhesion of pathogens. Adherence to intestinal mucosa is important to enable probiotic cells to persist and multiply in the host. To date, three different proteins that can interact with mucus components have been identified from lactobacilli, *viz.* the collagen binding protein (CnBP) from *Lactobacillus reuteri* that interacts with mucin, the mucus adhesion promoting protein (MapA) from *Lactobacillus reuteri* 104R (Satoh *et al.*, 2000) and the mucus-binding protein (Mub) from *Lactobacillus reuteri* 1063 (Roos and Jonsson, 2002). The genome sequence of *Lactobacillus acidophilus* NCFM revealed five predicted proteins homologus to the Mub protein of *Lactobacillus reuteri* 1063 (Roos and Jonsson, 2002). Many copies of Mub homologs are also present in *Lactobacillus gasseri* (seven), *Lactobacillus johnsonii* (four) and *Lactobacillus plantarum* (two) (Alterman *et al.*, 2005). The frequency of these proteins suggests an important role in mucus adhesion and colonisation of the host (Alterman *et al.*, 2005).

The elongation factor Tu (EF-Tu) was also recognised as an adhesin-like factor in *Lactobacillus johnsonii* NCC533 (Granato *et al.*, 2004). EF-Tu is a small GTP-binding protein that functions in protein synthesis in prokaryotes (Gaucher *et al.*, 2001). The mechanism of interaction of this molecule to mucin, or its role in adhesion, is not well understood, but it mediates attachment of *Lactobacillus johnsonii* La1 to human intestinal cell lines and mucin (Granato *et al.*, 2004). The EF-Tu protein of *Lactobacillus johnsonii* La1 displayed 84% homology with

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TABLE 1 - Co-aggregation values recorded for *Lactobacillus plantarum* ST26MS, ST28MS, ST23LD, ST341LD, 423 and ST8KF, *Enterococcus faecium* ST311LD and *Leuconostoc mesenteroides* subsp. *mesenteroides* ST33LD with *Enterococcus faecium* HKLHS, *Lactobacillus sakei* DSM 20017, *Lactococcus lactis* subsp. *lactis* HV219, *Listeria ivanovii* subsp. *ivanovii* ATCC 19119 and *Listeria innocua* UWS N27 and LMG 13568 as partners

Bacteriocin producer	Co-aggregation partners					
	Enterococcus faecium HKLHS	*Lactobacillus sakei* DSM 20017	*Lactococcus lactis* subsp. *lactis* HV219	*Listeria innocua* LMG 13568	*Listeria innocua* UWC N27	*Listeria ivanovii* subsp. *ivanovii* ATCC 19119
ST26MS	79.9 ± 2.3	85.6 ± 1.7	75.6 ± 1.6	85.9 ± 2.1	65.3 ± 2.3	65.2 ± 1.6
ST28MS	88.5 ± 0.4	94.2 ± 0.5	86.5 ± 1.1	93.2 ± 0.3	85.7 ± 0.1	84.7 ± 1.6
ST23LD	90.0 ± 2.5	96.3 ± 1.1	88.9 ± 3.9	94.3 ± 1.1	74.0 ± 2.2	75.4 ± 0.6
ST341LD	68.4 ± 1.4	83.8 ± 1.0	79.4 ± 0.7	86.5 ± 1.3	71.3 ± 2.0	74.0 ± 0.2
423	71.8 ± 5.5	76.7 ± 1.2	58.0 ± 0.4	69.1 ± 1.4	38.9 ± 1.1	42.8 ± 2.1
ST8KF	77.5 ± 1.0	91.1 ± 0.2	75.8 ± 0.4	86.1 ± 1.8	67.9 ± 1.5	68.7 ± 2.9
ST311LD	91.8 ± 1.1	95.5 ± 1.1	90.4 ± 0.9	93.7 ± 0.2	68.6 ± 0.5	75.9 ± 1.9
ST33LD	83.2 ± 0.4	87.5 ± 1.2	80.8 ± 1.8	90.4 ± 1.5	70.8 ± 0.9	73.9 ± 3.6

the EF-Tu protein of *Lactobacillus plantarum* (Granato *et al.*, 2004). More recently, EF-Tu and the surface-bound proteins glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and triosephosphate isomerase (TPI) have also been implicated in playing a role in adhesion of *Lactobacillus plantarum* 423 to Caco-2 cells (Ramiah *et al*., 2008).

Apart from competition for binding sites, production of hydrogen peroxide and bacteriocins (antimicrobial peptides) play a key role in competitive exclusion and probiotic properties (Velraeds *et al.*, 1998; Boris and Barbes, 2000; Lepargneur and Rousseau, 2002; Reid and Burton, 2002). Although the role of bacteriocins and their significance in controlling the proliferation of pathogenic bacteria in the intestinal tract is questionable (Brink *et al.*, 2006), recent reports on bacteriocins active against Gram-negative bacteria (Ivanova *et al.*, 1998; Messi *et al.*, 2001; Caridi, 2002; Todorov and Dicks, 2005a, 2005b) placed a renewed interest in these peptides and their interaction with intestinal pathogens.

In the current study, we report on eight bacteriocins of lactic acid bacteria with activity against Gram-positive and Gram-negative bacteria and studied the survival of the strains in the presence of low pH and elevated levels of ox-bile, adhesion to Caco-2 cell lines, growth in the presence of antibiotics and commercially available medicaments, and the presence of genes encoding surface proteins involved in cellular adhesion. *Lactobacillus plantarum* ST26MS and ST28MS have been isolated from molasses (Todorov and Dicks, 2005a), *Lactobacillus plantarum* ST23LD, *Enterococcus faecium* ST311LD, *Leuconostoc mesenteroides* subsp. *mesenteroides* ST33LD and *Lactobacillus plantarum* ST341LD from spoiled black olives (Todorov and Dicks, 2005b), *Lactobacillus plantarum* 423 from beer (van Reenen *et al.*, 1998) and *Lactobacillus plantarum* ST8KF from kefir (Powell *et al.*, 2007).

MATERIALS AND METHODS

Bacterial strains and growth conditions. *Lactobacillus plantarum* ST26MS, ST28MS, ST23LD, ST341LD, 423 and ST8KF, *Enterococcus faecium* ST311LD and *Leuconostoc mesenteroides* subsp. *mesenteroides* ST33LD have been described by Van Reenen *et al.* (1998), Todorov and Dicks (2005a, 2005b) and Powell *et al.* (2007). *Enterococcus faecium* HKLHS, *Lactobacillus sakei* DSM 20017, *Lactococcus lactis* subsp. *lactis* HV219, *Listeria innocua* LMG 13568 and UWC N27, and *Listeria ivanovii* subsp. *ivanovii* ATCC 19119 served as target strains for the screening of bacteriocin production and in co-aggregation studies. All lactic acid bacteria listed in Tables 1 and 2 were cultured in MRS broth (Biolab, Biolab Diagnostics, Midrand, SA) and the other strains in BHI broth (Biolab) at 37 °C. Pure cultures were stored at −80 °C in growth medium, supplemented with glycerol (15%, v/v, final concentration).

TABLE 2 - Spectrum of activity of *Lactobacillus plantarum* ST26MS, ST28MS, ST23LD, ST341LD, 423 and ST8KF, *Enterococcus faecium* ST311LD and *Leuconostoc mesenteroides* subsp. *mesenteroides* ST33LD against co-aggregation partners *Enterococcus faecium* HKLHS, *Lactobacillus sakei* DSM 20017, *Lactococcus lactis* subsp. *lactis* HV219, *Listeria ivanovii* subsp. *ivanovii* ATCC 19119 and *Listeria innocua* UWS N27 and LMG 13568

Bacteriocin producer	Test microorganisms					
	Enterococcus faecium HKLHS	*Lactobacillus sakei* DSM 20017	*Lactococcus lactis* subsp. *lactis* HV219	*Listeria innocua* LMG 13568	*Listeria innocua* UWC N27	*Listeria ivanovii* subsp. *ivanovii* ATCC 19119
ST26MS	+	+	+	+	+	-
ST28MS	+	-	+	+	+	-
ST23LD	+	+	+	+	+	+
ST341LD	+	+	+	+	+	+
423	+	+	-	+	-	+
ST8KF	+	-	-	+	+	+
ST311LD	+	+	-	+	+	+
ST33LD	+	+	-	-	-	+

+ = inhibition zones > 3 mm in diameter; - = no inhibition zone

TABLE 3 - Effect of antibiotics on growth of *Lactobacillus plantarum* ST26MS, ST28MS, ST23LD, ST341LD, 423 and ST8KF, *Enterococcus faecium* ST311LD and *Leuconostoc mesenteroides* subsp. *mesenteroides* ST33LD

Antibiotic	ST26MS	ST28MS	ST23LD	ST341LD	423	ST8KF	ST311LD	ST33LD
Nalidixic acid	0	0	0	0	0	0	0	0
Sulpfamethoxazole	21	0	0	0	0	0	0	0
Neomycin	13	8	15	16	10	14	13	0
Tobramycin	0	0	0	0	0	0	0	0
Nitrofurantoin	35	24	32	33	30	30	25	32
Ciprofloxacin	0	0	0	0	0	0	24	0
Cefuroxime	24	22	23	23	25	23	0	20
Fusidic acid	18	15	15	15	12	13	20	14
Clindamycin	35	34	35	33	11	35	15	35
Furazolidone	31	22	26	22	24	24	20	27
Cefotaxime	22	23	25	23	22	23	0	20
Rifampicin	25	24	26	28	26	24	14	24
tetracycline	27	30	30	26	27	30	37	28
Compound sulphonamides	22	0	0	0	0	0	0	20
Ofloxacin	12	13	14	13	12	12	20	10
Oxacillin	0	0	12	0	0	0	0	13
Cefepime	28	26	24	22	24	27	0	22
Amikacin	10	13	15	11	0	13	12	0
Cephazolin	21	27	25	18	27	19	13	20
Ceftazidime	15	18	15	14	15	15	0	15
Ceftriaxone	24	25	19	20	24	21	0	18
Streptomycin	11	0	10	10	0	0	0	0
Metronidazole	0	0	0	0	0	0	0	0
Erythromicin	32	28	28	27	28	27	11	25
Chloramphenicol	27	30	32	27	30	31	30	30
Vancomycin	0	0	0	0	0	0	23	0
Sulphamethoxazole/Trimethoprim	30	27	28	30	25	28	15	28
Sulphafurazole	25	20	15	27	15	21	0	0
Trimethoprim	33	23	26	28	24	25	20	25

Growth at different pH and bile concentrations. *Lactobacillus plantarum* ST26MS, ST28MS, ST23LD, ST341LD, 423 and ST8KF, *Enterococcus faecium* ST311LD and *Leuconostoc mesenteroides* subsp. *mesenteroides* ST33LD were grown at 37 °C in MRS broth adjusted to pH 3.0, 4.0, 5.0, 7.0, 9.0, 10.0 and 13.0, respectively. The pH was adjusted with 1 N HCl or 1 N NaOH before autoclaving and re-adjusted with sterile 1 N HCl or 1 N NaOH after autoclaving as required.

Resistance to bile was tested by growing the cells at 37 °C in MRS broth, supplemented with 0.3, 0.6, 0.8, 1.0, 2.0 and 5.0% (w/v) ox-bile (Oxoid, Basingstoke, England). All tests were conducted in sterile STERELIN™ microtiter plates. Each well was filled with 180 µl bile-containing medium and inoculated with 20 µl culture (OD_{600nm} = 0.3). Optical density readings (at 600 nm) were recorded every hour for 10 h. Growth in MRS broth, without bile, served as control.

Auto-aggregation and co-aggregation. *Lactobacillus plantarum* ST26MS, ST28MS, ST23LD, ST341LD, 423 and ST8KF, *Enterococcus faecium* ST311LD and *Leuconostoc mesenteroides* subsp. *mesenteroides* ST33LD were studied for their ability to auto-aggregate and co-aggregate. Co-aggregation was studied in the presence of *Listeria innocua* LMG 13568 and UWC N27, *Listeria ivanovii* subsp. *ivanovii* ATCC 19119, *Lactobacillus sakei* DSM 20017, *Enterococcus faecium* HKLHS and *Lactococcus lactis* subsp. *lactis* HV219. The cultures were grown in 10 ml MRS broth or BHI broth at 37 °C for 24 h and then harvested (7400 × *g*, 10 min, 4 °C). The cells were washed, re-supended in sterile saline and adjusted to OD_{660nm} = 0.3.

Auto-aggregation was determined spectrophotometrically as described by Malik *et al.* (2003) and by using the formula:

$$\% \text{ Auto-aggregation} = \frac{OD_0 - OD_{60}}{OD_0} \times 100$$

where OD_o is the initial OD at 660 nm, OD_{60} refers to the optical density measured after the cells were incubated for 60 min and centrifuged for 2 min at 300 × *g.*

Co-aggregation was monitored by determining the OD_{660nm}-readings of paired cell suspensions after incubation for 60 min and centrifuged for 2 min at 300 × *g* (Malik *et al.,* 2003). The level of co-aggregation was calculated using the equation:

$$\% \text{ Co-aggregation} = \frac{OD_{Tot} - OD_S}{OD_{Tot}} \times 100$$

where the OD_{Tot} value refers to the initial OD at 660 nm, taken immediately after the strains were paired, OD_S refers to the OD_{660nm} of the supernatant after the cells were centrifuged for 60 min (Malik *et al.*, 2003). The experiment was conducted twice, each time in triplicate.

Bacteriocin bioassay. Strains were inoculated (2%, v/v) into 100 ml MRS broth and incubated at 30 and 37 °C for 24 h. The cells were harvested (1000 × *g* , 15 min, 4 °C), the pH of the cell-free culture supernatants containing bacteriocins adjusted to 6.0 with sterile 1 M NaOH, heated for 10 min at 80 °C, and then filter-sterilised (0.20 µm, Minisart®, Sartorius).

TABLE 4 - Susceptibility of *Lactobacillus plantarum* ST26MS, ST28MS, ST23LD, ST341LD, 423 and ST8KF, *Enterococcus faecium* ST311LD and *Leuconostoc mesenteroides* subsp. *mesenteroides* ST33LD to substances in commercially available medicaments

Active substance*	Concentration (mg/ml)	Inhibition zone (diameter in mm)							
		ST26MS	ST28MS	ST23LD	ST341LD	423	ST8KF	ST311LD	ST33LD
Ibuprofen	40	21	17	20	20	12	18	18	21
Triamterene, hydrochlorothiazide	7.5	35	18	15	18	15	20	13	22
Sodium diclofenac	10	0	0	0	10	0	13	12	12
Thioridazine hydrochlorid	2	12	8	10	10	10	9	7	6
Dimenhydrinate	10	0	0	0	0	0	10	0	0

* None of the strains were inhibited by N-acetyl-L-cystein (120 mg/ml), ambroxol (20 mg/ml), aminophylline (10 mg/ml), antazoline (100 mg/ml), aspirin (60 mg/ml), bisacodyl (1 mg/ml), cinarizin (3 mg/ml), heptaminol (14 mg/ml), codeine hydrate (2 mg/ml), Na-hydrogen carbonate (50 mg/ml), dichlorhydrate hydroxyzine (5 mg/ml), famotidine (4 mg/ml), metamizole (100 mg/ml), metoclopramide hydrochloride (2.5 mg/ml), simethicone (2.5 mg/ml), methyl-4-hydroxybenzoate (8 mg/ml), silymarin (14 mg/ml), vitamin A (7.5 mg/ml), vitamin D_2 (7.5 mg/ml), Oleum Jecoris (7.5 mg/ml), and N-(4-hydroxyphenyl) acetamide (100 mg/ml).

Sensitivity of cells to antibiotics and medicaments. Overnight cultures of *Lactobacillus plantarum* ST26MS, ST28MS, ST23LD, ST341LD, 423 and ST8KF, *Enterococcus faecium* ST311LD and *Leuconostoc mesenteroides* subsp. *mesenteroides* ST33LD were embedded in MRS soft agar (1.0%, v/v) to a final concentration of 10^6 CFU/ml. Antibiotic disks listed in 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.

In a similar experiment, the effect of medicaments and commercially available antibiotics listed in Table 4 on the growth of the strains was determined by spotting 10 µl of each medicament on the agar surface and incubating the plates at 37 °C for 24 h. Growth inhibition was recorded by measuring the diameter of the zones.

Adhesion of bacteria to Caco-2 cells. Caco-2 cells (Highveld Biological PTY LTD; Kelvin, Johannesburg) were grown in Minimal Essential Medium (MEM) Earle's Base (Highveld Biological), supplemented with 10% (v/v) foetal bovine serum (Sigma), 100 U/ml penicillin and 100 U/ml streptomycin (Sigma). Incubation was at 37 °C in the presence of 5% CO_2. Caco-2 cells were seeded at 1 × 10^5 cells per well in 12-well plates to obtain confluence. *Lactobacillus plantarum* ST26MS, ST28MS, ST23LD, ST341LD, 423 and ST8KF, *Enterococcus faecium* ST311LD and *Leuconostoc mesenteroides* subsp. *mesenteroides* ST33LD were grown in MRS broth for 18 h at 37 °C. The cells were harvested (10000 *x g*, 10 min, 4 °C), washed twice with sterile phosphate-buffered saline (PBS, 6.0 mM Na_2HPO_4, 1.5 mM KH_2PO_4, 0.14 M NaCl, 3.0 mM KCl, pH 7.3), diluted in MEM and adjusted to an OD_{600nm}-reading of 0.5, which is equivalent to approximately 1 x 10^6 CFU/ml.

Adherence to Caco-2 cells was studied by inoculating each well (1 x 10^5 Caco-2 cells) with 100 µl (approximately 1 x 10^5 viable cells) of *Lactobacillus plantarum* ST26MS, ST28MS, ST23LD, ST341LD, 423 and ST8KF, *Enterococcus faecium* ST311LD and *Leuconostoc mesenteroides* ST33LD. After 2 h of incubation at 37 °C on an orbital shaker, the bacterial cells were withdrawn from the wells and the Caco-2 cells washed twice with 1 ml sterile PBS, followed by 1 ml 0.5% (v/v) Triton X-100. The number of viable bacteria released from Caco-2 cells were determined by plating onto MRS medium (Biolab).

Identification of genes encoding adhesion proteins Map and Mub and elongation factor EF-Tu. DNA was isolated according to the method of Dellaglio *et al.* (1973). Primers Mub423F (5'-GTA GTT ACT CAG TGA CGA TCA ATG-3'), Mub423R (5'-TAA TTG TAA AGG TAT AAT CGG AGG-3'), Map423F (5'-TGG ATT CTG CTT GAG GTA AG-3'), Map423R (5'-GAC TAG TAA TAA CGC GAC CG-3'), EFTu423F (5'-TTC TGG TCG TAT CGA TCG TG-3') and EFTu423R (5'-CCA CGT AAT AAC GCA CCA AC-3'), designed to amplify *mapA*, *mub* and *EF-Tu* (Ramiah *et al.*, 2007), were used. PCR reactions were performed using a GeneAmp® PCR Instrument System 9700 (Applied Biosystems, Foster City, USA). The following conditions were used: an initial denaturation step of 94 °C for 5 min, followed by 35 cycles of 30 s at 94 °C, 30 s at 55 °C and 30 s at 72 °C, and final extension at 72 °C for 7 min. The amplified product was visualised in a 2.0% (w/v) agarose gel stained with ethidium bromide. A band corresponding to the correct size was purified from the gel using the QIAquick PCR purification kit (QIAGEN GmbH). PCR purified products were ligated into pGEM-T® Easy Vector (Promega, Madison, USA) and transformed to *Escherichia coli* DH5α, according to instructions of the manufacturer. Plasmids were isolated using a QIAGEN Plasmid Mini Kit and fragments sequenced via an automatic sequencer (ABI Genetic Analyzer 3130XI, Applied Biosystems) using bigdye terminator chemistry (Biosystem, Wanington, England). Sequences were analysed using DNAMAN *for Windows*® (Lynnon Biosoft, Quebec, Canada).

RESULTS AND DISCUSSION

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

Lactobacillus plantarum has a long history of natural occurrence in a variety of food products and has GRAS status. Clinical studies underline the safe use of *Lactobacillus plantarum* in humans (De Vries *et al.*, 2006). *Enterococcus* spp. are present in artisan food products (Torres-Llanez *et al.*, 2006; Fontán *et al.*, 2007; Ruiz-Barba *et al.*, 2007) and most species have GRAS status. However, a few strains of *Enterococcus faecalis* and *Enterococcus faecium* with transferable antibiotic resistance genes have been reported and are considered opportunistic pathogens (Erdinc *et al.*, 2006; Furtado *et al.*, 2006). *Leuconostoc mesenteroides* subsp. *mesenteroides* is used as starter culture in several food products (Drosinos *et al.*, 2006; Herreros *et al.*, 2007; Sajur *et al.*, 2007).

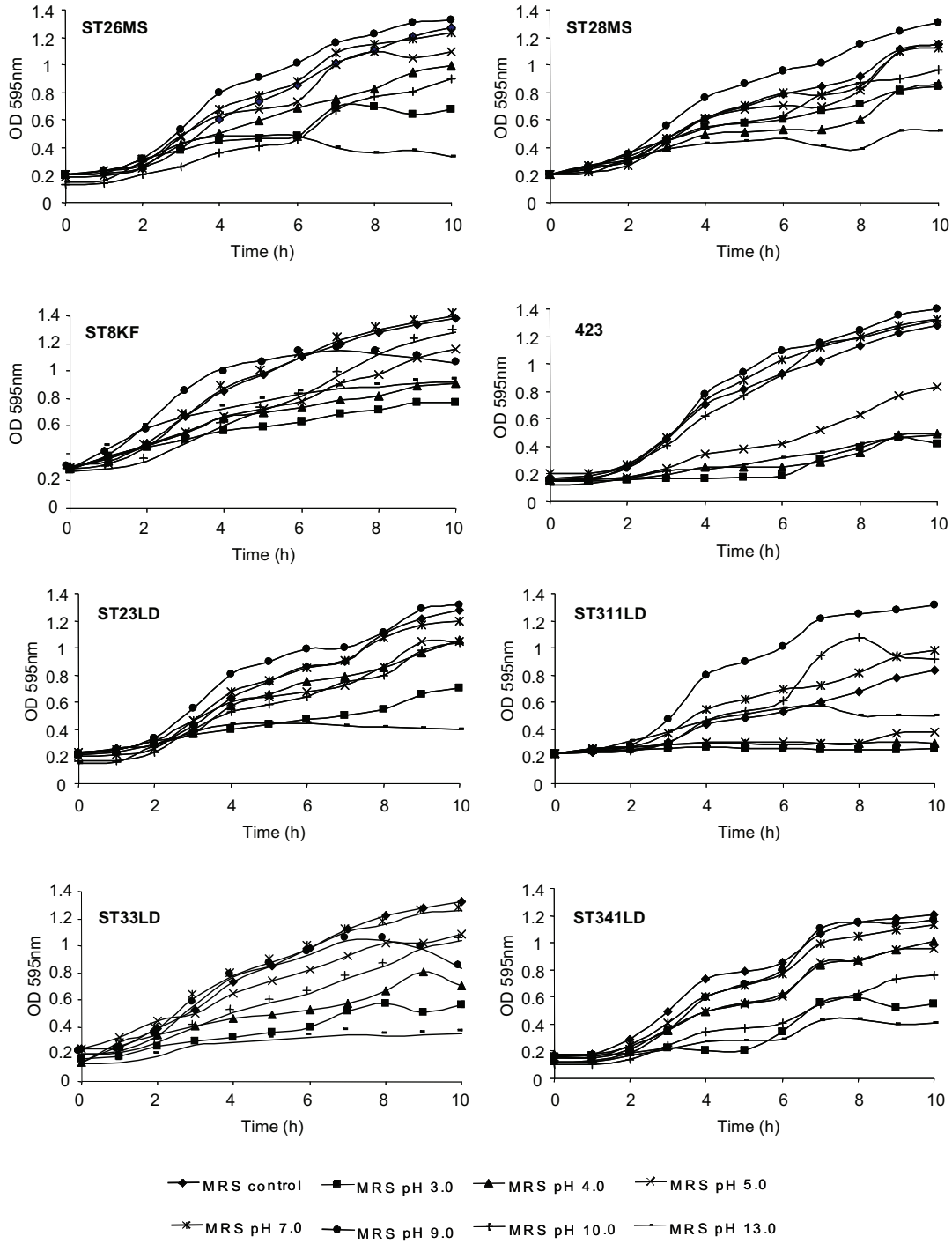

FIG. 1 - Comparison of growth of *Lactobacillus plantarum* ST26MS, ST28MS, ST8KF, 423, ST23LD and ST341LD, *Enterococcus faecium* ST311LD and *Leuconostoc mesenteroides* subsp. *mesenteroides* ST33LD in MRS (Biolab) at different initial pH levels. The experiment was done in triplicate.

Growth at different pH and bile concentrations

All strains grew well in MRS broth adjusted to pH 7.0. An increase to pH 13.0 retarded growth. *Lactobacillus plantarum* ST341LD and 423, and *Enterococcus faecium* ST311LD grew in MRS broth with an initial pH adjusted to 10.0 (Fig. 1). *Enterococcus faecium* ST311LD, *Leuconostoc mesenteroides* subsp. *mesenteroides* ST33LD and *Lactobacillus plantarum* 423 did not grow in MRS broth with an initial pH of pH 3.0, but grew in the same medium with an initial pH of 4.0 (Fig. 1). Similar results were recorded for *Lactobacillus salivarius* 241, *Lactobacillus curvatus* DF38 (Brink *et al.*, 2006) and *Lactobacillus acidophilus* (Park *et al.*, 2006). Certain strains of *Lactobacillus plantarum* are tolerant to repeated exposure to HCl (pH 2.0) and bile salts (Haller *et al.*, 2001). This was observed for strains isolated from intestinal samples and fermented foods (Haller *et al.*, 2001). A relatively high percentage of *Lactobacillus plantarum* (10%) survived this low pH, whilst none or only 0.001% of *Lactobacillus sakei* and *Lactobacillus paracasei* survived these conditions (Haller *et al*., 2001). *Enterococcus faecium* ST311LD and *Leuconostoc mesenteroides* subsp. *mesenteroides* ST33LD may be used as probiotics. Incorporation into alginate beads may protect the cells from gastric acids (Chan and Zhang, 2005).

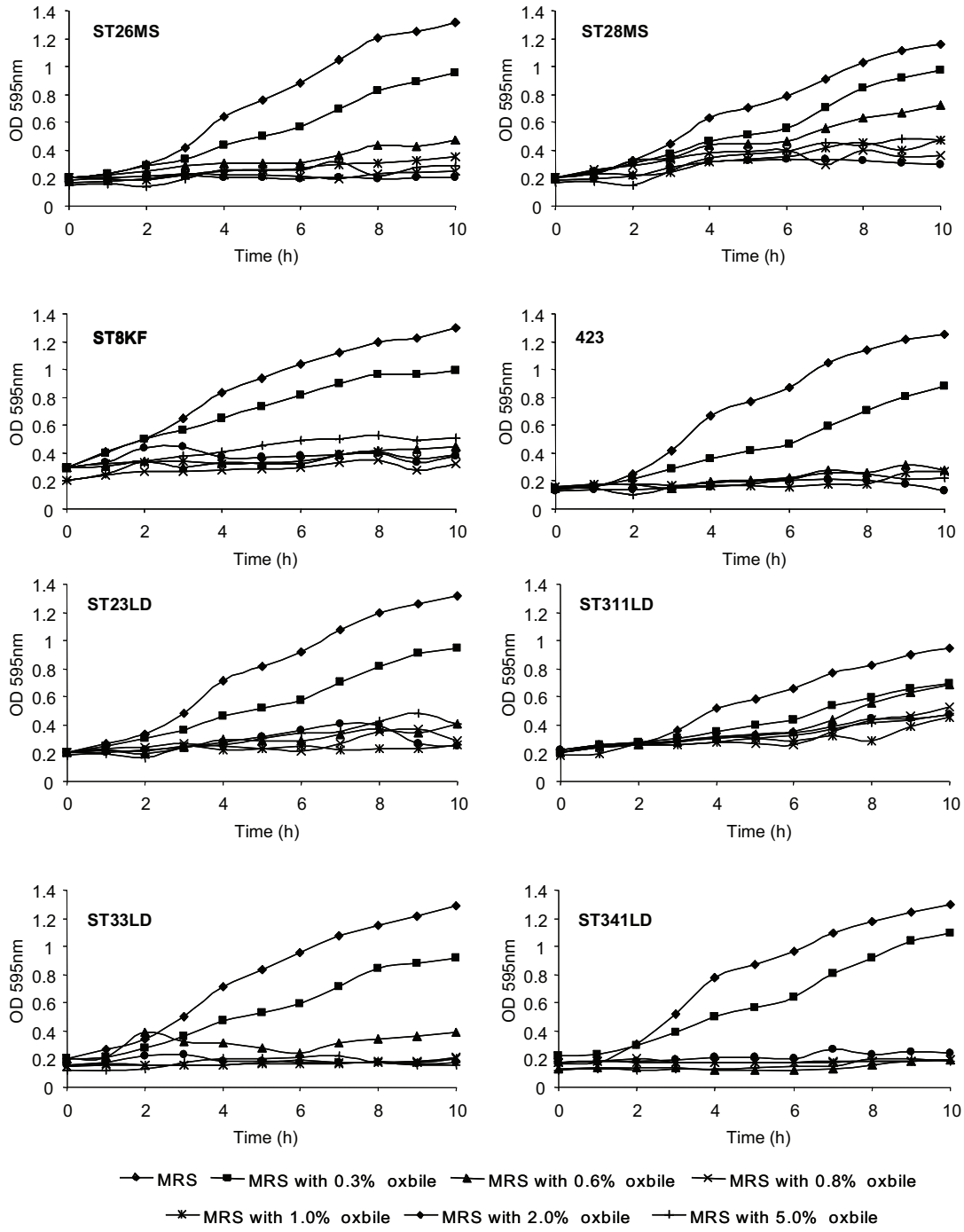

FIG. 2 - Comparison of growth of *Lactobacillus plantarum* ST26MS, ST28MS, ST8KF, 423, ST23LD and ST341LD, *Enterococcus faecium* ST311LD and *Leuconostoc mesenteroides* subsp. *mesenteroides* ST33LD in MRS broth supplemented with different levels of ox-bile. The experiment was done in triplicate.

Growth of *Lactobacillus plantarum* ST26MS, ST28MS, ST23LD, ST341LD, ST8KF and 423, *Enterococcus faecium* ST311LD and *Leuconostoc mesenteroides* subsp. *mesenteroides* ST33LD in MRS broth supplemented with 0.3% (w/v) ox-bile compared well with growth in MRS broth in the absence of ox-bile (Fig. 2). *Lactobacillus plantarum* ST28MS and *Enterococcus faecium* ST311LD grew well in the presence of 0.6% (w/v) ox-bile. Relatively good growth of *Lactobacillus plantarum* ST26MS and *Leuconostoc mesenteroides* subsp. *mesenteroides* ST33LD was recorded in the presence of 0.6% (w/v) ox-bile (Fig. 2). Ox-bile concentrations of 1.0% and higher inhibited the growth of all strains (Fig. 2). Resistance to ox-bile has been recorded for *Lactobacillus acidophilus* (Park *et al.*, 2006), *Lactobacillus salivarius* 241, *Lactobacillus plantarum* 423 and *Lactobacillus curvatus* DF38 (Brink *et al.*, 2006).

Probiotic strains have to survive harsh conditions in the gastrointestinal tract (GIT), e.g. pH values ranging from 1.0 to 3.0 in the stomach and bile salt levels of approximately 0.3% in the small intestine (Mainville *et al.*, 2005). Resistance to low pH and elevated concentrations of bile salts are thus important criteria for the selection of probiotic bacteria (Havenaar *et al.*, 1992).

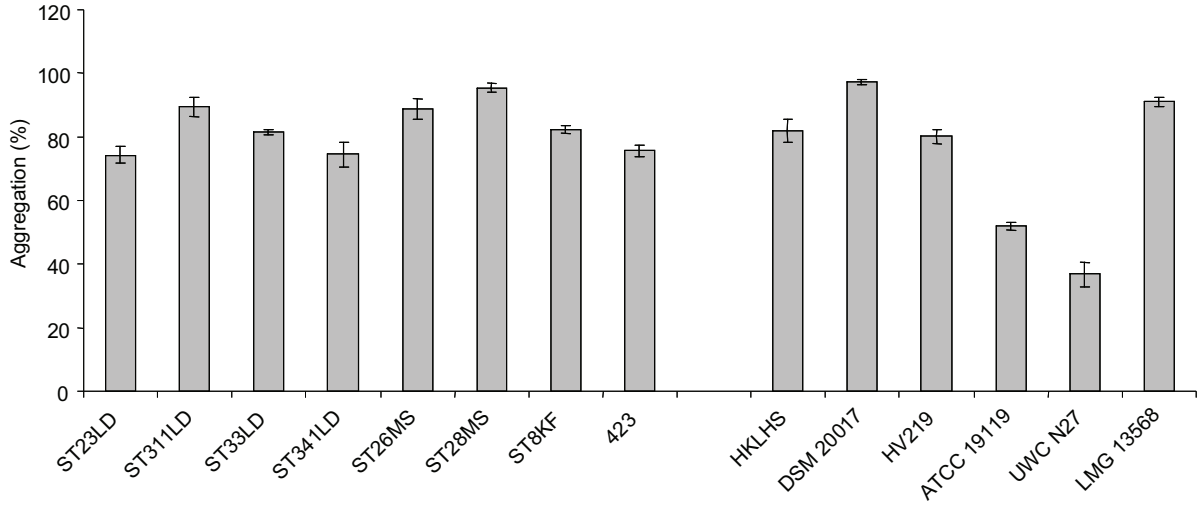

FIG. 3 - Auto-aggregation of *Lactobacillus plantarum* ST26MS, ST28MS, ST8KF, 423, ST23LD and ST341LD, *Enterococcus faecium* ST311LD and *Leuconostoc mesenteroides* subsp. *mesenteroides* ST33LD and co-aggregation with *E. faecium* HKLHS, *Lactobacillus sakei* DSM 20017, *Lactococcus lactis* subsp. *lactis* HV219, *Listeria ivanovii* subsp. *ivanovii* ATCC 19119, and *Listeria innocua* UWS N27 and LMG 13568. The experiment was done in triplicate.

Auto-aggregation and co-aggregation

Auto-aggregation is strain-specific. Values ranging from 74.3% for *Lactobacillus plantarum* ST23LD to 95.4% for *Lactobacillus plantarum* ST28MS were recorded (Fig. 3). Aggregation is important for biofilm formation and may assist probiotic bacteria to adhere to mucus and epithelial cells and survive harsh conditions in the GIT (Lepargneur and Rousseau, 2002; Reid and Burton, 2002). *Lactobacillus plantarum* has a number of genes encoding production of surface proteins that function in recognition of, or binding to, certain components in the environment. Several of these genes share homology with mucus-binding, aggregation-promoting and intracellular adhesion proteins (Kleerebezem *et al.*, 2003).

Different levels of co-aggregation between the eight strains and *Enterococcus faecium* HKLHS, *Lactobacillus sakei* DSM 20017, *Lactococcus lactis* subsp. *lactis* HV219, *Listeria innocua* LMG 13568 and UWC N27, and *Listeria ivanovii* susbp. *ivanovii* ATCC 19119 were obtained (Table 1). High levels of co-aggregation with *Enterococcus faecium* HKLHS, *Lactobacillus sakei* DSM 20017 and *Lactococcus lactis* subsp. *lactis* HV219 were recorded (Table 1). Co-aggregation with other lactic acid bacteria is important for facilitating the gastro-intestinal tract of the host. *Lactococcus lactis* subsp *lactis* HV219 produces a bacteriocin active against a number of pathogens, including *Escherichia coli* (Todorov *et al.*, 2006). Reduced levels of co-aggregation were observed in mixed cultures with *Listeria innocua* UWC N27 and *Listeria ivanovii* susbp. *ivanovii* ATCC 19119 (Table 1). High levels of co-aggregation were documented between *Listeria innocua* LMG 13568 and *Lactobacillus plantarum* ST23LD (94.3% compared with auto-aggregation of 74.3% for strain ST23LD and 91.1% for *Listeria innocua* LMG 13568), and *Lactobacillus plantarum* ST311LD (93.7% co-aggregation and 89.5% auto-aggregation for ST311LD). Co-aggregation of *Listeria innocua* LMG 13568 with *Lactobacillus plantarum* 423 was only 69.1%, compared to 75.6% and 91.1% auto-aggregation for *Lactobacillus plantarum* 423 and *Listeria innocua* LMG 13568 (Table 1, Fig. 3).

Auto-aggregation and co-aggregation are strain-specific and most probably involves species-specific surface proteins. The co-aggregation assay did not, however, identify isolates capable of forming moderate to strong biofilm structures as being more efficient at co-aggregation than non-biofilm-forming isolates. Co-aggregation between *Lactobacillus plantarum* and other cells, especially *Listeria innocua* LMG 13568, may be considered a positive characteristic, since it is one of the steps required for elimination of non-desirable strains from the gastro-intestinal tract.

Antimicrobial activity

pH neutralised cell-free supernatants from 24-h-old cultures of *Lactobacillus plantarum* ST26MS, ST28MS, ST23LD, ST341LD, ST8KF and 423, *Enterococcus faecium* ST311LD and *Leuconostoc mesenteroides* subsp. *mesenteroides* ST33LD inhibited the growth of *Enterococcus faecium* HKLHS*, Lactobacillus sakei* DSM 20017*, Lactococcus lactis* subsp. *lactis* HV219*, Listeria innocua* LMG 13568*, Listeria innocua* UWC N27 and *Listeria ivanovii* subsp*. ivanovii* ATCC 19119 (Table 2). Identical results were recorded with agar-spot and well-diffusion methods.

To survive harsh conditions in the GIT, probiotic bacteria have to defend themselves against other microbial cells and pathogens. This may be by done by production of hydrogen peroxide and bacteriocins (Lepargneur and Rousseau, 2002; Reid and Burton, 2002). Uncontrolled proliferation of pathogenic bacteria may lead to diarrhoea and other clinical disorders such as cancer, inflammatory disease and ulcerative colitis (Fooks *et al*., 1999).

Sensitivity of cells to antibiotics and medicaments

Patients taking probiotics are often treated for other illnesses. It is thus important to determine the affect of medicaments on the growth of probiotic strains. Growth of *Lactobacillus plantarum* ST26MS, ST28MS, ST23LD, ST341LD, ST8KF and 423, *Enterococcus faecium* ST311LD and *Leuconostoc mesenteroides* subsp. *mesenteroides* ST33LD were inhibited by several antibiotics (Table 3), anti-inflammatory medicaments, moderate diuretic and neuroleptic medicaments containing ibuprofen, hydrochlorothiaziden and thioridazine hydrochlorid (Table 4). Sodium diclofenac inhibited the growth of *Lactobacillus plantarum* ST8KF and ST341LD, *Enterococcus faecium* ST311LD and *Leuconostoc mesenteroides* subsp.

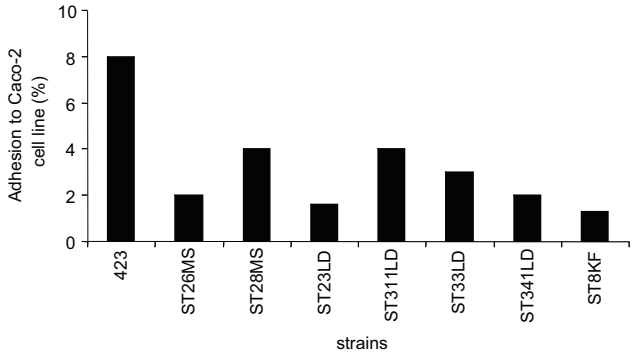

FIG. 4 - Adhesion of *Lactobacillus plantarum* ST26MS, ST28MS, ST8KF, 423, ST23LD and ST341LD, *Enterococcus faecium* ST311LD and *Leuconostoc mesenteroides* subsp. *mesenteroides* ST33LD to Caco-2 cells. The experiment was done in triplicate.

mesenteroides ST33LD. Dimenhydrinate inhibited the growth only of *Lactobacillus plantarum* ST8KF. In a previous study, diclofenac and ibuprofen inhibited the growth of *Lactococcus lactis* subsp. *lactis* HV219 (Todorov *et al.*, 2007). Anti-inflammatory medicaments, moderate diuretic and neuroleptic containing diclofenac, ibuprofen, triamterene hydrochlorothiaziden and thioridazine hydrochlorid acted as inhibitors of growth of *Lactobacillus plantarum*, *Lactobacillus rhamnosus*, *Lactobacillus paracasei* and *Lactobacillus pentosus* strains isolated from boza and evaluated as a probiotic (Todorov *et al.*, 2008). Dimenhydranat inhibited the growth of *Lactobacillus rhamnosus* ST462BZ and *Lactobacillus plantarum* ST664BZ (Todorov *et al.*, 2008). It is, however, important to mention that the concentration of these substances is critical. Growth of the same cells treated with ibuprofen produced by a different company was not inhibited (Todorov *et al.*, 2007, 2008).

The antibiotic resistance profile of 55 probiotic strains isolated in Europe revealed resistance to kanamycin (79% of the isolates), vancomycin (65%), tetracycline (26%), penicillin G (23%), erythromycin (16%) and chloramphenicol (11%). Overall 68.4% of the isolates showed resistance against multiple antibiotics, including intrinsic resistance (Temmerman *et al.*, 2002). Multi-resistant probiotic strains may transfer antibiotic resistance to commensal microbiota in the gastro-intestinal tract. This is of particular concern in immuno-compromised patients (Courvalin, 2006).

Adhesion of bacteria to Caco-2 cells

All eight strains adhered to Caco-2 cells, ranging from 1.3 to 8.0% (Fig. 4). Higher levels of adherence was recorded for *Lactobacillus plantarum* 423 (8%), *Lactobacillus plantarum* ST28MS and *Enterococcus faecium* ST311LD (4%) and *Leuconostoc mesenteroides* subsp. *mesenteroides* ST33LD (3%). Adhesion values as high as 14% to Caco-2 cells have been reported for some probiotic lactobacilli (Tuomola and Salminen, 1998). Caco-2 cells express several markers that are distinctive of normal small intestinal villi and have served as excellent *in vitro* models for understanding the mechanisms involved in adherence of probiotic bacteria and invasion of pathogens (Granato *et al.*, 2004).

Identification of genes encoding adhesion proteins Map and Mub and elongation factor EF-Tu

All *Lactobacillus plantarum* strains used in this study contain the *mub, mapA* and *EF-Tu* genes, as determined by PCR analysis (Fig. 5). The presence of these adhesion genes in the *Lactobacillus plantarum* strains are not surprising due to the high adhesive properties of the microorganism. Whole genome sequence data of *Lactobacillus plantarum* WCFS1 revealed the presence of 223 extracellular proteins of which a large proportion is involved in the adherence of the cell to its environment (Kleerebezem *et al.*, 2003). Seven of these proteins facilitate adhesion to mucus. Some of these possible mucus binding proteins contain the Mub domain, a trait unique to lactic acid bacteria (Boekhorst *et al.*, 2006). The presence of these genes in lactic acid bacteria may be imperative, particularly for probiotic properties.

CONCLUSIONS

The *Lactobacillus plantarum*, *Enterococcus faecium* and *Leuconostoc mesenteroides* subsp. *mesenteroides* strains isolated from molasses, olives, beer and kefir survived low pH conditions and ox-bile levels above normal (0.3%, w/v) concentrations. Although *Enterococcus faecium* ST311LD, *Leuconostoc mesenteroides* subsp. *mesenteroides* ST33LD and *Lactobacillus plantarum* 423 did not grow in MRS broth

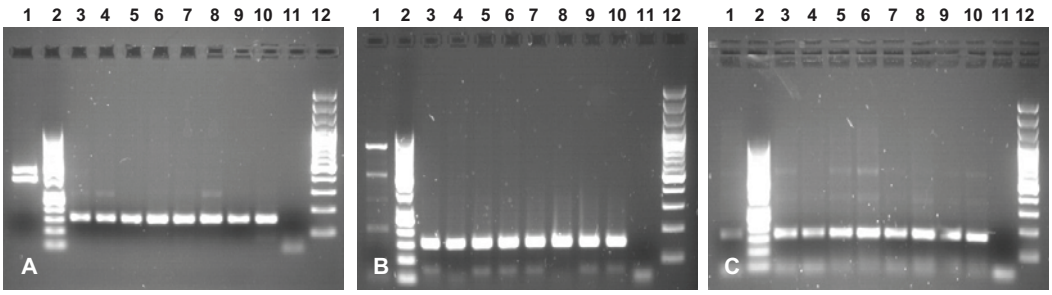

FIG. 5 - Agarose gels showing DNA fragments characteristic for (A) *MapA,* (B) *Mub* and (C) *EF-Tu* amplified by PCR. Lane 1: negative control (*Streptococcus macedonicus* ST91KM), lane 2: 50-bp molecular marker, lane 3: *Lactobacillus plantarum* 423, lane 4: *Lactobacillus plantarum* ST26MS, lane 5: *Lactobacillus plantarum* ST28MS, lane 6: *Lactobacillus plantarum* ST23LD, lane 7: *Enterococcus faecium* ST311LD, lane 8: *Leuconostoc mesenteroides* susbp. *mesenteroides* ST33LD, lane 9: *Lactobacillus plantarum* ST341LD, lane 10: *Lactobacillus plantarum* ST8KF, lane 11: no DNA, lane 12: 100-bp molecular marker.

with an initial pH of pH 3.0, they grew in the same medium with an initial pH of 4.0, suggesting that they will survive conditions in the large intestinal tract. Furthermore, the strains produced bacteriocins and may remove pathogens from the intestinal tract by mere aggregation. These characteristics and adherence to mucus and epithelial cells, facilitated by the presence of *Mub, MapA* and *EF-Tu* suggests that the strains may be excellent probiotics. Growth of the strains was, however, inhibited by several antibiotics and anti-inflammatory medicaments, suggesting that they may not be used by patients exposed to these medicaments.

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